Maintenance of Beta Cell Identity and Function

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ABSTRACT

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The acquisition of beta cell identity and function is a multistage process that involves the sequential regulation of specific factors and signals. The maintenance of beta cell identity and function is a process of comparable importance that requires persistent and continuous regulation. Loss of beta cell identity and/or reprogramming represents an important feature of beta cell dysfunction in genetic models of diabetes, as well as in patients with type 1 and type 2 diabetes. The factors and mechanisms involved in the acquisition and maintenance of beta cell identity are still not well understood. Nevertheless, several beta cell developmental transcription factors have been found to be important in the maintenance of its functional identity during the postnatal stage.

Nkx2.2 is a transcription factor that is critical for the development and differentiation of beta cells both in mice and humans. In adults, Nkx2.2 is expressed in the entire beta cell population. However, due to the perinatal lethality of the Nkx2.2 null mice, the study of its function in adult beta cells has remained elusive. For my dissertation work, I explored the function and mechanism of action of Nkx2.2 in the adult beta cell. I deleted *Nkx2.2* specifically in beta cells during their maturation and in adults. Deletion of *Nkx2.2* in beta cells caused rapid onset of diabetes due to the loss of insulin and the down-regulation of many beta cell functional genes. Concomitantly, *Nkx2.2*-deficient beta cells acquired non-beta cell endocrine features, resulting in populations of completely reprogrammed cells and bi-hormonal cells that have hybrid endocrine cell morphological characteristics.

Molecular analysis in mouse and human islets revealed that Nkx2.2 is a conserved master regulatory protein that controls the acquisition and maintenance of a functional monohormonal beta cell identity by directly activating critical beta cell genes, and actively repressing genes that specify the alternative islet endocrine cell lineages. This study demonstrates the highly volatile nature of the beta cell; it is necessary to actively maintain expression of genes involved in beta cell function, but to also maintain repression of closely related endocrine gene programs. These findings have potential applications that include the optimization of iPS cell differentiation protocols that aim to differentiate functional beta cells that remain safely locked into that identity state; as well as in future therapies that attempt to restore beta cells into a functional state.

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List of Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
AldoB	Aldolase B fructose-biphosphate
BMI	Body mass index
CCK	Cholecystokinin
ChIP-Seq	Chromatin immunoprecipitation-Sequencing
Dnmt1	DNA Methyltransferase 1
DNMT3a	DNA Methyltransferase 3a
ESCs	Embryonic stem cells
ER	Endoplasmic reticulum
GIP	Gastric inhibitory polypeptide
GLP-1	Glucagon-like-peptide 1
GSIS	Glucose stimulates insulin secretion
Glut2	Glucose transporter 2
G6P	Glucose-6-phosphate
Hk1	Hexokinase 1
Hk2	Hexokinase2
HDAC1	Histone deacetylase 1
HDAC2	Histone deacetylase 2
HD	Homeodomain
iPSc	Induced pluripotent stem cells
IIDP	Integrated islet distribution program
Ldha	Lactate dehydrogenase A
Ngn3	Neurogenin 3
SD	NK2 Specific domain
SNP	Single nucleotide polymorphism
PP	Pancreatic Polypeptide
PYY	Peptide YY
PRC2	Polycomb Repressive Complex 2
PC1	Pro-Hormone Convertase 1
PC2	Pro-Hormone Convertase 2
RER	Rough Endoplasmic reticulum
RIPA	Radio Immunoprecipitation assay
TEM	Transmitted electron microscopy
TN	Tinman Domain
TCA	Tricarboxylic Acid Cycle
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes

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Dedication

I dedicate this work to my family and my husband Juan Luis.

Chapter 1

Introduction

Diabetes

Diabetes is a condition where the body is unable to produce or utilize enough insulin to cope with elevated blood glucose levels. This chronic condition has become a very important healthcare concern, positioning itself as a major cause of death in most countries around the globe. According to the International Diabetes Federation, there are currently an estimated 415 million adults living with diabetes. In addition, there are also 318 million additional individuals who present with glucose intolerance and are therefore at risk of developing this disease. The number of diabetic individuals is expected to increase to 642 million people by 2040 (Federation., 2015). Furthermore, the financial cost associated with the prevention or management of diabetes and its complications was estimated to range between $672 billion to $1,197 billion, which represents 12% of the global healthcare expenditure. More importantly, this condition imposes a significant burden on the patient lives and their families, by having to cope with the different complications that are associated with diabetes, including cardiovascular disease, kidney, nerve and eye disease.

There are two major forms of diabetes, type I and type II diabetes (American Diabetes, 2014). Type I diabetes (T1D), is a chronic condition characterized by immune dysregulation, where the pancreatic insulin-producing beta cells are attacked and destroyed by the immune system. As a result of the autoimmunity, patients are unable to produce sufficient amounts of insulin and are therefore dependent on taking multiple doses of this hormone daily.

The onset of T1D primarily occurs in children and young adults. In addition, the incidence of T1D is increasing around the globe, and although there is a strong underlying genetic component, the trigger for the development of T1D remains unknown (Federation., 2015). There is still much debate in the etiology of this disease; both environmental and genetic factors are believed to contribute to its development and progression. Additionally, recent studies have suggested that insulin-producing beta cells could play a critical role in their own demise (Atkinson, 2012; Soleimanpour and Stoffers, 2013).

Type 2 diabetes (T2D) is the most prevalent form of diabetes, and is mainly characterized by insulin resistance in the peripheral organs and/or defects in pancreatic beta cell function. The origin of T2D is associated with a combination of genetic and environmental factors. Some of the environmental factors include a lack of proper nutrition and physical activity (Olokoba et al., 2012). Although T2D occurs more commonly in adults, it is now increasingly seen in children and adolescents (IDF –atlas ref). Metabolic dysfunction of peripheral tissues, such as liver and fat can contribute to the development of T2D. However, a decrease in beta cell function has been identified as a very important hallmark in both the development and progression of disease (Prentki and Nolan, 2006; Tahrani et al., 2011; Ward et al., 1984). Accordingly, recent studies have identified genes important for beta cell development, function and identity to be associated with T2D risk variants. These genes are not only expressed in beta cells, but are also differentially expressed in patients with T2D compared to healthy controls (Taneera et al., 2012).

One of the main features observed in both T1D and T2D is a gradual loss of beta cell mass, which had previously been thought to occur primarily through beta cell death. (Butler et al., 2003; Clark et al., 1988; Eisenbarth, 1986; Eizirik and Mandrup-Poulsen, 2001; Sakuraba et al., 2002). Recent studies, however, have led to alternative explanations for the apparent loss of beta cells. Talchai and colleagues (Talchai et al., 2012) used genetically modified mice to ablate *FoxO1* in beta cells, and found that loss of beta cell identity and not apoptosis was in fact the trigger for the development of diabetes in these mice. Interestingly, beta cells lacking *FoxO1* were found to first dedifferentiate, followed by the expression of other islet hormones, such as glucagon, somatostatin and pancreatic polypeptide. Furthermore, Talchai et al. showed that beta cell dedifferentiation was not a unique feature of *FoxO1* mutant mice, but was also present in several other diabetic mouse models. These findings provide strong evidence that maintenance of beta cell identity is crucial for the proper function of the beta cell during physiological and pathophysiological conditions. Moreover, it sheds light on the high level of plasticity that the beta cell possesses, a feature that provides immense therapeutic potential.

Current Treatments

The discovery of insulin in 1921-1922 represented a major advancement in the treatment of diabetes, especially for T1D (Rosenfeld, 2002). Currently, insulin is given in daily doses to patients either through injections or via insulin pumps, which have become more widely used in the past decade. Although, great advances have been made in the optimization of insulin therapy, precise metabolic control that can avoid all of the complications that arise from diabetes has not yet been achieved (Atkinson et al., 2014). For many years, significant effort has also been put into the development of immunotherapies to prevent or attenuate the autoimmune response.

These therapies range from antigen specific approaches and cell therapy to cytokine and anti-inflammatory treatments. Although several therapies have been tested in clinical trials, any success in disease attenuation have been transient and have not yet resulted in complete remission and insulin independence. Additionally, toxicity side effects remain a central concern (Ben Nasr et al., 2015).

An alternative treatment for patients with diabetes is islet transplantation, for which the first successful experiments in rats date back to the 1970s (Ballinger and Lacy, 1972) and subsequent trials in humans occurred in 1980 (Najarian et al., 1980). The success of this procedure culminated in 2000 when seven T1D patients attained normoglycemia after this procedure, widely known as the Edmonton protocol (Shapiro et al., 2000). Since this breakthrough, scientists have made significant advances in the optimization of this protocol. Patients who receive such transplants are able to reduce or eliminate insulin injections for a period of time that can vary from months to years, thereby preventing disease-related complications (Froud et al., 2005; O'Connell et al., 2006; Toso et al., 2006). Nonetheless, there is a very short supply of human islets, limiting the availability of treatments for all the patients, particularly since each patient generally requires islets from more than one donor. Additionally, one of the biggest challenges associated with this procedure is the rejection of the transplanted beta cells by the immune system, which can reduce patient lifespan significantly. In an effort to overcome this issue, patients have to continually take immunosuppressant drugs post-transplantation, which can have deleterious systemic side effects. Furthermore, the long-term side effects of this immunosuppressive regimen remain unknown (Hafiz et al., 2005; Hirshberg et al., 2003).

The management and development of therapies for diabetes poses a great challenge to both doctors and scientists alike. The current treatment for T2D involves lifestyle interventions in combination with different kinds of pharmacological agents. Lifestyle interventions mainly include a proper diet and regular exercise, which can be helpful in managing diabetes (Jeon et al., 2007; Sigal et al., 2006); however, it is difficult for many patients to adhere to these regimens. Therefore, in many cases the treatment relies almost entirely on the use of pharmaceutical agents. In this regard, there are two categories of the most commonly used drugs. The first focuses on increasing insulin sensitivity in the peripheral organs using drugs such as roziglitazone, pioglitazone and metformin. The second drug category increases the output of insulin secretion in beta cells, with sulfonylureas being the most frequently used. Although these medications are the conventional line of treatment, they are not able to provide the precise metabolic regulation needed to avoid the complications associated with diabetes. Also, the success of these medications varies between patients, and their effectiveness in some cases is only temporary. Furthermore, many of these drugs have several associated risks, which can include fluid retention, lactic acidosis, increased cardiovascular risk as well as dangerous episodes of hypoglycemia (Inzucchi et al., 2015).

Beta cell development

Pancreas

The pancreas is an organ that plays a critical role in the metabolic homeostasis of the body, and as discussed above, plays a major role in T1D and T2D. The pancreas is comprised of three main compartments: the exocrine and ductal tissues that are responsible for secreting digestive enzymes into the gut; and the endocrine compartment that is comprised of the islets of

Langerhans, which secrete hormones into the blood stream. The adult islet contains four endocrine cell populations, each secreting a unique hormone with an individual function. Beta cells secrete insulin, which drives glucose uptake in peripheral tissues and decreases renal and hepatic glucose production. Alpha cells secrete glucagon, a hormone that increases the blood glucose levels by stimulating hepatic glycogenolysis and gluconeogenesis (Quesada et al., 2008). Delta cells secrete somatostatin, a hormone found to potentially inhibit insulin and glucagon secretion (Hauge-Evans et al., 2009). Finally, PP cells secrete pancreatic polypeptide, a hormone associated with food intake regulation (Katsuura et al., 2002).

In mice, the insulin-producing beta cells are found at the core of the islet, surrounded by the delta, alpha and pancreatic polypeptide cells (Shih et al., 2013). In contrast, human islet architecture has been intensely debated. Recently, Bonner-Weir revisited this subject using novel technologies and found that surprisingly, small islets resemble the rodent architectural pattern; however, larger islets consist of subunits of cell clusters of aggregates that nevertheless also contain a mantle-core arrangement (Bonner-Weir et al., 2015). The effect that such differences might have in the overall regulation of metabolism will need to be further explored.

Pancreas development and beta cell differentiation

The pancreas begins to form during mouse fetal development around embryonic day (e)8.5, with the evagination of the two epithelial buds that arise from the foregut endoderm. These buds expand and merge at e12.5, during a stage referred to as the primary transition. At this stage of development, the pancreatic epithelial buds predominantly contain multipotent pancreatic progenitor cells.

These pancreatic progenitor cells will give rise to the tip and trunk domain, marking the first lineage commitment and the secondary transition. The trunk cells will give rise to the endocrine and ductal lineage and the tip will form the acinar cells. During this transition, a population of cells from the trunk will start expressing Neurogenin (Ngn3), which marks the onset of endocrine differentiation; cells that do not express Ngn3 contribute to the ductal fate. The Ngn3-expressing endocrine precursors will undergo dynamic changes in gene expression, independently giving rise to the five endocrine cell types: alpha, beta, delta, epsilon and PP cells (Figure 1-1) (Shih et al., 2013).

Studies in mice have successfully mapped the overlapping of expression patterns of many transcription factors found to be important for pancreas development, and beta cell differentiation and maturation. In humans, limited information exists about the earliest stages of pancreas specification from the foregut endoderm. However, early development of the PDX1 expressing pancreatic buds is comparable to rodents, nevertheless a primary transition stage has not been characterized. During subsequent stages, the transcription factor gene expression pattern between these two species has been found to be very similar. For example, the expression of PDX1, NGN3, NEUROD1 and NKX2.2 has been found to be just as important in human endocrine and beta cell lineage commitment as it is in rodents (Flanagan et al., 2014; Pinney et al., 2011; Piper et al., 2004; Rubio-Cabezas et al., 2010; Stoffers et al., 1997; Thomas et al., 2009; Wang et al., 2006). Interestingly, one particular difference in regard to the timing of expression applies to NKX2.2; which appears to be absent from the progenitor population in humans, and instead appears after the induction of NGN3, at the initial maturation stage (Jennings et al., 2013; Jennings et al., 2015; Lyttle et al., 2008).

An interesting feature found in human islets, was the existence of polyhormonal cells during the majority of the islet developmental process. These polyhormonal cells were observed to resolve over time, establishing the appearance of monohormonal functional cells as an important step in maturation (Bocian-Sobkowska et al., 1999). The specific factors or mechanisms involved in this maturation step as well as in helping maintain a monohormonal identity are still unresolved.

The comparative analysis between mouse and human pancreatic development has provided useful information. However, the limited access to human samples has hampered progress. Alternative approaches to find factors or signals that are involved or can potentially promote beta cell maturation in humans are being developed. In the meantime, significantly valuable information has mostly been acquired from studies performed in rodents.

Stem cells

As discussed above, the current treatments for diabetes are only able to manage symptoms, and haven't yet been able to prevent or reverse the disease. Presently, there are major efforts focused on providing a renewable supply of healthy and functional beta cells. Stem cell research has made great strides in accomplishing this goal. Stem cells represent ideal candidates for generating surrogate beta cells, due to their special capacity to grow almost indefinitely and their ability to give rise to cells from the three germ layers, including pancreatic islets (Thomson et al., 1998; Yu et al., 2007). The first attempts to differentiate human embryonic stem cells into insulin producing cells resulted in the differentiation of a limited number of insulin expressing cells.

Even though this was a significant achievement, the efficiency and secretory capacity of the cells was very limited. Additionally, most of the insulin expressing cells co-expressed other islet endocrine hormones, highlighting their immature state (Assady et al., 2001; Blyszczuk et al., 2003; Segev et al., 2004).

Subsequent studies focused their efforts at improving the differentiation process, mainly by recapitulating the different stages of beta cell development during embryogenesis. Through this approach, the efficiency of the protocol was increased, achieving a greater number of insulin expressing cells, albeit still with limited secretory response (Jiang et al., 2007; Kroon et al., 2008). In 2014, Pagliuca and colleagues (Pagliuca et al., 2014), as well Rezania and colleagues (Rezania et al., 2014) simultaneously optimized the differentiation process, obtaining cells that more closely resembled human beta cells. These cells expressed important markers of beta cell function and identity and were able to secrete insulin at levels that closely resemble human beta cells.

Generation of higher quality beta-like cells represents a milestone in the field, and holds great promise for the development of future therapies, patient disease modeling and drug screening. There are still important limitations to be considered, such as the immune system response to these cells. This concern may possibly be addressed by using the patient's own cells through the differentiation of induced pluripotent stem cells. In the case of T1D, novel encapsulation technologies are being developed to protect the surrogate beta cells from the immune system attack (An et al., 2015; Vegas et al., 2016a; Vegas et al., 2016b)

One caveat of *in vitro* differentiation protocols is their variable efficiency: they tend to yield an estimated 10-30% of cells with beta cell characteristics, a small population of polyhormonal cells and a large population of undifferentiated cells. These results raise important points to consider. First, there appears to be heterogeneity within the population of differentiated cells, which results in one population being more responsive to the signals during the differentiation process. Second, beta cell identity and metabolic maturation has not been completely achieved yet, as compared to human beta cells. These are important issues, not only with regards to the physiological importance of a stable functional identity, but also to prevent the development of teratoma formation. Therefore, it will be essential to acquire more information on the factors and signaling pathways that help induce the final steps of the maturation process and lock beta cells into their identity, so that we can efficiently differentiate insulin-producing beta cells that acquire and retain their function over time and post-transplantation.

Beta cell maturation

The maturation stage represents an important step in the differentiation process of the beta cell, and establishes the cell's glucose sensing ability and fully developed metabolic function. This process has become an area of great interest in the last decade, due to the difficulties associated with differentiating fully functional beta cells from embryonic stem cells (ESCs). Although there has been some success, as described above, the correct and efficient functional maturation of surrogate beta cells has remained elusive(Pagliuca et al., 2014; Rezania et al., 2014).

Functional Maturation

The most important process during the functional maturation of a beta cell is the acquisition of tightly regulated glucose stimulated insulin secretion (GSIS).

Traditionally, GSIS has been defined as the differential insulin secretion response between low (2.8mM) and high (> 10mM) glucose concentrations. Studies in rodents have consistently found that in the early postnatal period, beta cells secrete high basal levels of insulin at low glucose concentrations, and have a blunted insulin response when stimulated with a higher glucose concentration (Blum et al., 2012; Boschero et al., 1988; Freinkel et al., 1984). Furthermore, it has also been observed that beta cells during the neonatal period contain fewer insulin granules compared to mature cells. As beta cells mature, their glucose response evolves such that they secrete basal insulin at low glucose concentrations, and enhance their insulin secretion in response to higher glucose levels. This results in efficient glycemic regulation and prevention of hypoglycemia.

During the early postnatal period, important epigenetic changes in histone methylation have been found to set the stage for subsequent maturation. More specifically, genes involved in the activation of the glycolytic flux at low glucose levels have been found to become highly methylated during this period. They include: Hexokinase 1 (Hk1), hexokinase 2 (Hk2), lactate dehydrogenase A (Ldha), as well as aldolase B fructose-biphosphate (AldoB)(Dhawan et al., 2015). Concomitantly, these enzymes are expressed during development and subsequently become repressed as the cell continues to mature. Repression of this gene program ensures that pyruvate is shuttled into the tricarboxilic acid cycle (TCA) cycle, and therefore couples blood

glucose levels with proper insulin secretion. These enzymes have previously been categorized as islet disallowed genes, which are genes specifically repressed in beta cells compared to other tissues. Accumulating evidence has shown that in general, repression of disallowed genes is necessary for proper beta cell function (Pullen et al., 2010; Thorrez et al., 2011). Furthermore, their negative regulation has been shown to take place through active repression (Thorrez et al., 2011). In this regard, de-novo DNA methyltransferase 3a (DNMT3a) has been found to be responsible for the initiation of methylation of important regulatory regions in these genes (Dhawan et al., 2015). However, DNMT3a expression also decreases overtime, suggesting that active repression of such genes must be enforced by other independent factors or mechanisms.

Transcription factors involved in beta cell development have also been implicated in the acquisition of beta cell maturation. Neurod1, a transcription factor critical for insulin expression and beta cell differentiation, was also found to play an important role during maturation. In support of this, mice devoid of Neurod1 expression have a defective response to glucose, accompanied by the expression of glycolytic genes and Ldha. Furthermore, the beta cell gene expression profile in these mice was found to be comparable to immature neonatal beta cells (Gu et al., 2010). Additional factors involved in beta cell maturation are MafA, a transcription factor that becomes active right after birth, and Tshz1 a recently discovered maturation factor. Deletion of either factor renders mice with significant glucose intolerance due to beta cell maturation defects and defective insulin secretion (Hang et al., 2014; Raum et al., 2015). Although these factors have been found to be important for the establishment of beta cell maturation, more information is needed on the mechanisms employed and the signals that regulate them.

It has recently become more evident that beta cell insulin secretory function continues to develop beyond 3 weeks of age. At this stage, basal insulin secretion is further reduced and insulin secretion response to high glucose concentrations is enhanced. Recent studies have found that maternal weaning represents an important event in furthering such improvements. The transition from a high fat diet to a high carbohydrate diet was found to be the most influential factor(Jacovetti et al., 2015; Stolovich-Rain et al., 2015). Interestingly, additional studies have implicated microRNAs in the maturation of beta cells during such transition. Global expression analysis during this period revealed significant microRNA expression changes. Furthermore, when expression of these microRNAs was mimicked in newborn beta cells, premature GSIS was acquired. However, when these microRNAs were inversely modified in the adult stage, a reversion of GSIS to an immature state was not attained (Jacovetti et al., 2015). This highlights the absolute importance that context and timing have in the maturation process.

There have been important attempts to find markers of maturation that help in the development of beta cell differentiation protocols. In this regard, Ucn3 is one of such markers that becomes highly expressed during the early maturation stage (Blum et al., 2012). Although it cannot induce maturation on its own, it was recently found to be co-secreted with insulin to function as a potentiator of somatostatin release, causing insulin inhibition and better glycemic control (van der Meulen et al., 2015).

Overall, functional maturation appears to be acquired in several consecutive stages. Evidence suggests that factors and signals both in the environment and at the molecular level play very specific and controlled functions in this process.

There is a pressing need for more information that helps identify those factors and their mechanisms. Such information will be very valuable in the optimization of beta cell differentiation protocols, as well as in other alternative therapies.

Beta cell function

Insulin biosynthesis and secretion

The most important functional feature of the beta cell is its ability to secrete insulin in a tightly regulated manner in response to the body's metabolic demands. Insulin is a peptide hormone whose primary function is to regulate the blood glucose levels by promoting glucose uptake in several organs of the body.

Insulin is first translated as pre-proinsulin, and is then transported across the rough endoplasmic reticulum in the lumen where it is cleaved, giving rise to its product proinsulin (Chan et al., 1976; Patzelt et al., 1978). After acquiring three-disulfide bonds that allows it to properly fold, proinsulin moves to the Golgi and into immature secretory granules; where it is cleaved into insulin and C-peptide by the pro-hormone convertases Pcsk1 (PC1) and Pcsk2 (PC2). The end product insulin is then stored in mature secretory granules (Huang and Arvan, 1995; Itoh et al., 1996; Kemmler et al., 1972; Malide et al., 1995)

There are different molecules that can stimulate insulin secretion, such as the glucagon-like-peptide 1 (GLP-1), gastric inhibitory polypeptide (GIP), cholecystokinin (CCK), peptide YY (PYY) and oxyntomodulin. However, the main potentiator for insulin secretion is glucose (Poitout et al., 2006). In rodents, glucose is identified and transported into the beta cell through the Glut2 transporter (McCulloch et al., 2011; Thorens et al., 1988).

Subsequently, glucose is phosphorylated by the rate limiting enzyme glucokinase. This enzyme is a member of the hexokinase family; its low affinity for glucose allows it to continuously control insulin secretion with a threshold that falls within normal glucose levels (Xu et al., 1995). Although glucose-6-phosphate (G6P) can be metabolized through a different mechanism, the predominant route for insulin secretion involves G-6-P incorporation into the glycolytic pathway. The pyruvate end product of glycolysis is then oxidized in the mitochondria through the TCA cycle, resulting in increased cellular ratios of free adenosine triphosphate (ATP) to adenosine diphosphate (ADP) (Kennedy et al., 1999). This process ultimately leads to closure of the K+ (K_{ATP}) channels that results in membrane depolarization (Ashcroft et al., 1984; Cook and Hales, 1984). Voltage gated calcium channels then open to trigger the fusion of insulin granules with the membrane, releasing insulin (Yang and Berggren, 2006).

Maintenance of beta cell identity

Throughout the endocrine differentiation process, a combination of signals and regulatory mechanisms elegantly outline and shape what will become the identity of the functional beta cell. It was previously believed that this process was strictly unidirectional, giving rise to cells with an irreversibly "locked" identity. However, important breakthroughs such as the ability to reprogram differentiated cells into pluripotent stem cells by the introduction of key factors have shown otherwise (Takahashi and Yamanaka, 2006). Therefore, it has become more evident that a sufficient level of plasticity remains in the differentiated cell, and that active regulation must exist in order to maintain its functional identity. Finding the factors and underlying mechanisms that maintain such identity has become a main priority, due to their important implications in the therapies that are currently under development.

Although a big gap of information still exists in this area, several studies have begun to show the implication of developmental transcription factors in the maintenance of adult beta cell identity and function.

Transcription factors

Loss of function studies have started to shed light on the different roles that these factors play in the adult stage of the beta cell, as well as the consequences that result when this regulation is disturbed. For example, one of the main outcomes observed when beta cell identity is lost is the absence of hormone expression.

In this regard, Talchai and colleagues (Talchai et al., 2012) showed that loss FoxO1 during stressful conditions caused loss of insulin expression in a big population of islet beta cells. These cells were found to de-differentiate to a progenitor like state, as shown by the expression of progenitor markers that included: Ngn3, Oct4, Nanog and L-Myc. Furthermore, a portion of these cells were found to express other islet endocrine hormones such as somatostatin, pancreatic polypeptide and glucagon. Interestingly, no co-expression of insulin and these other hormones was found, further supporting that in this case de-differentiation to a progenitor state must happen in order to acquire these other endocrine identities.

Similarly, loss of identity followed by the expression of other islet endocrine hormones such as glucagon or somatostatin has also been found after the loss of Grg3, Nkx6.1 and Pdx1(Gao et al., 2014; Metzger et al., 2014; Taylor et al., 2013). However, unlike the FoxO1 mutant beta cells, these cells did not regress to a progenitor state, as shown by the absence of progenitor markers.

Instead this cells co-expressed insulin with either glucagon or somatostatin, therefore exhibiting a partial trans-differentiation. These findings highlight the versatility of the beta cell, capable of responding in different ways according to the different circumstances.
Although these cells were able to co-express these other hormones, it is unknown whether those new identities were completely functional.

Another important outcome that commonly occurs when beta cell identity is perturbed is the up-regulation of the disallowed genes. *Ldha, AldoB, Hk1, Hk2* and *Pcx* are some of the genes that most frequently become dysregulated. Due to their inherent characteristics and functions, their up-regulation is translated into a dysregulated GSIS response that in some cases resembles the one found in immature beta cells. This particular response has consistently been found in loss of function studies of Neurod1, MafA, Insm1 and Rfx6 (Gu et al., 2010; Jia et al., 2015; Nishimura et al., 2015; Piccand et al., 2014). Of these factors, Rfx6 was shown to be a critical regulator of this gene program. Loss of this transcription factor resulted in the significant up-regulation of 54 of 68 disallowed genes (Piccand et al., 2014).

Ultimately, loss of functional identity results in glucose intolerance and rapid progression to diabetes. These findings show that functional features acquired during the maturation stage, such as the repression of disallowed genes and of other alternative identities, also have to be actively maintained during the adult stage. Interestingly, even though the aforementioned transcription factors have shown both specific and independent functions; the ablation of their expression consistently results in the dysregulation of overlapping targets. This overlap can be explained by the formation of transcription factor complexes, where different combinations of these factors

serve to regulate specific gene programs. Evidence for this combinatorial network formation was brought by Pasquali and colleagues (Pasquali et al., 2014) through a detailed analysis of mapped chromatin states, DNA binding sites of key beta cell transcription factors, as well as gene expression profiles specific for human islets. Remarkably, it was shown that this novel regulation of islet specific gene expression by clusters of transcription factors was done through enhancer binding. In this regard, NKX2.2, PDX1, NKX6.1, MAFB and FOXA2 were found to directly bind these enhancer regions. Additionally, these enhancers were enriched for motifs that are recognized by HNF1a, RFX, FOXA, NEUROD and MAFA.

Further confirmation of this kind of network regulation was recently provided by a loss of function study of Insm1 in mouse beta cells (Jia et al., 2015). Loss of this factor resulted in beta cells reversing to an immature state, with essential beta cell function genes becoming dysregulated. Most importantly, regulatory regions associated with such targets genes were found to be cooperatively bound by Insm1, Neurod1 and Foxa2.

Epigenetic regulatory mechanisms

The mechanisms employed by transcription factors to induce and maintain beta cell identity are still largely unknown. However, it has become more evident that such factors can interact with specific chromatin regulators in order to influence the epigenetic landscape and ultimately regulate gene expression. Recent findings have demonstrated the importance that chromatin modifications play in the development and acquisition of functional identity in beta cells. In this regard, it was shown that prior to lineage induction, pioneer factors such as FOXA1 and FOXA2 open the chromatin and bind to enhancers that then become poised by acquiring the H3k4me chromatin mark.

This priming of enhancers allows subsequent binding of lineage determinant factors in a stepwise manner (Wang et al., 2015). Ultimately, these epigenetic modifications pave the way for the acquisition of different endocrine lineages.

Several of these epigenetic marks have been intensely studied in the beta cell, which is the case of H3k27me3; a chromatin mark associated with repression. Arensberguen and colleagues (van Arensbergen et al., 2010) found that H3k27me3 arises de novo during beta cell differentiation in a very tissue specific manner. It was shown to repress regulators of inappropriate cell fates such as neural, skeletal and heart development. Additionally, it actively repressed genes that were detrimental for proper beta cell function during the adult stage, therefore maintaining its functional identity. In support of these findings, several disallowed genes that are actively repressed during adulthood are also marked by H3k27me3 (Thorrez et al., 2011). Furthermore, Pasquali and colleagues (Pasquali et al., 2014) found that many of the enhancers associated with beta cell function and identity, posses not only repression (H3k27me3) chromatin marks but also marks associated with activation (H3k27Ac) and priming (H3k4me).

Disruption of these chromatin marks has been shown to have very deleterious effects in gene programs important for beta cell identity. In support of this, histone deacetylase (HDAC) inhibitors caused dysregulation of the insulin secretion pathway when applied to BTC3 cells. Interestingly, two classes of HDAC inhibitors were used in this study. The first class made of hydroxamic acids, which inhibited HDAC1, HDAC2, HDAC3, HDAC6 and HDAC8 caused the down-regulation of important beta cell regulators that included Nkx6.1, Nkx2.2, Pdx1 and Neurod1. The second class, composed of orthoamino anilides, which inhibits HDAC1, HDAC2 and HDAC3, resulted in the increase of alpha cell regulators such as MafB and Arx.

In turn, this resulted in the significant up-regulation of glucagon expression in these cells (Kubicek et al., 2012).

Overall, it is becoming more evident that epigenetic marks play a very important role in preserving the functional identity of beta cells. During the differentiation process, it is likely that epigenetic marks are involved in the assembly of transcription factors complexes but also in their reinforcement too. This can be inferred from the different outcomes obtained when transcription factors are deleted at different stages of life. It is possible that the subsequent activation of other factors and further conformational rearrangement of such complexes renders their original function in them, redundant at a later stage.

Nevertheless, it has become clear that during the adult stage, epigenetic chromatin marks still need to be actively maintained in order to preserve beta cell identity and function. It relation to this, several transcription factors can interact with chromatin remodeler complexes and regulate gene expression. A recent study found Pdx1 to interact with the Swi/Snf chromatin remodeler. Interestingly, the activation or repression activities of this factor were mediated by its core components, Brg1 and Brm respectively. Binding to either one of these components was influenced by environmental signals, allowing for a dual and dynamic response (McKenna et al., 2015). Even though such kinds of interactions have started to be found for other transcription factors such as Isl1 and Nkx2.2, more information is needed to understand this kind of regulation (Papizan et al., 2011; Wang et al., 2016).

Loss of identity in beta cells, apart from causing disturbed functional features can also initiates events such as dedifferentiation or even direct reprogramming to other different identities. This ability to acquire other identities when a transcription factor is lost raises two important points, the inherent plasticity still conserved in the adult stage, and the existence of factors that guard the identity of the beta cell by tightly regulating such plasticity. Furthermore, the identities acquired by beta cells commonly pertain to the islet endocrine lineage. Bramswig and colleagues (Bramswig et al., 2013) found that in humans, a large fraction of genes important for beta cell function and identity were bivalently marked (H3k4me3+/H3k27me3+) in the adult stage in other cell pancreatic types, particularly alpha cells. These surprising results show that plasticity exists in the endocrine islet populations, and the similarities in the chromatin landscape allows them to interconvert under certain circumstances. Understanding how these factors and their regulation of the epigenetic landscape influences these events will be of great importance in the development of future diabetes therapies.

Loss of beta cell identity during disease

Loss of beta cell identity during disease conditions has been found in mouse models of diabetes, including the insulin resistant GIRKO and the db/db mice. Here, beta cell loss of identity leads to dedifferentiation, and it is accompanied by a decrease in beta cell markers that include MafA, Nkx6.1 and Pdx1 (Guo et al., 2013; Talchai et al., 2012). The extent of beta cell de-differentiation is positively correlated with the degree of hyperglycemia.

In humans, evidence has been found for the loss of beta cell identity in T1D and T2D patients. Novel findings have shown that during T1D, in addition to beta cell apoptosis due to the immune

system attack, beta cells can undergo a process of neogenesis from alpha cells, which is then followed by trans-differentiation into delta cells. Here, T1D patients show an increase in delta cells that correlates as a direct function with the duration of the disease. Cells co-expressing insulin with somatostatin as well as insulin with glucagon were consistently found. Additionally, cells co-expressing insulin and somatostatin were found to express NKX6.1, further supporting that trans-differentiation between the beta and delta cell lineage was taking place. Interestingly, the amount of co-expressing cells varied greatly between patients, with some patients presenting islets where all the insulin expressing cells co-expressed somatostatin (Piran et al., 2014). More patient samples will be needed to assess the frequency of this event in this population, and most importantly the signals that influence those changes in cell identity.

Loss of beta cell identity in T2D has also been confirmed in several studies. Islets from T2D patients present a significant increase population of cells that contain no endocrine hormone expression. Due to the fact that these cells contain mislocalized expression of important beta cell factors such as NKX6.1, FOXO1 and MAFA, it is likely that these cells were once beta cells that have now lost their identity. Interestingly, co-expression of NKX6.1 and somatostatin was found in a population of cells in these patients. These findings could potentially imply that these cells have not only lost their identity but have also gained a different endocrine identity. It is unlikely that this population of cells is functional; nonetheless that remains to be confirmed.

In contrasts to findings from diabetes mouse models, no expression of progenitor markers has been found so far in these cells, which could be the result of mechanistic differences between these two different species (Guo et al., 2013; Talchai et al., 2012).

Lastly, beta cell partial trans-differentiation has also been observed in these patients, with cells co-expressing insulin-glucagon as well as insulin-somatostatin (White et al., 2013; Yoneda et al., 2013).

Overall, these studies demonstrate the critical role that beta cell identity plays during pathophysiological conditions. Whether it has a causative role in the development of diabetes, and or influences its progression remains to be determined. Furthermore, the nature of the signals, and how they render beta cell transcription factors dysfunctional and unable to maintain its identity is still unresolved. In this thesis work, I will focus on a transcription factor named Nkx2.2, which I have found to be essential for the maturation and maintenance of beta cell identity and function; and whose function points to be conserved in mice and humans.

Nkx2.2

Nkx2.2 is part of the NK2 homeodomain transcription factor gene family that was first identified in Drosophila. This factor contains 273 aminoacids and contains three protein domains: the tinman domain (TN), the homeodomain (HD) and the NK2 specific domain (SD). All of these domains posses conserved homology, and among them the homeodomain is responsible for binding DNA (Kim and Nirenberg, 1989). There are five other members in the NK2 family that have been identified in mice: Nkx2.1, Nkx2.3, Nkx2.4, Nkx2.5 and Nkx2.6.

Several of them have been shown to have important functions in the specification and regulation of cell fate decisions in a variety of organs (Caprioli et al., 2011; Czompoly et al., 2011; Du et al., 2008).

Nkx2.2 is found in the intestine, the central nervous system and the pancreas (Arnes et al., 2012). Within the pancreas, Nkx2.2 is first expressed at the onset of pancreatic bud evagination, becoming overtime more restricted to the core in the endocrine precursors. Finally, in the mature islet, it is expressed in all of the beta cells, in the majority of the alpha and PP cells, and remains absent from the somatostatin expressing delta cells (Arnes et al., 2012; Sussel et al., 1998).

In order to analyze Nkx2.2 developmental function within the pancreas, Sussel and colleagues (Sussel et al., 1998) deleted this factor in mice. Loss of Nkx2.2 caused overt hyperglycemia, resulting in mice dying shortly after birth. At the cellular level, deletion of this factor resulted in an ablation of the beta cell population, with an accompanying decrease in the alpha and PP cell populations. Interestingly, a population of cells that did not express any insulin, but that contained some beta cell markers remained. It was proposed at the time that loss of Nkx2.2 had arrested the beta cell population in an undifferentiated state. However, in a later study Prado and colleagues (Prado et al., 2004) found that this cell population was in fact expressing ghrelin; a hormone associated with the regulation of food intake, which is expressed in several tissues including the brain and the stomach (Klok et al., 2007). Therefore, loss of Nkx2.2 prompted an increase of the ghrelin cell population at the expense of the beta cell population. Collectively, these findings showed that Nkx2.2 plays a critical part during pancreas development, and more specifically in specifying the beta cell population.

In humans, it was recently found that NKX2.2 plays a comparatively important function during development. Patients with homozygous null mutations of NKX2.2 presented with very similar

phenotypes as the ones observed in the Nkx2.2 null mice. These patients had defects in insulin secretion, diabetes at an early age with no evident exocrine dysfunction, as well as affected motor and intellectual functions (Flanagan et al., 2014). The similarity in the phenotypes of both of these species suggests a conservation of function of this factor during development.

To address the mechanism behind the function of Nkx2.2 in the developing pancreas, Doyle and colleagues used an Nkx2.2 dominant activator or repressor derivative under the Pdx1 promoter as a substitution for endogenous Nkx2.2 in the mouse developing islets. Although no significant effect was found with the Nkx2.2-activator derivative, the Nkx2.2-repressor was able to partially rescue the Nkx2.2-null phenotype previously observed in the pancreas. There was a full recovery of the alpha cell population, a decrease in the ghrelin population, and a recovery of a small number of beta cells. Although these cells expressed insulin, they did not express Glut2 and MafA, which are important beta cell markers acquired during the maturation stage (Doyle et al., 2007). These results showed that Nkx2.2 repressor activity during differentiation serves to specify the alpha cell population, and to suppress the ghrelin lineage. However, its inability to fully rescue the beta cell population, and activate maturation markers pointed to a possible activator function during the maturation and adult stage.

The activator function of Nkx2.2 and its ability to regulate the mature beta cell was tested using a transgenic mouse line that expressed the Nkx2.2-repressor in the presence of endogenous Nkx2.2. These mice became severely diabetic at weaning and presented with disrupted islet architecture, a decrease in maturation factors, and a dysregulated beta cell function (Doyle and Sussel, 2007).

These important findings showed that Nkx2.2 is likely to function both as repressor and as an activator, and is required not only during development but also during the adult stage of the beta cell. This dual function has been observed in the drosophila orthologue of Nkx2.2, where the nature of its molecular interactions allows it to function as an activator or a repressor (Yu et al., 2005). Furthermore, Papizan and colleagues (Papizan et al., 2011) found that Nkx2.2, through its TN domain can interact with factors such as DNMT3a, Grg3 and HDAC1 and form a repressive complex that silences the Arx promoter. Interestingly, mutations in this domain not only perturb beta cell differentiation but also cause beta to alpha cell trans-differentiation.

Overall, these results highlight the essential function that Nkx2.2 plays during beta cell development, and provide evidence for a possible differential function of equal importance in the maturation and maintenance of the adult beta cell identity. This function had not been able to be explored due to the lethality of the Nkx2.2-null phenotype. However, using an Nkx2.2 conditional allele (Mastracci et al., 2013), I have deleted Nkx2.2 specifically in beta cells during the maturation stage as well as during the adult stage. This thesis work aims to address the requirement of Nkx2.2 function once the beta cell has differentiated. Furthermore, it will explore the regulatory mechanisms behind this function and the main targets involved.

In humans, it has been shown that NKX2.2 binds to enhancers associated with beta cell specific gene targets; such genes have been associated with islet cell identity and function.
Additionally, it has also been revealed that enhancers bound by NKX2.2 in these cells, contain single nucleotide polymorphisms associated with T2D (Pasquali et al., 2014).

To evaluate the possible involvement of NKX2.2 in the human adult beta cell, I deleted this factor using a sh-NKX2.2 adenovirus and assessed the expression changes of its aforementioned targets. This thesis will present evidence for a conservation of function in the maintenance of beta cell functional identity in both mice and humans.

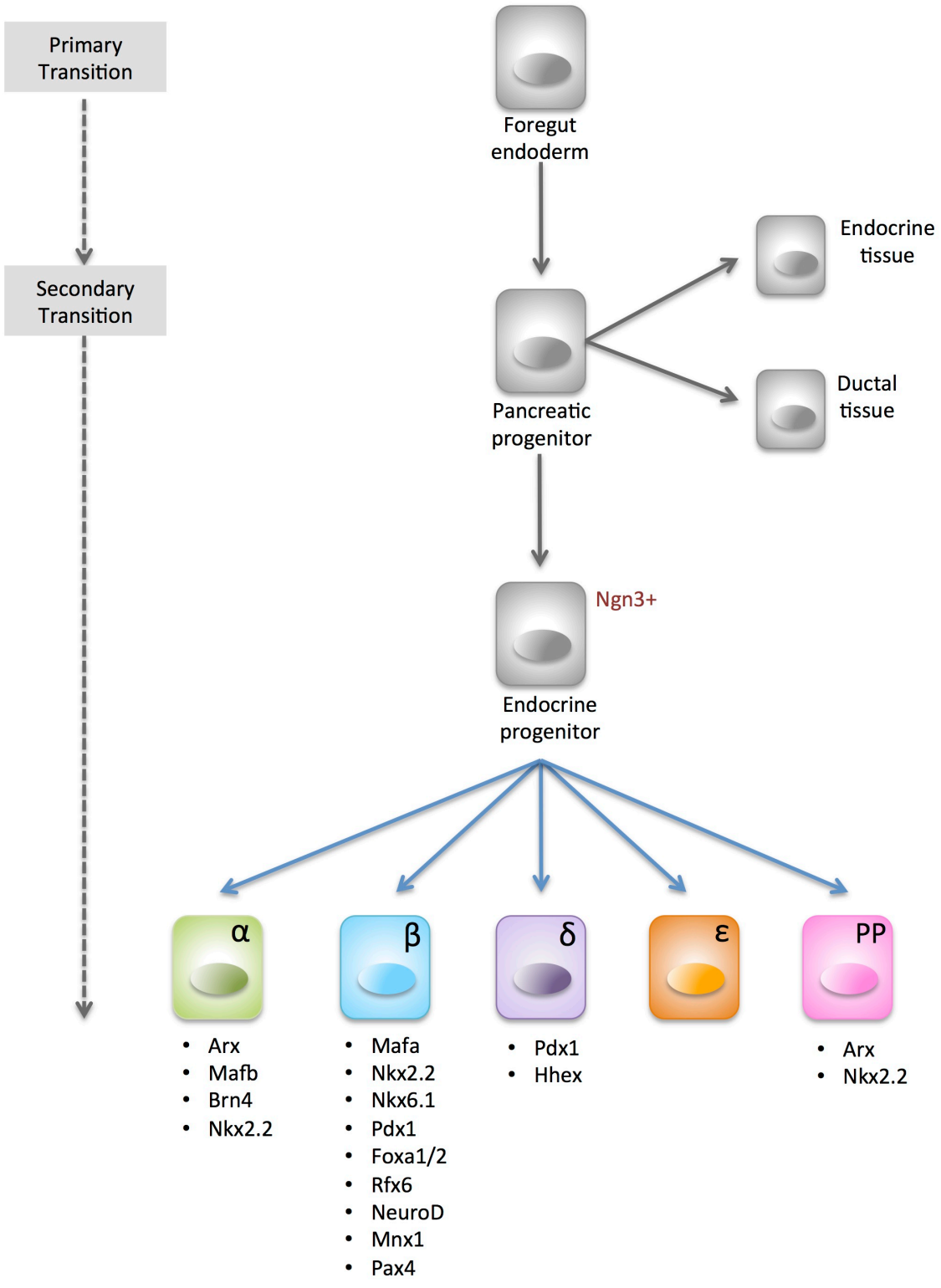


Figure 1-1: Pancreatic Endocrine Development. Figure adapted from (Shih et al., 2013)

Chapter 2

Maintenance of pancreatic beta cell identity requires active repression of non-beta cell endocrine genes

Lori Sussel conceived the mouse study. Giselle Dominguez Gutierrez and Teresa L. Mastracci collected and analyzed the mouse data. Giselle Dominguez Gutierrez conceived and executed the human islet study. Aaron Bender assisted with the human islet studies. Vincenzo Cirulli performed the EM studies and assisted with their interpretation. Aristotelis Tsirigos assisted with the computational analysis of ChIP-Seq and RNA-Seq data. Klaus H. Kaestner provided the raw data for the mouse chromatin marks for comparative analysis. Lori Sussel and Giselle Dominguez Gutierrez assembled and analyzed the data and wrote the manuscript.

Introduction

Type 1 and Type 2 diabetes mellitus (T1D and T2D) are chronic conditions in which glycemic control becomes severely dysregulated. Although there are many causes of diabetes, one of the main contributors to the progression of disease is the loss of beta cell function and beta cell mass, which ultimately leads to an inability to meet metabolic demand (Prentki and Nolan, 2006). Recently, Talchai et al. (2012) proposed that loss of beta cell identity rather than beta cell death accounted for a significant portion of the beta cell loss reported during diabetes progression. This landmark study raised the possibility that permanent regulatory mechanisms must be sustained to maintain the fully differentiated functional state of the beta cell.

Therefore, understanding the mechanisms required to actively maintain beta cell identity during adverse metabolic conditions that could lead to diabetes will be essential for the development of interventions and/or therapies to treat the disease.

Studies from many labs have identified the extrinsic signaling pathways and intrinsic transcriptional networks needed to generate functional insulin producing beta cells *in vitro* and *in vivo* (reviewed in (Pan and Wright, 2011). More recently, there has been increasing evidence that several of the developmental regulatory factors that are essential for endocrine lineage specification, including Nkx6.1, NeuroD1 and Rfx6, are also required for the maintenance of beta cell function in the adult (Schaffer et al., 2013); (Taylor et al., 2013); (Naya et al., 1997);(Gu et al., 2010);(Smith et al., 2010);(Piccand et al., 2014). This suggests that many of the transcriptional regulatory networks that are necessary for the initial specification of beta cells may continue to be expressed in the adult beta cell to actively maintain beta cell identity and function.

Nkx2.2 is a highly conserved homeobox transcription factor that has been shown to be a critical regulator of cell fate decisions in the pancreas, intestine and central nervous system (Sussel et al., 1998); (Prado et al., 2004); (Desai et al., 2008);(Anderson et al., 2009);(Papizan et al., 2011);(Mastracci et al., 2011);(Clark et al., 2014);(Zhu et al., 2014). During embryogenesis, Nkx2.2 is expressed throughout the developing pancreatic epithelium and gradually becomes restricted to the Neurogenin3 (Ngn3)–expressing endocrine progenitor population, and subsequently to the alpha, beta, PP and epsilon cell lineages (Arnes et al., 2012). Within the adult islet, Nkx2.2 expression is maintained in the alpha, beta and PP cells.

Global deletion of *Nkx2.2* in mice demonstrated that Nkx2.2 is essential for endocrine lineage specification; deletion of *Nkx2.2* resulted in decreased formation of alpha and PP cells, as well as a complete abrogation of beta cell specification, leading to severe hyperglycemia and neonatal lethality (Prado et al., 2004);(Sussel et al., 1998). In humans, loss of function mutations in *NKX2.2* also resulted in the development of permanent neonatal diabetes, suggesting that NKX2.2 is also important for beta cell formation during human fetal development (Flanagan et al., 2014).

Although these studies demonstrated a critical role for Nkx2.2 in beta cell development in mice and humans, the complete absence of beta cells precluded assessment of its function in adult islets. To determine whether Nkx2.2 is necessary for beta cell maturation and/or function, we generated mouse models that allowed constitutive and inducible deletion of the *Nkx2.2* gene. Disruption of *Nkx2.2* in maturing beta cells resulted in the rapid development of diabetes, with a significant decrease in insulin expression and content. Strikingly, the loss of genes associated with beta cell identity and function was accompanied by an increase in the expression of genes from alternative islet cell fates. Furthermore, beta cells appeared to trans-differentiate to acquire other non-beta cell endocrine identities. Deletion of *Nkx2.2* in fully differentiated adult beta cells also resulted in the very rapid onset of diabetes and the islets of these mice were also characterized by a loss of beta cell identity and the acquisition of alternative endocrine cell characteristics, confirming the importance of Nkx2.2 in both establishing and maintaining beta cell identity. These results demonstrate that Nkx2.2 is a master regulator that plays a critical role in mouse and human beta cells to maintain beta cell identity and function in the adult by directly

activating beta cell genes and preventing reprogramming by actively repressing non beta cell endocrine genes.

Materials and Methods

Mouse Strains

Mice carrying a conditional allele of *Nkx2.2*$^{fl/fl}$ (Mastracci et al., 2013) were bred to the *RIP:Cre* mice (Herrera, 2000) and to the tamoxifen inducible *MIP-CreERT* mice (Wicksteed et al., 2010). To induce the *MIP-CreERT* allele, tamoxifen (Sigma) was dissolved in corn oil, and a dose of 100mg/kg body weight was given via intraperitoneal injection every other day for 3 days. All mice used in physiologic tests were male, unless otherwise stated in the text.

Tissue Preparation and Immunohistochemistry

Mouse pancreata was fixed for either 2 hours or overnight in 10% neutral-buffered formalin (VWR) at 4°C. Tissue was then transferred to 70% ethanol and subsequently embedded in paraffin and sectioned at 5 μm thickness. Antigen retrieval was performed using 10mM sodium citrate buffer pH=6 at boiling temperature in a water bath for 15 min. After a 20 min cool down period at room temperature, samples were incubated with primary antibodies overnight and further stained with secondary antibodies the following day. Details of primary and secondary antibodies utilized are listed in Tables 2-1 and 2-2 respectively.

Transmitted Electron Microscopy (TEM)

Ultra structural analysis of wild type and *Nkx2.2* mutant pancreatic islets was performed by transmitted electron microscopy (TEM), as previously described with minor modifications (Miller et al., 2008; Diaferia et al., 2013). Briefly, islets were fixed immediately after isolation at 4°C in 0.1M sodium cacodylate buffer pH=7.4, 2% paraformaldehyde, 2.5% glutaraldehyde

(Electron Microscopy Sciences (Fort Washington, PA), and 3 μM CaCl2. Samples were then post-fixed with osmium tetraoxide (1% (w/v) in H2O) and counterstained with uranyl acetate (2% (w/v) in H_2O). Following gradual dehydration in ethanol, samples were embedded in Durcupan resin (Sigma Immunochemicals) and polymerized overnight at 60°C and –20mm Hg, as described (Staubli, 1963). Ultrathin sections (70 nm) were then cut using a 35° angle Diatome diamond knife, and mounted on 300 mesh gold grids (Electron Microscopy Sciences, Fort Washington, PA). Following counterstaining with uranyl acetate (1% (w/v) in H2O) and Sato lead [1% (w/v) in H2O] sections were imaged at 80 keV using an electron microscope (JEOL JEM-1400, Akashima, Japan), equipped with a Gatan Ultrascan 1000XP with 2K x 2K resolution CCD camera, using the image acquisition software Gatan Digital Micrograph (Gatan, Inc. Pleasanton, CA).

Chromatin Immunoprecipitation and Sequencing

MIN6 cells were grown to confluency in a 15 cm culture dish and were formaldehyde-cross-linked for 10 min. Cross-linked chromatin was fragmented by sonication using the Diagenode BioRuptor for 3 cycles of 5min each (30 sec on/off). Two micrograms of rabbit anti-Nkx2.2 antiserum was added to 80 μg of sheared chromatin. The antibody/chromatin complex was left to rotate end-to-end overnight at 4°C, and subsequently pulled with protein G Dynabeads (Fisher). Chromatin was washed, eluted and reversed cross-linked, followed by protease treatment.

ChIP-seq libraries were prepared as per instructions of the Kapa Hyper Prep kit for Illumina. Sequencing was performed using the Illumina HiSeq 2500 system as per manufacture's instructions.

Peak annotation was performed using the MACS peak caller (version 1.4.2 20120305). The q-value cutoff we used was 0.05. Binding peaks were annotated with genes with a transcription start site present within 100kb of an identified Nkx2.2 binding peak. *De novo* motif analysis was performed with Hypergeometric Optimization of Motif EnRichment (HOMER v4.6). GEO Accession number# GSE79725.

Western Blot Analysis

Pancreatic islets were isolated out from approximately 21week old mice. Protein was extracted using RIPA (Radio-Immunoprecipitation Assay) buffer during a 5 min sonication period (30sec on/off). 13μg of protein were used for analysis as previously described (Borok et al; 2015). Primary and secondary antibodies used are listed in Table 2-1 and 2-2 respectively.

Glucose Tolerance Test, Plasma Insulin and Insulin Content

Glucose tolerance tests: Mice were fasted overnight, followed by an intraperitoneal injection with glucose (2mg/g body weight). Tail vein blood samples were collected at several time-points after the injection. Glucose concentration was determined using the Accu-Chek Compact Plus Blood Glucose Meter.

Acute plasma insulin assays: Mice were fasted overnight, followed by intraperitoneal injections with glucose (2mg/g body weight). Blood plasma samples were collected at different time-points after the injection through heart puncture, and individual cohorts were used per time-point. Plasma insulin was measured by ultrasensitive insulin ELISA kit (Crystal Chem).

Pancreatic insulin content: pancreata was dissected and disrupted using a homogenizer. Insulin was extracted by acid ethanol. The insulin concentration was analyzed by insulin ELISA (Mercodia) and normalized to pancreas weight (gm).

Islet Isolation and Glucose Stimulated Insulin Secretion Assays (GSIS)

Mouse pancreatic islets were isolated by perfusing the pancreata with Collagenase P (Roche) through the common hepatic bile duct at a concentration of 1mg/ml of M199 medium (Invitrogen). Pancreata was removed and dissociated at 37°C for 16 min. After several washes using M199 medium supplemented with 10% FBS (Gemini Bio Products), islets were separated onto a gradient using a combination of serum free M199 medium and Histopaque (Sigma). Islets were then handpicked to avoid exocrine contamination and processed for different applications.

Human islets from 3 donors were obtained through the NIH-supported Integrated Islet Distribution Program (IIDP) via Mt Sinai Human Islet Core facility. The islets were harvested from deceased donors without any identifying information at NIH-approved centers with informed consent and IRB approval at the islet-isolation centers. Donors ranged in age from 32-64 years old (mean 45.7); two were females and one was a male. Mean BMI was 28.03 (range 23-32.8).

GSIS assays: 20 islets/well were cultured in Kreb's buffer with a glucose concentration of 2.8mM for one hour in a 12 well plate, supernatant was collected to measure basal insulin secretion. Islets were then transferred to Kreb's buffer with 16.7mM glucose, and the supernantant was collected after one hour.

Islets were disrupted using a homogenizer in 50 ul of lysis buffer (150mM NaCl, 50mM Tris-HCL [pH8.0], 1% IGEPAL), and insulin content was extracted by acid ethanol. Insulin in supernatant and islet lysates was measured by ultrasensitive insulin ELISA (CrystalChem). Secreted insulin was calculated as percentage of total insulin content per hour.

Quantitative Real Time RT-PCR and RNA-Seq

Total mRNA was prepared from P0 pancreata or isolated islets at 4 weeks of age using the RNAEasy micro kit (Qiagen) and reverse transcribed with Superscript (Roche). Real time RT-PCR was carried out with either SYBR Green (Bio-Rad) or Taqman probes (Applied Biosystems) and measured on a BioRad CFX96 Real Time System. Expression levels were normalized to *Cyclophilin b* for mouse samples and to *36B4* for human samples. Primers and probes are listed in tables 2-3 and 2-4 respectively.

For RNA-Seq analysis, RNA from mouse and human islets was obtained using the RNEasy micro kit (Qiagen). RNA quantity and quality was assessed using the Agilent 2100 Bioanalyzer and 400ng of total RNA were sent to the Columbia University Genome Center for library preparation, sequencing (Illumina 2500) and bioinformatics analysis. Expression changes were considered significant if they had a p value of at least 0.05 and a log2 fold change of at least 0.5. GEO Accession number# GSE79725.

Adenovirus

Ad.shRNA directed against human Nkx2.2, targeting GCCGACGAGTCACCGGACAA was obtained from Welgen Inc.

Ad.Scrambled was provided by Dr. Andrew Stewart (Icahn School of Medicine at Mount Sinai). Adenovirus were packaged and produced in HEK-293A cells. Titers were determined by plaque assay (PFU). Dispersed human islets were transduced with either experimental or control (Ad.Scrambled) at 100 and 300 MOI in serum free media for 2 hrs. Transduction was terminated by adding complete medium containing 10% FCS and cultured for 96 hrs.

Cell Lines

The adenovirus packaging cell line, HEK-293A (Life Technologies), was cultured in DMEM medium supplemented with 10% FCS, and 1% penicillin- streptomycin, and 1X MEM containing non-essential amino acids. The MIN6 cell line was cultured in DMEM medium supplemented with 10%FBS and 1% penicillin- streptomycin.

Morphometric analysis

For all morphometric analysis, quantification was done on 6 evenly spaced sections through the whole pancreas. To determine beta cell mass and islet number, tiles of each of these sections were taken at 10X using a Leica fluorescent microscope (DM5500). To calculate beta cell mass, each section was stained with insulin (beta cell area) and amylase (pancreas area). Islet area was divided by the pancreas area and then multiplied by the weight of the pancreas (gms). To determine the islet number, all islets were quantified in each section and then divided by the pancreas area (cm^2). To determine the amount of insulin cells per islet area as well as the percent of polyhormonal cells, images of 5 islets per section in all 6 levels were captured at 40X on a Zeiss Confocal LSM 710 microscope.

Percent of polyhormonal cells was calculated by dividing the insulin positive cells by the cells co-positive for insulin and either somatostatin, glucagon or pancreatic polypeptide and multiplied by 100. To determine the insulin cells per islet area, insulin positive cells were counted and divided by the islet area. At least 3000 cells were counted to calculate insulin cells per islet area, and at least 3500 for the percent of polyhormonal cells. All areas and cell quantification were processed with Image J 1.46R software

Image Analysis

All immunofluorescent images were processed using Adobe Photoshop CS5.1 and ImageJ 1.46R.

Statistical analysis

Statistical analysis was calculated using a 2-tailed Student's unpaired t test. Results are expressed as means ± SEM and a $p \leq 0.05$ was considered significant.

Primary Antibodies				
Antigen	***Host***	***Dilution***	***Source***	***Catalogue #***
Glucagon	Goat	1:200	Santa Cruz	sc-7780
Insulin	Guinea Pig	1:1000	Dako	A0564
Nkx2.2	Rabbit	1:200	Sigma	hpa003468
Pancreatic Polypeptide	Goat	1:150	Sigma	SAB2500747
RFP	Rabbit	1:1000	Rockland Immunochemicals	600-401-379
Somatostatin	Rat	1:500	Abcam	ab30788
Cleaved Caspase 3	Rabbit	1:500	Cell Signaling	9661

Table 2-1: List of primary antibodies used in western blot analysis and immunohistochemistry

Secondary Antibodies			
Antigen	***Conjugation***	***Dilution***	***Source***
Guinea Pig/Rabbit	Alexa-488	1:500	Jackson Immunoresearch
Rabbit	Cy3	1:500	Jackson Immunoresearch
Goat/ Guinea Pig/ Rat	Alexa-647	1:500	Jackson Immunoresearch
Rabbit	HRP	1:10,000	Jackson Immunoresearch

Table 2-2: List of secondary antibodies used in western blot analysis and immunohistochemistry

Gene	AOD
Insulin 2	Mm00731595_gh
Slc2a2	Mm00446229_m1
Nkx6.1	Mm00454962_m1
Somatostatin	Mm00436671_m1

Gene	Sybr green FWD	Sybr green REV
Hhex	TCAGAATCGCCGAGCTAAAT	CTGTCCAACGCATCCTTTTT

Gene	Probe	FWD	REV
Nkx2.2	CCATTGACTCTGCCCCATCGCTCT	CCTCCCCGAGTGGCAGAT	GAGTTCTATCCTCTCCAAAAGTTCAAA
Cyclophilin B	TGGTACGGAAGGTGGAG	GCAAAGTTCTAGAGGGCAGGA	CCCGGCTGTCTGTCTGGT

Table 2-3: List of primer sequences, probes and AODs used for qRT-PCR analysis in mouse samples

Gene	AOD
NKX2.2	Hs00159616_m1

Gene	Sybr green FWD	Sybr green REV
36B4	GGCGACCTGGAAGTCCAACT	CCATCAGCACCACAGCCTTC

Table 2-4: List of primer sequences and AODs used for qRT-PCR analysis in human samples

Results

Deletion of *Nkx2.2* in beta cells causes glucose intolerance and impaired insulin secretion

To determine the function of Nkx2.2 specifically in beta cells, we generated *RIP:Cre*; *Nkx2.2*$^{fl/fl}$ (hereafter referred to as Nkx2.2$^{\Delta Beta}$) mice (Herrera, 2000) (Mastracci et al., 2013). For the metabolic studies, we included cohorts of mice carrying each single allele as controls to ensure these individual mutations were not contributing to the observed metabolic defects (Figure 2-1). Nkx2.2$^{\Delta Beta}$ mice displayed efficient deletion of *Nkx2.2*; greater than 80% of the *Nkx2.2* RNA transcript was deleted in the insulin-producing cells of four week old Nkx2.2$^{\Delta Beta}$ mice versus littermate controls (Figure 2-1A). Western blot analysis confirmed there was a corresponding reduction of Nkx2.2 protein (Figure 2-1B). Since Nkx2.2 is also expressed in several islet endocrine cell populations, we used co-immunofluorescence staining for Nkx2.2 and insulin in adult islets to demonstrate the efficient recombination of Nkx2.2 specifically in the beta cell lineage (Figures 2-1C-D).

In contrast to the *Nkx2.2* null mice, which develop severe hyperglycemia and die shortly after birth (Sussel et al., 1998), both female and male Nkx2.2$^{\Delta Beta}$ mice survive into adulthood. The adult mice appear overtly indistinguishable from their littermate controls (data not shown) and exhibit no apparent difference in body weight (Figure 2-2A-B). However, by three weeks of age, the male Nkx2.2$^{\Delta Beta}$ mice displayed significantly higher blood glucose levels than controls. The hyperglycemic phenotype became exacerbated with age, reaching mean glucose levels of 339.3 mg/dL at 11 weeks (Figure 2-1E). Fasting blood glucose levels in the Nkx2.2$^{\Delta Beta}$ mice was also significantly elevated (≥ 217.3 mg/dL) at 11 weeks of age (Figure 2-1F), indicating their progression to diabetes.

Consistent with the elevated *ad libitum* and fasting blood glucose levels, three week old Nkx2.2$^{\Delta Beta}$ mice were unable to effectively clear a glucose bolus (Figure 2-1G). The metabolic impairment worsened with age, suggesting there was a progressive loss of beta cell function in Nkx2.2$^{\Delta Beta}$ mice (Figure 2-1H). Similar phenotypes were observed in female Nkx2.2$^{\Delta Beta}$ mice, however, hyperglycemia and glucose intolerance were manifested at slightly older ages and with less intensity (Figure 2-2C-D). To eliminate the possibility that the observed glucose intolerance was due to an inability of the peripheral tissues to take up glucose, we performed an insulin tolerance test at 3 weeks of age. As expected for a beta cell-specific genetic modification, no difference in insulin resistance was detected between Nkx2.2$^{\Delta Beta}$ mice and controls (Figure 2-2E).

Nkx2.2$^{\Delta Beta}$ mice display reduced numbers of insulin-producing cells

Consistent with previous studies demonstrating that Nkx2.2 directly regulates insulin expression (Cissell et al., 2003), beta cell deletion of Nkx2.2 resulted in a significant reduction of *insulin* gene expression and total insulin content in four week old Nkx2.2$^{\Delta Beta}$ mice (Figure 2-3A-B). Furthermore, there was a 33.6% decrease in beta cell mass in the Nkx2.2$^{\Delta Beta}$ mice compared to their littermate controls (Figure 2-3C). Surprisingly, however, despite the large reduction in beta cell mass, there was no significant difference in the number of islets or islet size (Figure 2-3D-E). A loss of insulin content without a corresponding change in islet size could suggest the retention of a beta cell population that no longer produced insulin. To explore this possibility, we introduced to *R26R:Tomato* allele into the Nkx2.2$^{\Delta Beta}$ mice to genetically label the beta cell lineage regardless of its insulin-producing status.

Interestingly, although the Tomato reporter was co-expressed with insulin in the majority of control beta cells, the Nkx2.2$^{\Delta Beta}$ mice contained a significant number of Tomato-expressing cells that had little or no detectable insulin expression (Figure 2-3F-G). This observation was validated by quantitative determination of the pixel intensity of insulin-specific immunoreactivity that revealed a significant reduction in the median distribution of pixel brightness in the Nkx2.2$^{\Delta Beta}$ islets (Figure 2-3H-I). Furthermore, enumeration of the number of insulin-expressing cells per islet area confirmed the loss of insulin without a corresponding reduction in islet cell numbers (Figure 2-3J), suggesting that a loss of insulin content per cell and not cell loss causes the observed decrease in beta cell mass.

Although the Nkx2.2$^{\Delta Beta}$ mice display a significant reduction in the number of insulin producing cells, this degree of beta cell loss does not usually result in hyperglycemia (Peshavaria et al., 2006); (Liu et al., 2000). To determine whether islet function was affected, we performed a GSIS on young and old Nkx2.2$^{\Delta Beta}$ mice. Surprisingly, although these islets contain significantly less insulin-producing cells, they were able to secrete a higher percentage of insulin during a one-hour glucose challenge *in vitro* (Figure 2-2F). However, the ability of Nkx2.2$^{\Delta Beta}$ islets to secrete insulin declined with age (data not shown). This phenomenon has been previously observed in obese models of diabetes, such as the diabetic *obese/obese* (*ob/ob)* mouse, where an increased GSIS occurs as an adaptation to an increased insulin demand and obesity, which is thought to represent a pre-diabetic state (Irles et al., 2015). Although the Nkx2.2$^{\Delta Beta}$ islets are able to compensate in response to a single dose of glucose *in vitro*, they are unable to mount an insulin response to an *in vivo* glucose challenge (Figure 2-3K), indicating the Nkx2.2$^{\Delta Beta}$ mutant beta cells can only mount a transient response to glucose and are metabolically dysfunctional.

Nkx2.2 is essential for the acquisition of monohormonal beta cell identity

To identify the primary molecular changes that were causing beta cell dysfunction after deletion of Nkx2.2, we assessed the transcriptome of isolated islets extracted from Nkx2.2$^{\Delta Beta}$ mice and their littermate controls at 4 weeks of age. This analysis was performed shortly after the onset of hyperglycemia to minimize secondary affects caused by a chronically impaired metabolic environment. Consistent with the observed beta cell dysfunction, we found several essential beta cell genes, including those encoding beta cell transcription factors, *glucose transporter 2* (*glut2*), insulin granule proteins and components of the insulin secretory pathway to be significantly down-regulated (Figure 2-4A). We validated a subset of these gene changes in islets at the protein level, including the loss of two of the most functionally important beta cell factors, Glut2 and Nkx6.1 (Figure 2-5). Notably, in addition to the loss of many essential beta cell genes in the Nkx2.2$^{\Delta Beta}$ mice, there was a significant up-regulation of the non-beta cell pancreatic endocrine hormones, including somatostatin, pancreatic polypeptide and glucagon (Figure 2-4A). Since we had not observed an overall increase in islet size that could account for increased numbers of non-beta endocrine cells that would express these hormones, we immunostained islets with insulin and somatostatin, pancreatic polypeptide or glucagon to determine their cell localization. In addition, we lineage traced the mutant beta cells using the *R26R:Tomato* reporter to assess whether the beta cell population in the Nkx2.2$^{\Delta Beta}$ mice had acquired expression of the non-beta cell endocrine hormones and/or novel cellular identities. This analysis revealed that not only were the Nkx2.2$^{\Delta Beta}$ islets losing beta cell gene expression, but a significant number of beta cells were co-expressing insulin and either glucagon, pancreatic polypeptide or somatostatin.

In addition, there were populations of lineage-labeled beta cells that appeared to be completely reprogrammed; they no longer expressed insulin, but now expressed an alternative endocrine hormone (Figures 2-4B-K). These data revealed that loss of *Nkx2.2* is sufficient to cause partial and/or complete trans-differentiation of beta cells into the other islet cell types.

Quantification of the bihormonal and polyhormonal populations at 4 weeks of age revealed that approximately 10% of the beta cells had acquired multi-hormonal features (Figure 2-4L-N). Detailed immunofluorescence analysis of the Nkx2.2$^{\Delta Beta}$ mutant islets also revealed extraordinary heterogeneity in the Nkx2.2$^{\Delta Beta}$ islets. At any given age, we observed constant relative ratios of Tomato lineage-labeled beta cells that were expressing only insulin, expressing no insulin, expressing insulin with other hormones and expressing other endocrine hormones without insulin (Figure 2-4K). Interestingly, we did not observe evidence of beta cell dedifferentiation; Ngn3, Sox9 or other progenitor markers were not upregulated in the beta cell lineage (data not shown). To further characterize the different beta cell populations at the single cell level, electron microscopy was performed on control and Nkx2.2$^{\Delta Beta}$ mutant islets. As predicted by the light microscopic analysis, control islets contained endocrine cells displaying insulin granules with characteristic electron-dense crystal cores (Figure 2-4O and data not shown). In contrast, the mutant islets were filled with beta cells containing both insulin granules and either glucagon or somatostatin-like granules (Figure 2-4P,Q). Remarkably, these results suggest that many of the Nkx2.2$^{\Delta Beta}$ beta cells have acquired a fully hybrid identity that is characterized not only by the expression of more than one endocrine hormone, but also by the likely expression of their corresponding granule packaging machineries. There were also a large number of cells that were devoid of granules and displayed features of altered endoplasmic

reticulum (ER) with numerous enlarged cisternae (Figure 2-4R and Figure 2-6, B and C, red stroked arrows), whose membranes exhibited reduced ribosome content and increased frequency of free ribosomes in the cytosol (Figure 2-6, C, blue stroked arrows). Many beta cells from Nkx2.2$^{\Delta Beta}$ mice also contained insulin granules that exhibited an altered crystallization pattern (Figure 2-6, D, red stroked arrows), and numerous coalescent granules (D, inset, arrowheads), suggesting early stages of increased secretory vesicle autophagy. Collectively, these ultrastructural alterations indicate that in the absence of Nkx2.2, many of the beta cells became severely dysfunctional. It was not possible to determine whether these cells derived from the polyhormonal populations or arise independently. Surprisingly, despite the high degree of observed beta cell dysmorphogenesis and apparent dysfunction, there was little evidence of beta cell death by Caspase3 staining (data not shown), which was consistent with the maintenance of normal islet size in the Nkx2.2$^{\Delta Beta}$ mice (Figure 2-1E) and the preservation of wild type levels of *Chromogranin A* expression.

Nkx2.2 directly activates beta cell genes and represses non beta cell features

The transdifferentiation phenomenon observed in the Nkx2.2$^{\Delta Beta}$ mice could be directly caused by the deletion of Nkx2.2 or could be secondary to the loss of beta cell identity. To distinguish these possibilities, we performed ChIP-Seq analysis in MIN6 cells to determine how Nkx2.2 directly impacts the regulatory networks required to maintain beta cell identity. Similar to previous studies in human beta cells (Pasquali et al., 2014), Nkx2.2 predominantly bound distal intergenic regions of the genome, rather than at promoter elements (Figure 2-7A). *De novo* motif analysis showed CACTC to be the core motif sequence that is preferably bound by Nkx2.2 in 46.42% of its targets ($p<1e-217$) (Figure 2-7B).

Consistent with the Nkx2.2$^{\Delta Beta}$ phenotype, Nkx2.2 binds and activates beta cell genes that are essential for glucose uptake and insulin secretion *(Slc2a2*, *G6pc2, Ucn3, and Slc30a8*), insulin granule homeostasis (*Sytl4, Ptprn2, and Rab37*) and key beta cell transcription factors (*Nkx6.1*, *Insm1,* and *Tle3*). Interestingly, Nkx2.2 was also found to directly bind and *repress* the non-beta cell endocrine genes, including the endocrine hormones (*Sst, Ppy, Pyy,* and *Npy)*, islet disallowed genes (*Oat, Acot7, and Higd1a*), as well as the alpha and delta cell determination factors *Arx* and *Hhex* (Figure 2-7C-G). In agreement with previous studies that have suggested that Nkx2.2 functions as an activator and repressor (Doyle and Sussel, 2007), this analysis revealed that within the beta cell, Nkx2.2 simultaneously activates beta cell gene programs and represses non-beta cell programs. This data is consistent with the polyhormonal phenotype observed in the Nkx2.2$^{\Delta Beta}$ mice and clarifies that Nkx2.2 maintains beta cell identity and function by directly activating beta cell genes and repressing non-beta cell endocrine genes.

The ability of Nkx2.2 to function both as an activator and repressor in a single cell may be dependent on the underlying chromatin features present in the vicinity of Nkx2.2 binding. To characterize the regions that appeared to be activated or repressed by Nkx2.2, we compared the association of Nkx2.2 binding peaks with chromatin marks present in young islets (Avrahami et al., 2015). Interestingly, consistent with the relatively equal number of activated (49.2%) and repressed (50.8%) targets (Figure 2-8A), Nkx2.2 appeared to equally associate with active enhancers (H3K4me+ and H3K27ac+) and poised enhancers (H3K4me+, H3k27ac-, H3k27me3-). Only a small number of Nkx2.2 bound regions were associated with repressive marks (H3k4me+ and H3k27e3+) (Figure 2-8B). This would suggest, not unexpectedly, that Nkx2.2 binds to active enhancers to regulate the expression of beta cell-specific genes.

However, it also suggests that Nkx2.2 function is equally necessary to maintain repression of the lineage-related non-beta cell endocrine genes that are associated with poised enhancers that have been implicated in facilitating islet cell plasticity (Wang et al., 2015).

Nkx2.2 maintains beta cell function and identity in mature beta cells

Although analysis of the Nkx2.2$^{\Delta Beta}$ mice was performed in adult mice, it is possible that constitutive deletion of *Nkx2.2* in the beta cells during their normal maturation process compromises the initial acquisition and "locking in" of a stable beta cell identity. To assess the importance of Nkx2.2 in maintaining beta cell function and identity not only during the maturation process of the beta cell but also after they have fully differentiated, we deleted *Nkx2.2* in adult beta cells. Adult *Nkx2.2$^{fl/fl}$*; *MIP-CreERT* (Wicksteed, 2010) (hereafter referred to as Nkx2.2$^{\Delta AdultBeta}$) mice were administered intraperitoneal injections of tamoxifen every other day for five days. (Figure 2-9A). We were able to consistently achieve ~77% deletion of *Nkx2.2* mRNA from the treated mice (Figure 2-9B).

Interestingly, depletion of *Nkx2.2* in the adult beta cell resulted in the very rapid onset of diabetes. *Ad libitum* blood glucose levels of Nkx2.2$^{\Delta AdultBeta}$ mice were significantly increased within 1 week of the last injection, and continued to worsen with age (Figure 2-9C). Correspondingly, a significant increase in the fasting blood glucose levels was observed; although, similar to the Nkx2.2$^{\Delta Beta}$ mice (Figure 2-1F), this increase occurred at a slightly later stage (Figure 2-9D). Furthermore, the Nkx2.2$^{\Delta AdultBeta}$ mice were unable to effectively clear glucose following a glucose challenge (Figure 2-9E). This phenotype also became more severe with age (Figure 2-9F).

To investigate whether the observed islet dysfunction in the Nkx2.2$^{\Delta AdultBeta}$ mice was also due to the development of polyhormonal cells, we introduced the *R26R:Tomato* reporter to lineage trace the beta cell lineage and immunostained the mutant islets with insulin and either glucagon, somatostatin or pancreatic polypeptide. Surprisingly, we were only able to identify cells co-expressing insulin and somatostatin (Figure 2-9G-I); however, there were no cells co-expressing insulin and the other endocrine hormones (data not shown). These data suggest that Nkx2.2 is essential in the acquisition *and* maintenance of beta cell identity, during development and in the adult. However, it also implies that the level of beta cell plasticity becomes more restricted with age, and only retains the ability to acquire delta cell characteristics.

Nkx2.2 function is maintained in human islets

To investigate whether NKX2.2 is also important for the function and identity of human beta cells, islets from three individuals were infected with adenovirus encoding an shRNA directed against human *NKX2.2* or a scrambled shRNA control. We were able to achieve an ~80% reduction in *NKX2.2* expression, which led to significant ($p<0.05$) gene expression changes of which approximately 38% were down-regulated and ~62% were up-regulated (Figure 2-10A). Comparison of the gene expression changes with previously published ChIP-Seq analysis of NKX2.2 binding sites in human islets (Pasquali et al., 2014) demonstrated that, similar to mouse Nkx2.2, human NKX2.2 functions both as an activator and repressor. Notably, several important NKX2.2 gene targets were conserved, including *NKX6.1*, which was bound and down-regulated (Figure 2-10C) and *SOMATOSTATIN*, which was bound and up-regulated after loss of NKX2.2 (Figure 2-10D).

Collectively, these data demonstrate that a conserved role for NKX2.2 in maintaining beta cell function and identity by activating important beta cell genes and actively repressing non beta cells features.

Discussion

There is increasing evidence that loss of beta cell identity and the acquisition of bihormonal and polyhormonal cells is occurring in the islets of patients with T1D and T2D (White et al., 2013) (Yoneda et al., 2013); (Piran et al., 2014). However, the molecular basis of these reprogramming events have not been elucidated, and it is not known whether these events participate in the initiation of disease or represent a condition that is secondary to disease progression. In the present study, we uncover the essential role of Nkx2.2 in the maturation and maintenance of adult mouse and human beta cell function and identity. Beta cell deletion of *Nkx2.2* destabilized the beta cell phenotype, leading to a reduction in insulin expression and content, and the loss of some beta cell functional features (Figure 2-3A-C), which unexpectedly was not accompanied by cell death. Instead, we observed the presence of at least four different beta cells subpopulations within the mutant islets (Figure 2-4B-J): 1. Single positive insulin expressing cells; 2. Beta cells that co-express insulin and other islet hormones, suggesting the occurrence of a partial trans-differentiation event; 3. Cells that have lost insulin expression and only express other islet endocrine hormones, perhaps indicative of a complete cellular trans-differentiation; and 4. Cells which did not express any hormones. The existence of these different beta cell populations suggests that beta cells could gradually traverse stepwise through partial reprogramming, then to complete trans-differentiation, and finally to a complete loss of beta cell identity (Figure 2-11). However, we did not observe changes in the relative ratios of these four different subpopulations with age or pregnancy, perhaps suggesting that the islet contains heterogeneous beta cell populations that differentially respond to the loss of Nkx2.2. We cannot, however, rule out the possibility that there is some interconversion between the different phenotypic states (Figure 2-11).

In contrast to previous studies that have shown beta cells undergo de-differentiation prior to reprogramming (Talchai et al., 2012), we did not observe significant increase in the expression of progenitor markers to suggest deletion of *Nkx2.2* causes beta cell de-differentiation. Instead, our data suggests that loss of *Nkx2.2* results in direct reprogramming by the primary regulation of specific gene sets. Interestingly, we also observed more restricted reprogramming potential in adult versus young beta cells. Deletion of Nkx2.2 in beta cells shortly after the activation of insulin, resulted in all combinations of polyhormonal cell populations; however, deletion of Nkx2.2 in adult beta cells only led to the formation of insulin and somatostatin co-expressing cells (Figure 2-9G-I), implying that the plasticity of the beta cell is diminished over time. The role of Nkx2.2 function in maintaining beta cell identity by activating beta cell gene programs and repressing non-beta cell programs appears to be conserved in human islets. Similar to loss of Nkx2.2 in adult mouse beta cells, inactivation of Nkx2.2 in human islets predominantly led to the up-regulation of *SST* (Figure 2-10D). Interestingly, the predominance of INS and SST co-expressing cell populations has been described in several recent studies reporting the presence of polyhormonal cells in islets derived from diabetic individuals (Yoneda et al., 2013; Cinti et al., 2016).

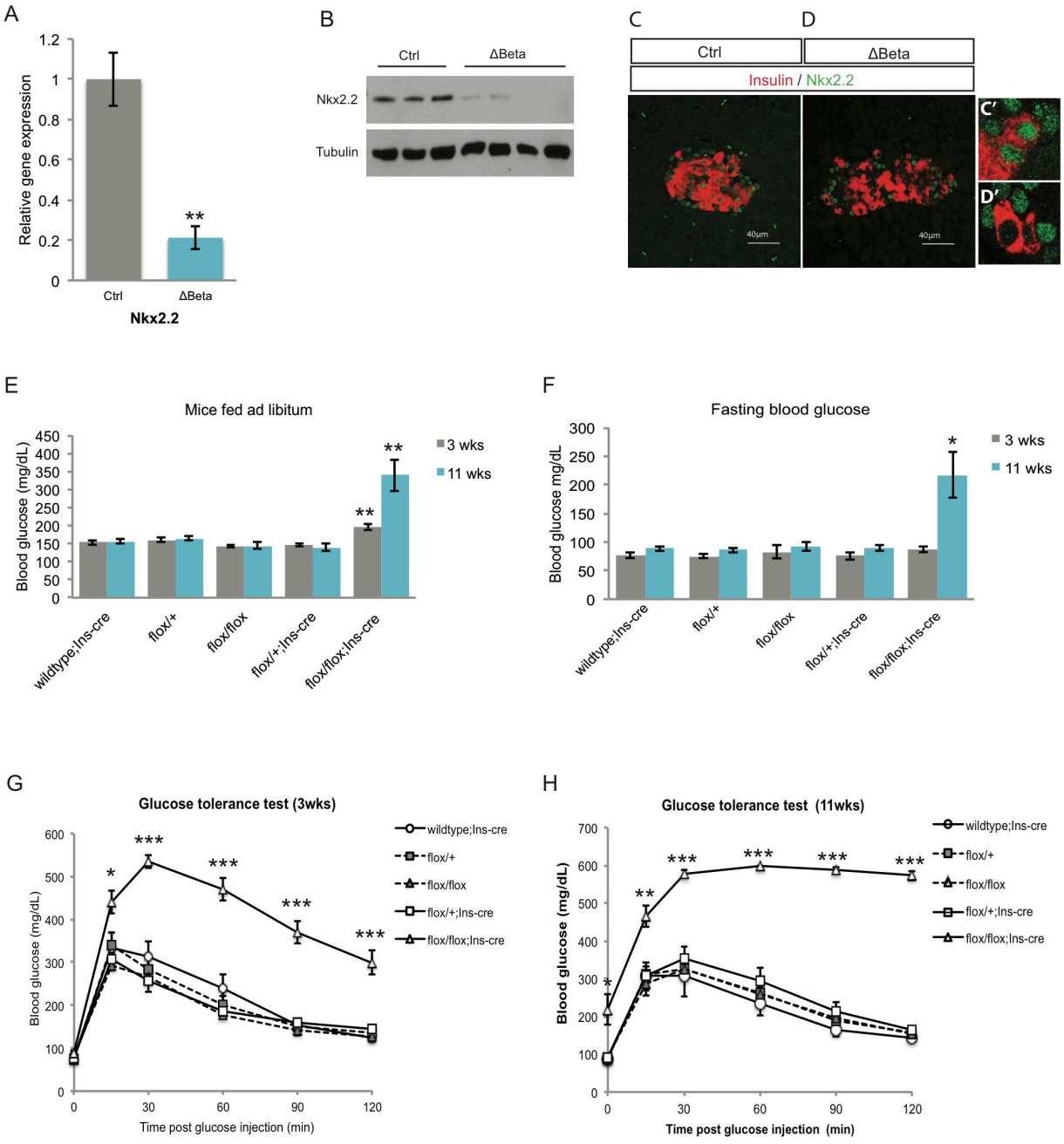
In this study, we have determined that Nkx2.2 directly regulates beta cell function and identity. Although previous studies have demonstrated the ability of Nkx2.2 to function as an activator and repressor in certain cellular and genomic contexts, this is the first evidence that Nkx2.2 simultaneously functions to directly activate the beta cell program and repress non-beta endocrine genes.

The unexpected finding that 47% of Nkx2.2 binding peaks were present at poised rather than repressed enhancers also provides an explanation for why beta cells are only able to revert to closely related endocrine cell fates, rather than a completely unrelated cell identity. Furthermore, it suggests a mechanism whereby Nkx2.2 is involved in the priming of enhancers during development and may be essential later for the direct activation of the genes involved in beta cell function as well as the active repression of gene sets that would otherwise activate non-beta islet related lineages. Furthermore, the direct regulation of both beta cell and non-beta cell gene programs by Nkx2.2 suggests that presence of bihormonal and/or polyhormonal cells is not secondary to the loss of beta cell identity. Importantly, these studies also demonstrate the highly unstable nature of the beta cell; there is an ongoing need to actively maintain the activation of genes involved in beta cell function, but to also maintain repression of closely related gene programs. Therefore, it is possible that in conditions of metabolic stress disruption of a single regulatory pathway will cause a simultaneous loss of beta cell function and identity.

The development of polyhormonal cells during T1D and T2D has been postulated to be indicative of a more primitive endocrine cell phenotype that is known to exist during human islet cell development (Riopel et al., 2014). Interestingly, during the early stages of human pancreas formation, the endocrine cells are initially polyhormonal and only gradually resolve into monohormonal cell identities (Bocian-Sobkowska et al., 1999). Intriguingly, unlike in mouse pancreatic development where Nkx2.2 is expressed early and there are only minor populations of polyhormonal cells, in humans NKX2.2 is not detected until late in pancreas formation, during the stage when monohormonal cells are acquired (Jennings et al., 2013). This suggests that there may be an intrinsic function of Nkx2.2 for acquiring a functional monohormonal identity.

The role of Nkx2.2 in maintaining beta cell identity in T2D remains relatively unexplored. Whereas Nkx2.2 expression is down regulated more than 5 fold (padj < 1.19E-07) in Db/Db mouse islets and in primate models of diabetes induced by a high fat/sugar diet (Fiori et al., 2013), Guo et al., (2013) did not detect changed expression levels of Nkx2.2 in samples from human patients with T2D. However, given the polygenic nature of T2D, it remains possible that larger sample sizes will identify NKX2.2 dysregulation in subclasses of T2D patients. In support of this, Pasquali et al (2014) identified a large number of T2D SNPs present in islet enhancers that were occupied by NKX2.2.

In summary, it is becoming increasingly clear that beta cell death may not be the primary cause of the loss of beta cell mass during diabetes progression. Instead, there is emerging evidence that fully differentiated mature beta cells in mice and humans retains much more plasticity than previously appreciated. In the present study, we identified Nkx2.2 as master regulator of such plasticity, actively repressing non-beta cell lineages and directly activating beta cell functional programs. Understanding the molecular mechanisms allowing for the retained plasticity will be important for deriving fully differentiated beta cells with a "locked-in" identity *in vitro*. Furthermore, these findings indicate that blocking or reversing beta cell reprogramming could represent viable therapies for treating diabetes.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Relative gene expression
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1
0.8
0.6
0.4
0.2
0
**
Ctrl
ΔBeta
Nkx2.2
B
Ctrl
ΔBeta
Nkx2.2
Tubulin
C
D
Ctrl
ΔBeta
Insulin / Nkx2.2
C′
D′
40μm
40μm
E
Mice fed ad libitum
Blood glucose (mg/dL)
450
400
350
300
250
200
150
100
50
0
**
**
3 wks
11 wks
wildtype;Ins-cre
flox/+
flox/flox
flox/+;Ins-cre
flox/flox;Ins-cre
F
Fasting blood glucose
Blood glucose mg/dL
300
250
200
150
100
50
0
*
3 wks
11 wks
wildtype;Ins-cre
flox/+
flox/flox
flox/+;Ins-cre
flox/flox;Ins-cre
G
Glucose tolerance test (3wks)
Blood glucose (mg/dL)
600
500
400
300
200
100
0
*
***
***
***
***
0
30
60
90
120
Time post glucose injection (min)
wildtype;Ins-cre
flox/+
flox/flox
flox/+;Ins-cre
flox/flox;Ins-cre
H
Glucose tolerance test (11wks)
Blood glucose (mg/dL)
700
600
500
400
300
200
100
0
*
**
***
***
***
***
0
30
60
90
120
Time post glucose injection (min)
wildtype;Ins-cre
flox/+
flox/flox
flox/+;Ins-cre
flox/flox;Ins-cre

Figure 2-1: Deletion of *Nkx2.2* in beta cells results in diabetes

(A) qRT-PCR analysis of *Nkx2.2* expression in islets isolated from Nkx2.2$^{\Delta Beta}$ and control mice at 4 weeks of age (n=4). **p ≤0.01.

(B) Western Blot analysis of isolated islets from Nkx2.2$^{\Delta Beta}$ and control mice confirms a reduction of Nkx2.2 protein in islets isolated from four individual adult Nkx2.2$^{\Delta Beta}$ mice.

(C,D) Immunofluorescence staining of insulin (red) and Nkx2.2 (green) demonstrates the loss of Nkx2.2 expression in beta cells from Nkx2.2$^{\Delta Beta}$ compared to control mice at 4 weeks of age. The white boxes indicate regions of the islet that are shown in higher magnification in C' and D'.

(E) Ad-libitum blood glucose levels in 3 week old male Nkx2.2$^{\Delta Beta}$ mice compared to controls (n=5-22) and in 11 week old mice (n=6-18). **p≤0.01. Each control genotype was examined separately to ensure the individual *Cre* and *floxed* alleles did not cause metabolic phenotypes.

(F) Higher fasting blood glucose levels are evident in 11 week old Nkx2.2$^{\Delta Beta}$ mice compared to controls. (3 week old mice: n=6-23; 11 week old mice: n=8-21) *p≤0.05.

(G) Glucose intolerance is observed in Nkx2.2$^{\Delta Beta}$ male mice compared to controls at 3 weeks of age (n=6-23). *p≤0.05; ***p≤0.001.

(H) Glucose intolerance becomes more severe at 11 weeks of age in Nkx2.2$^{\Delta Beta}$ male mice compared to control mice (n=8-21). *p≤0.05; **p≤0.01; ***p≤0.001.

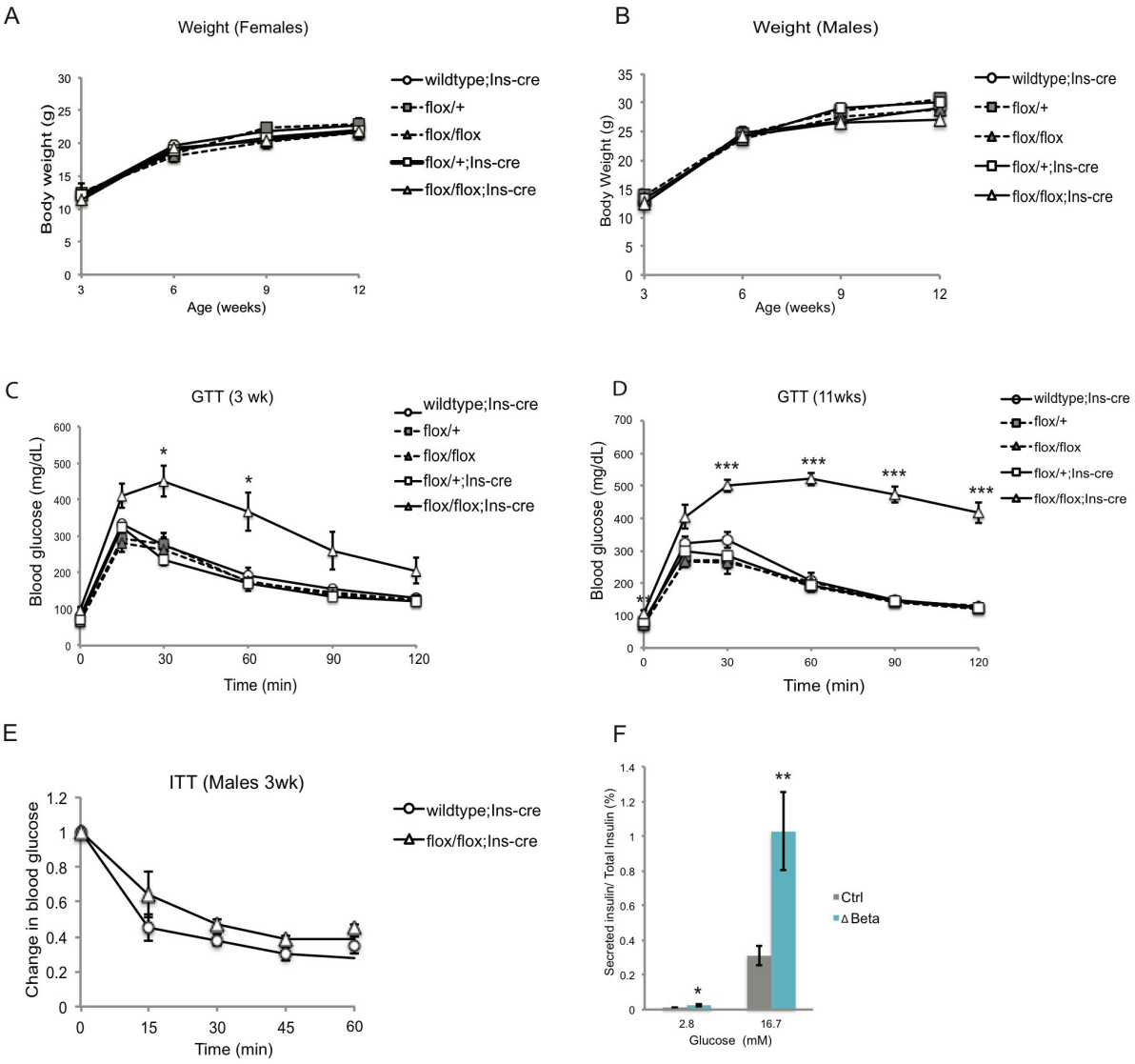

A
Weight (Females)
Body weight (g)
Age (weeks)
wildtype;Ins-cre
flox/+
flox/flox
flox/+;Ins-cre
flox/flox;Ins-cre
B
Weight (Males)
Body Weight (g)
Age (weeks)
C
GTT (3 wk)
Blood glucose (mg/dL)
Time (min)
D
GTT (11wks)
E
ITT (Males 3wk)
Change in blood glucose
F
Secreted insulin/ Total Insulin (%)
Glucose (mM)
Ctrl
Beta

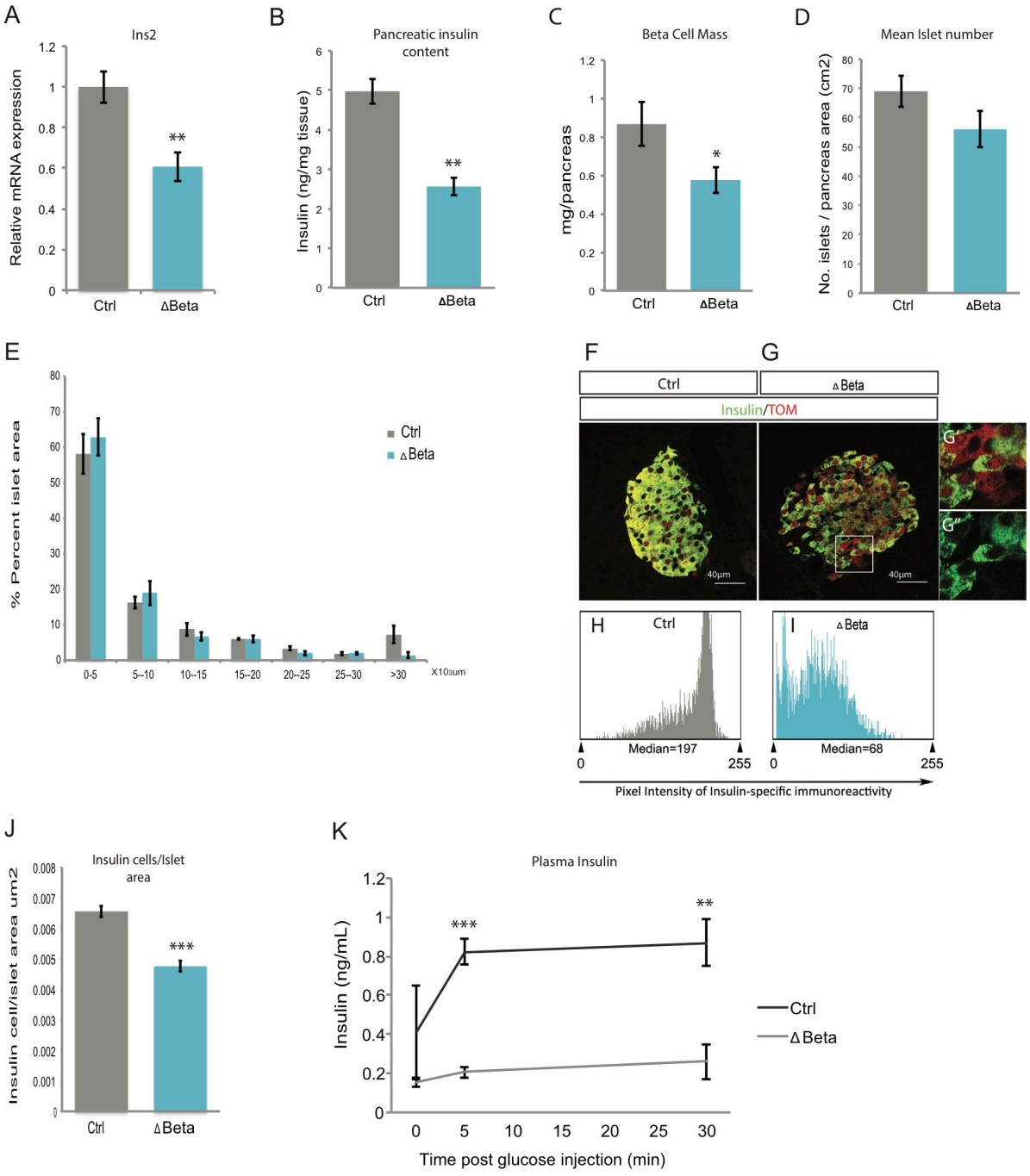
Figure 2-2: Development of diabetes is not influenced by changes in body weight or insulin sensitivity

(A-B) No significant weight differences were found in Nkx2.2$^{\Delta Beta}$ vs. littermate control females (n=5-20) and males (n=3-12).

(C-D) Female Nkx2.2$^{\Delta Beta}$ mice are glucose intolerant beginning at 3 weeks of age and become overtly diabetic by 11 weeks of age. *p≤0.05; **p≤0.01; ***p≤0.001.

(E) Insulin tolerance tests demonstrate that the Nkx2.2$^{\Delta Beta}$ mice do not have insulin sensitivity defects compared to controls at 3 weeks of age. (n=3-5).

(F) Glucose stimulated insulin secretion assays demonstrate Nkx2.2$^{\Delta Beta}$ mice are able to compensate at 4weeks of age compared to controls (n=3). *p≤0.05; **p≤0.01.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Ins2
Relative mRNA expression
1.2
1
0.8
0.6
0.4
0.2
0
**
Ctrl
ΔBeta
B
Pancreatic insulin content
Insulin (ng/mg tissue)
6
5
4
3
2
1
0
**
Ctrl
ΔBeta
C
Beta Cell Mass
mg/pancreas
1.2
1
0.8
0.6
0.4
0.2
0
*
Ctrl
ΔBeta
D
Mean Islet number
No. islets / pancreas area (cm2)
80
70
60
50
40
30
20
10
0
Ctrl
ΔBeta
E
% Percent islet area
80
70
60
50
40
30
20
10
0
Ctrl
ΔBeta
0-5
5--10
10--15
15--20
20--25
25--30
>30
X10sum
F
G
Ctrl
ΔBeta
Insulin/TOM
40μm
40μm
G'
G''
H
Ctrl
Median=197
0
255
I
ΔBeta
Median=68
0
255
Pixel Intensity of Insulin-specific immunoreactivity
J
Insulin cells/Islet area
Insulin cell/islet area um2
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
***
Ctrl
ΔBeta
K
Plasma Insulin
Insulin (ng/mL)
1.2
1
0.8
0.6
0.4
0.2
0
***
**
0
5
10
15
20
25
30
Time post glucose injection (min)
Ctrl
ΔBeta

Figure 2-3: Lineage tracing in Nkx2.2$^{\Delta Beta}$ mice indicates loss of insulin expression in Nkx2.2$^{\Delta Beta}$ beta cells

(A) qPCR analysis of *Insulin2* mRNA expression in islets from Nkx2.2$^{\Delta Beta}$ mice compared to control mice at 4weeks of age (n=4). **p ≤0.01.

(B) There is a ~50% reduction in pancreas insulin content in Nkx2.2$^{\Delta Beta}$ mice compared to control mice at 4 weeks of age (n=3). **p ≤0.01.

(C) Loss of Nkx2.2 results in decreased beta cell mass at 4 weeks of age (n=3). *p ≤0.05.

(D) There was no statistical difference found in islet numbers of Nkx2.2$^{\Delta Beta}$ compared to control mice at 4 weeks of age (n=3).

(E) Islet size of Nkx2.2$^{\Delta Beta}$ mice compared to control mice was not statistically different at 4 weeks of age (n=3). There was a trend down in the number of the largest islets, but the difference did not reach significance.

(F,G) Immunofluorescence staining of insulin (green) and Tomato (red) shows a decrease or absence of insulin expression in tomato expressing beta cell lineages. White box indicates region magnified in G' (with tomato channel) and G'' (without tomato channel).

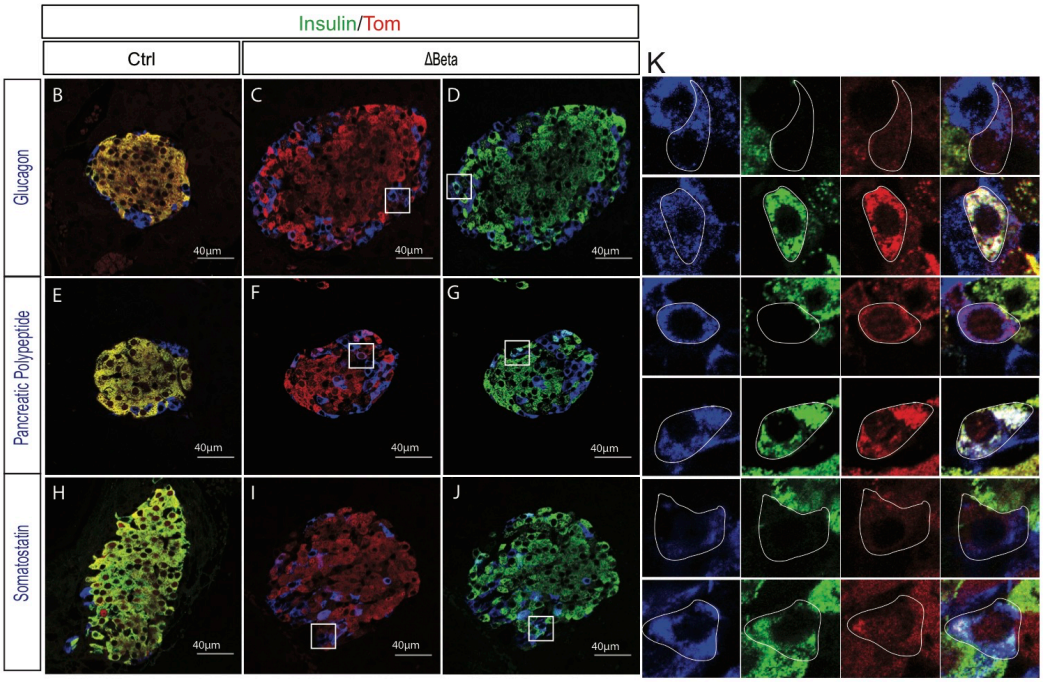
(H, I) Digital quantification of insulin-specific immunoreactivity (Adobe Photoshop CS4). There is a significant reduction in the median distribution of the brightest pixels from a value of 197 in control islets *versus* 68 in Nkx2.2$^{\Delta Beta}$ islets.

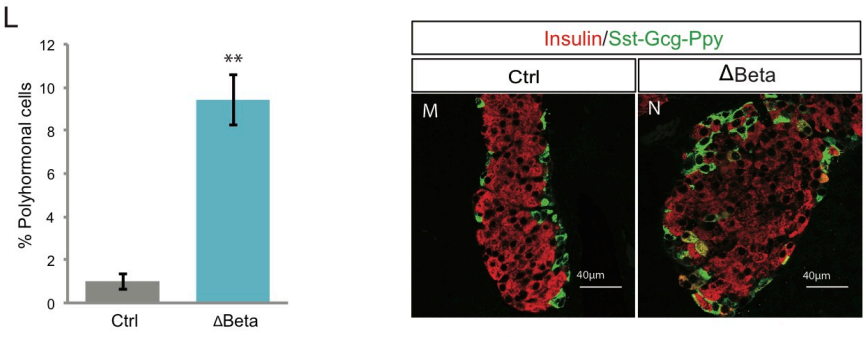
(J) Nkx2.2$^{\Delta Beta}$ 4 week old mice have less insulin positive cells per islet area compared to controls (n=3). ***p ≤0.001.

(K) Insulin levels are significantly lower in Nkx2.2$^{\Delta Beta}$ mice after a glucose stimulus compared with control mice (n=3-4). **p ≤0.01; ***p ≤0.001.

A

Category	Gene Symbol
Glycolytic Pathway	G6pc2, Pcx
Beta cell transcription factors	Nkx6.1, Pdx1, Insm1, Tle3
Insulin granule features	Cav1.3, Ptprn2, Rab37, Slc30a8, Sytl4
Glucose sensing and insulin secretion	Atp2a2, Atp2a3, Dpp4, Gipr, Glut2, Stxbp1, Ucn3
Non beta cell endocrine molecules	Sst, Ppy, Npy, Pyy, Arx, Hhex
Islet Disallowed	Acot7, Higd1a, Oat





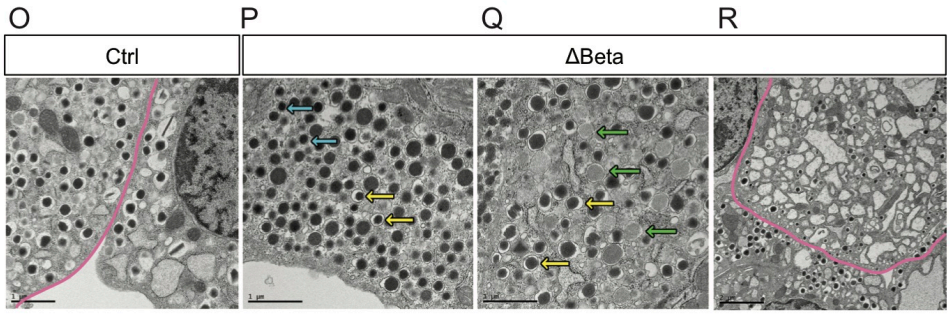


Figure 2-4: Nkx2.2 is essential for the establishment of beta cell identity

(A) A subset of genes and their corresponding functional categories that were identified in RNA-Seq analysis of islets isolated from Nkx2.2$^{\Delta Beta}$ mice compared to controls at 4 weeks of age. Genes that are significantly up-regulated are indicated in green and significantly down-regulated genes are indicated in red. All genes shown P≤0.05. The full dataset is available at GEO#GSE79725.

(B-K) Immunofluorescence staining of pancreata from Nkx2.2$^{\Delta Beta}$ and control mice at 4 weeks of age. White boxes indicate regions that are magnified in the panels in K.

(B,E,H) Representative images of control islets co-labeled with insulin (green), Tomato (red) and either glucagon (B), pancreatic polypeptide (E) or somatostatin (H) (blue).

(C,F,I) Representative images of Nkx2.2$^{\Delta Beta}$ islets co-labeled tomato (red) and either glucagon (C), pancreatic polypeptide (F) or somatostatin (I) (blue).

(D,G,J) Representative images of Nkx2.2$^{\Delta Beta}$ islets co-labeled with insulin (green) and either glucagon (D), pancreatic polypeptide (G) or somatostatin (J) (blue).

(K) Images of representative single lineage-labeled insulin-positive beta cells expressing either glucagon, pancreatic polypeptide, somatostatin and insulin-negative beta cells expressing either glucagon, pancreatic polypeptide, somatostatin. The channels are separated out from left to right: glucagon, pancreatic polypeptide and somatostatin (blue), insulin (green) and tomato (red). The final column shows the merged channels.

(L) Quantification analysis shows that an average of 10% of beta cells in Nkx2.2$^{\Delta Beta}$ compared to control mice at 4 weeks of age co-express insulin and either glucagon, somatostatin or pancreatic polypeptide.

(M,N) Representative images showing immunofluorescence analysis of pancreata from Nkx2.2$^{\Delta Beta}$ and control mice stained with guinea pig anti-insulin antibody (red) and a combination of the 3 rabbit anti-pancreatic polypeptide, rabbit anti-somatostatin and rabbit anti-glucagon antibodies (green).

(O-R) TEM of pancreatic islets reveals a combination of insulin and either glucagon or somatostatin-like granules in the same cell in Nkx2.2$^{\Delta Beta}$ mice. Magenta lines outline individual beta cells. Granule identity is indicated by colored arrows: glucagon (blue), insulin (yellow) and somatostatin (green) (P,Q). Representative image of a beta cell from Nkx2.2$^{\Delta Beta}$ islets with severely altered secretory granules (R).

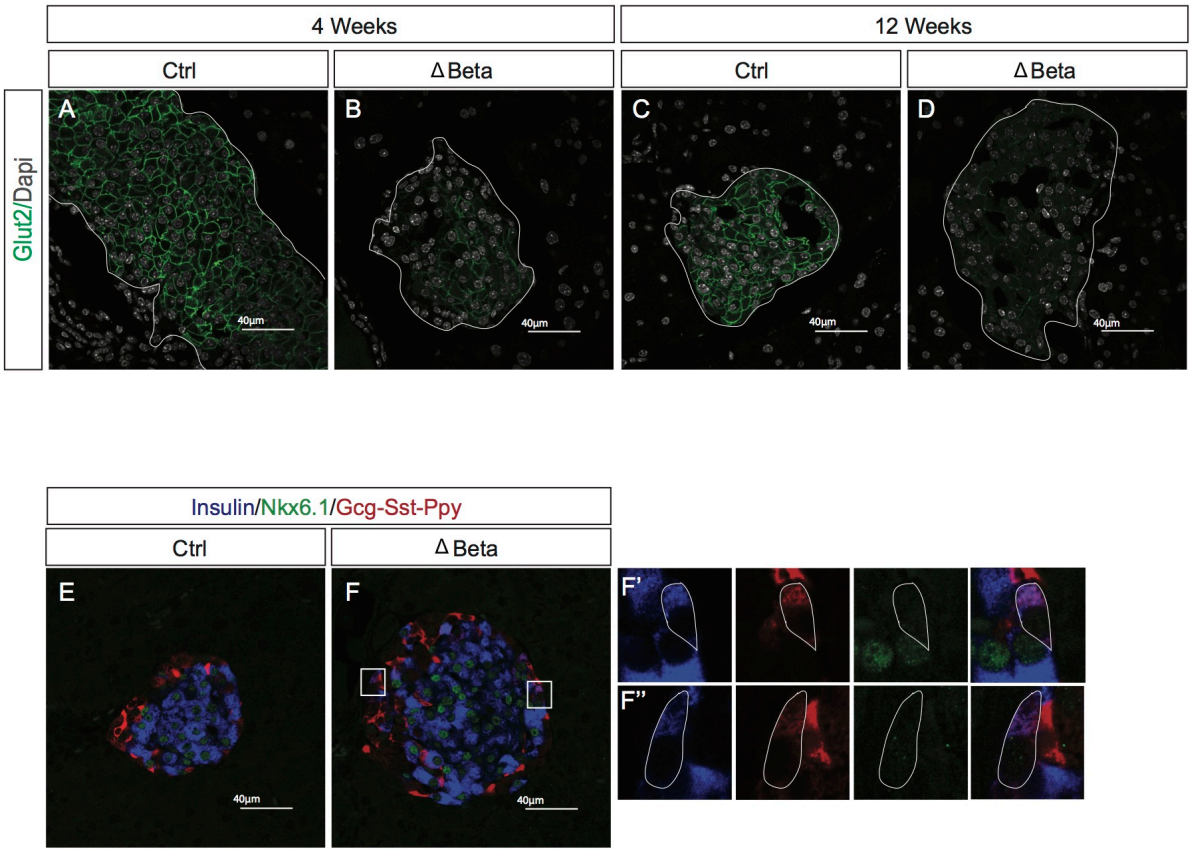


Figure 2-5: Loss of important beta cell protein expression in Nkx2.2$^{\Delta Beta}$ islets correlated with their decreased RNA expression.

(A-D) Representative images of immunostained islet sections from 4 week old (A,B) and 12 week old (C,D) Nkx2.2$^{\Delta Beta}$ mice (B,D) and their littermate controls (A,C). Dapi (grey) marks the nuclei and Glut2 (green) is on the cell membrane. Glut2 expression is decreased at 4 weeks of age and becomes almost undetectable at 12 weeks.

(E-F) Representative images of immunostained islet sections from 4 week old Nkx2.2$^{\Delta Beta}$ mice (E) and a littermate controls (F). Insulin (blue), glucagon, somatostatin and pancreatic polypeptide, combined (red), and Nkx6.1 (green). F' and F'' show higher magnification images of cells that are insulin+, express other hormones and are Nkx6.1 negative.

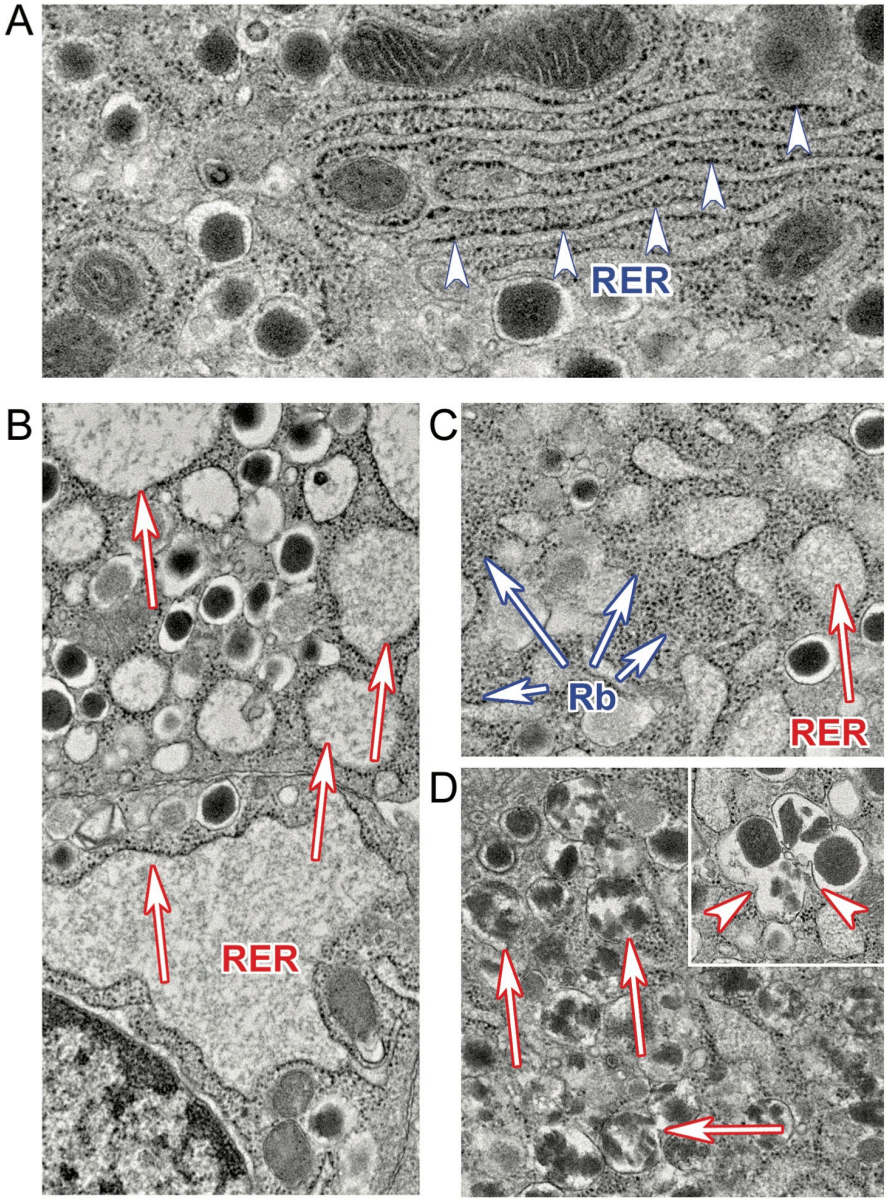

A
RER
B
RER
C
Rb
RER
D

Figure 2-6: Nkx2.2$^{\Delta Beta}$ islet cells exhibit ultra-structural alterations compatible with a disrupted secretory granules morphology and ER stress.

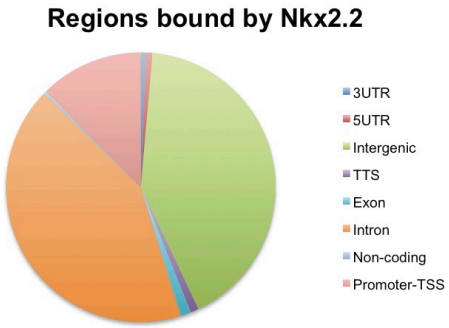
(A-D) TEM images of single cells from control and Nkx2.2$^{\Delta Beta}$ mice.

(A) Representative image of normal-looking rough endoplasmic reticulum (RER) in control islets (arrowheads).

(B,C) Islet cells from Nkx2.2$^{\Delta Beta}$ mice show enlarged RER cisternae (red stroked arrows) and increased free ribosomes not associated with RER (blue stroked arrows).

(D) Beta cells in Nkx2.2$^{\Delta Beta}$ mice contained large populations of insulin granules that exhibited an altered crystallization pattern (red stroked arrows), and numerous coalescent granules (inset, arrowheads).

A



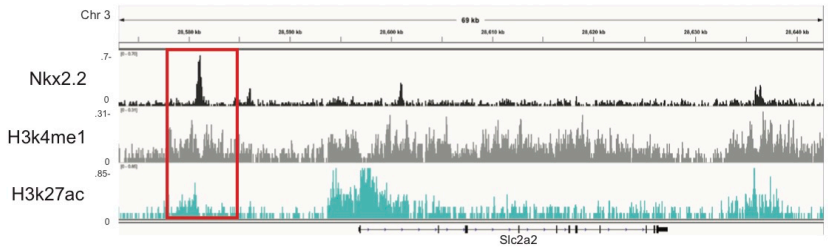
B

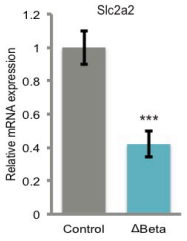
De-novo motif analysis

C

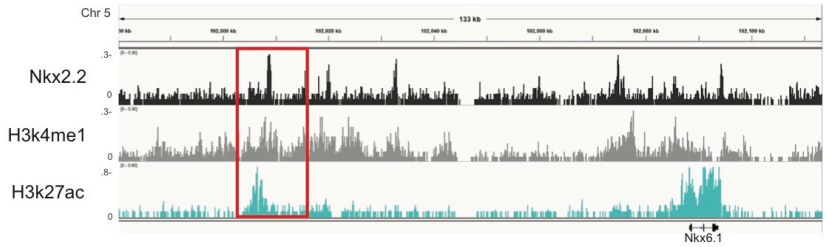
Category	Gene Symbol
Insulin Secretion	Slc2a2, G6pc2, Stxbp1, Slc30a8, Rims3, Ucn3, Pcx
Transcription factors	Nkx6.1, Insm1, Mlxipl, Tle3
Membrane channels/receptors	Kcnmb2, Gipr
Granule features	Sytl4, Ptprn2, Rab37
Non beta cell endocrine molecules	Sst, Ppy, Npy, Pyy, Arx, Hhex
Islet Disallowed	Acot7, Higd1a, Oat

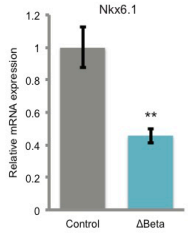
D



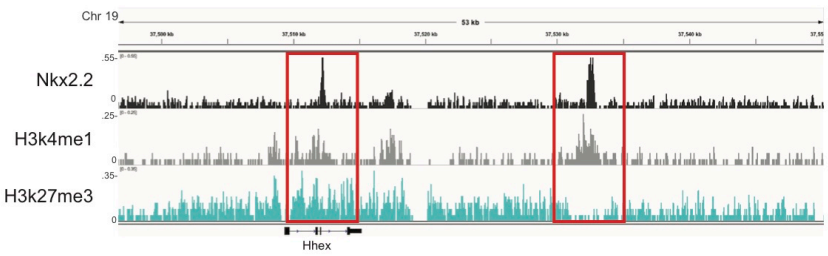


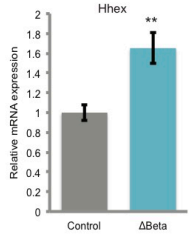
E



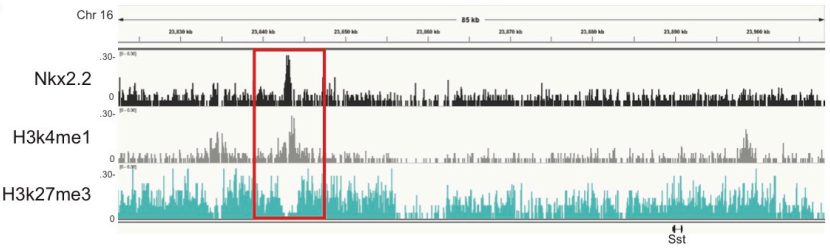


F





G



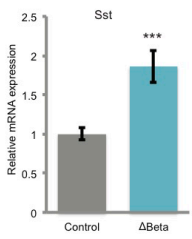


Figure 2-7: Nkx2.2 directly activates important beta cell genes and actively represses non-beta islet endocrine genes

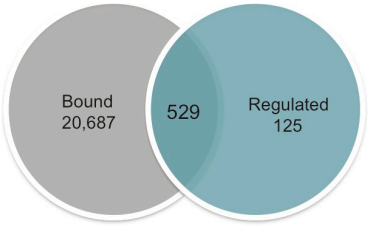
(A) Distribution of regions bound by Nkx2.2 within the genome obtained from ChIP-Seq analysis performed in a MIN6 cell line (n=3).

(B) The consensus Nkx2.2 binding motif identified through *de novo* motif analysis.

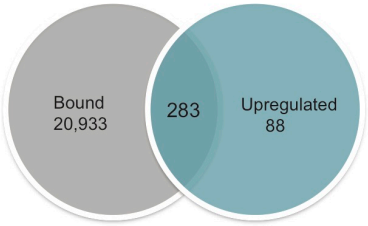
(C) Gene targets directly regulated by Nkx2.2 were identified by comparing the ChIP-Seq data with the RNA-Seq analysis from Nkx2.2$^{\Delta Beta}$ vs. control adult islets. Bound and activated genes are indicated in red and bound and repressed genes are indicated in green. Full data set is available at GEO#GSE79725.

(D-G) ChIP-Seq analysis identified binding of Nkx2.2 to active or repressed enhancers. qRT-PCR analysis confirms differential expression of direct Nkx2.2 gene targets in islets from Nkx2.2$^{\Delta Beta}$ compared to control mice. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

A



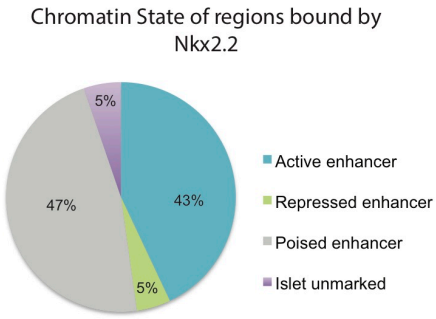
80.9% Bound and Regulated



53.5% Bound and Upregulated

46.5% Bound and Downregulated

B



C

Rank	Motif	P-value	% of Targets	% of Backround	Best Match
1		1 e-796	20.08	1.94	Rfx5
3		1 e-178	12.58	3.75	NeuroD1
6		1 e-146	21.46	10.1	FoxO1

Figure 2-8: Nkx2.2 activates and represses an equal amount of targets preferentially through active and poised enhancer binding

(A) Comparative analysis of ChIP-Seq data from MIN6 cells with RNA-Seq data from Nkx2.2$^{\Delta Beta}$ compared to control islets demonstrates that Nkx2.2 represses and activates relatively equal numbers of direct targets regulated by Nkx2.2.

(B) Chromatin state of regions bound by Nkx2.2 shows preferential binding to active enhancers (H3k427ac+/H3k4me+) and poised enhancers (H3k4me+). Repressed enhancers (H3k4me+/H3k27me3+) constitute a minor proportion. ChIP-Seq analysis done using Nkx2.2 binding peaks present in all triplicate samples.

(C) De novo motif analysis of Nkx2.2 binding shows motifs and targets matched to other known motifs. Information in the table is representative of one of the triplicate samples.

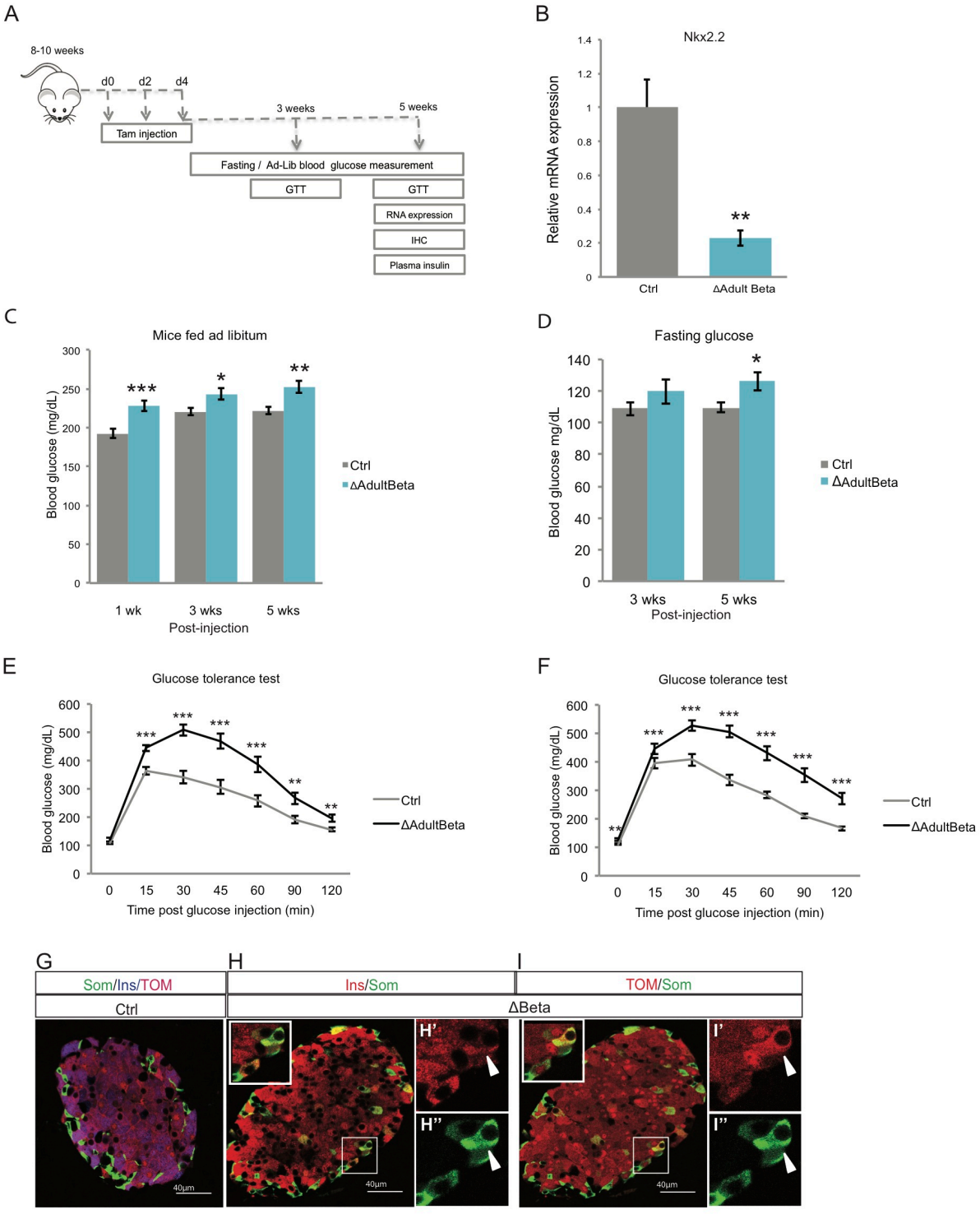

A
8-10 weeks
d0
d2
d4
3 weeks
5 weeks
Tam injection
Fasting / Ad-Lib blood glucose measurement
GTT
GTT
RNA expression
IHC
Plasma insulin
B
Nkx2.2
Relative mRNA expression
Ctrl
ΔAdult Beta
C
Mice fed ad libitum
Blood glucose (mg/dL)
1 wk
3 wks
5 wks
Post-injection
Ctrl
ΔAdultBeta
D
Fasting glucose
Blood glucose mg/dL
3 wks
5 wks
Post-injection
Ctrl
ΔAdultBeta
E
Glucose tolerance test
Blood glucose (mg/dL)
Time post glucose injection (min)
Ctrl
ΔAdultBeta
F
Glucose tolerance test
Blood glucose (mg/dL)
Time post glucose injection (min)
Ctrl
ΔAdultBeta
G
H
I
Som/Ins/TOM
Ins/Som
TOM/Som
Ctrl
ΔBeta
H'
H''
I'
I''
40μm

Figure 2-9: Nkx2.2 is essential for the maintenance of beta cell identity and function in adult beta cells.

(A) Schematic of tamoxifen treatment and experimental design.

(B) qRT-PCR analysis demonstrates significant deletion of *Nkx2.2* in isolated islets from Nkx2.2$^{\Delta AdultBeta}$ mice compared to control mice 5 weeks after the last injection (n=5-7). **p ≤0.01.

(C) Deletion of *Nkx2.2* increases *ad libitum* blood glucose levels in Nkx2.2$^{\Delta AdultBeta}$ compared to control mice starting at 1 week after the last injection(n=16-20). * p≤0.05; **p = ≤0.01; ***p ≤0.001.

(D) Fasting blood glucose levels begin to be significantly increased 5weeks after the last injection in Nkx2.2$^{\Delta AdultBeta}$ compared to control mice (n=12-15). * p ≤0.05.

(E) Glucose tolerance tests indicate the Nkx2.2$^{\Delta AdultBeta}$ are already glucose intolerant at 3 weeks after the last injection compared to control mice (n=12-15). **p ≤0.01; ***p ≤0.001.

(F) Glucose intolerance becomes more severe 5 weeks after the last injection in Nkx2.2$^{\Delta AdultBeta}$ compared to control mice (n=12-15).**p ≤0.01; ***p ≤0.001

(G-I) Immunofluorescence staining of pancreata from Nkx2.2$^{\Delta AdultBeta}$ and control mice 5 weeks after the last injection. Insets are magnifications of selected areas indicated by white boxes showing co-expression of insulin and somatostatin and co-expression of insulin and tomato.

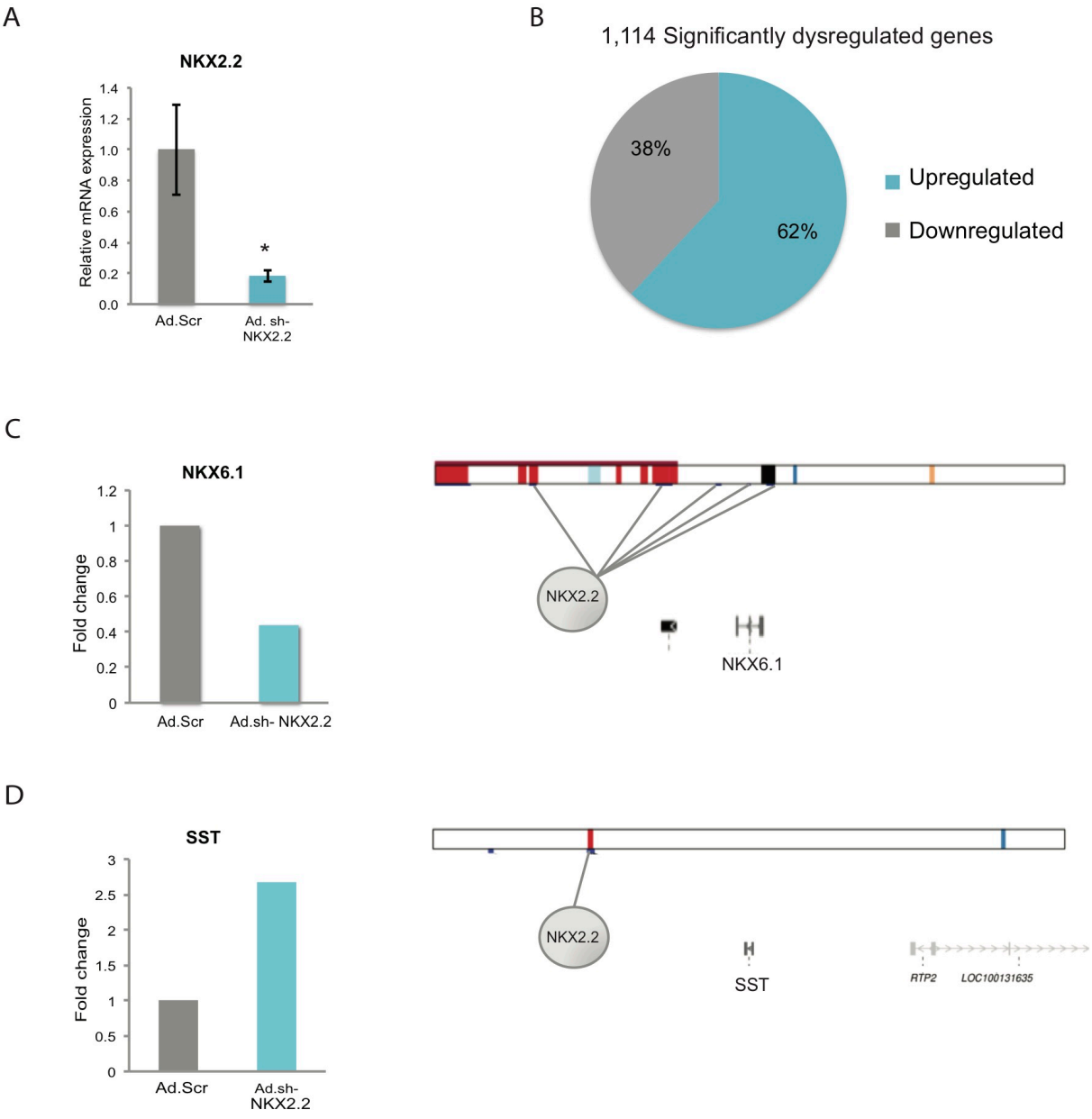

A
NKX2.2
Relative mRNA expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Ad.Scr
Ad. sh-NKX2.2
B
1,114 Significantly dysregulated genes
38%
62%
Upregulated
Downregulated
C
NKX6.1
Fold change
1.2
1
0.8
0.6
0.4
0.2
0
Ad.Scr
Ad.sh- NKX2.2
NKX2.2
NKX6.1
D
SST
Fold change
3
2.5
2
1.5
1
0.5
0
Ad.Scr
Ad.sh-NKX2.2
NKX2.2
SST
RTP2
LOC100131635

Figure 2-10: NKX2.2 function is conserved in human islets

(A) qRT-PCR analysis reveals significant deletion of NKX2.2 in human islets transduced with Ad.sh-NKX2.2 compared to scramble sh-RNA control vector (Ad.Scr) (n=3). *p≤0.05.

(B) 1,114 genes were differentially regulated in human islets when NKX2.2 was reduced. The graph indicates the percent distribution of genes differentially expressed (p≤0.05) from adenoviral mediated deletion of NKX2.2 in human islets compared to scramble control.

(C-D) Binding sites shown to be activated/repressed in human islets through ChIP-Seq analysis show direct targets of NKX2.2 that become dysregulated after its deletion. Fold change values obtained from RNA-Seq data. n=3.

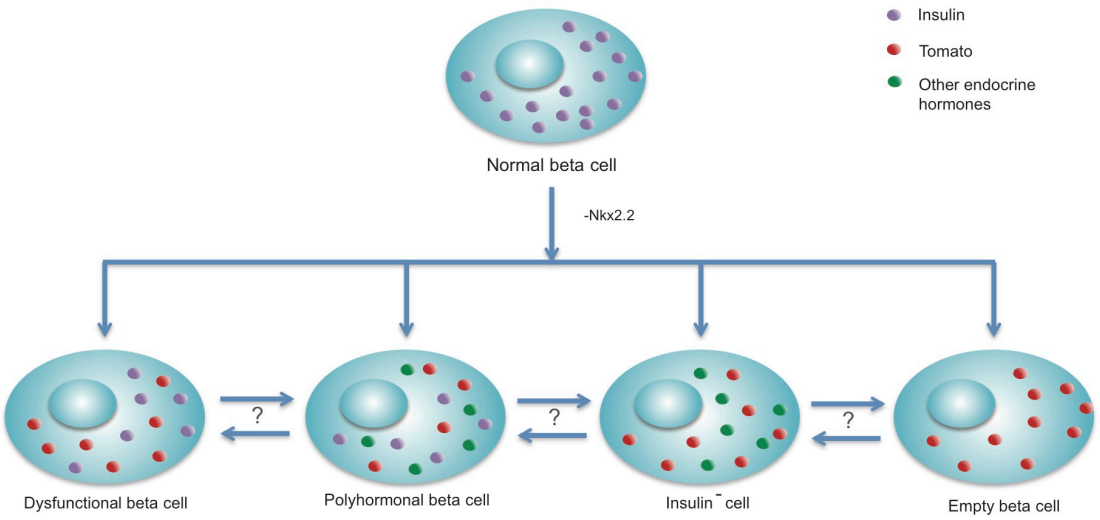

Insulin
Tomato
Other endocrine hormones
Normal beta cell
-Nkx2.2
?
?
?
Dysfunctional beta cell
Polyhormonal beta cell
Insulin - cell
Empty beta cell

Figure 2-11: Model of Nkx2.2 function in the adult beta cell

Nkx2.2 appears to be a master regulatory protein that is essential for the acquisition and maintenance of beta cell identity. Loss of Nkx2.2 in beta cell results the formation of different beta cell subpopulations that were identified by the expression of the beta cell-specific tomato reporter:

Dysfunctional β cells: This category includes lineage-labeled (red dots) insulin-expressing cells (purple dots) unable to mount a proper insulin response and represents the largest population of mutant beta cells.

Polyhormonal β cells: Lineage labeled (red dots), insulin positive (purple dots) beta cells that are mis-expressing the other islet endocrine hormones (green dots). It is possible that these cells represent incomplete transdifferentiation.

Insulin$^-$ β cells: Lineage labeled (red dots) beta cells that have lost insulin expression, but have acquired the expression of other non-beta endocrine hormones (green dots).

(Empty β cells): A rarer population of lineage labeled cells (red dots) without expression of any endocrine hormones.

It is unknown whether these populations originate independently or are able to introconvert. Arrowheads represent the possible transitioning between the different populations.

Chapter 3
Conclusions and Future Perspectives

Diabetes is a condition that has already reached epidemic proportions and there are millions of patients waiting for a cure. A very promising treatment approach is to identify a renewable source of beta cells either through replication or iPS cell differentiation. Another alternative is the development of therapies to stop and reverse the loss of beta cell functional features and identity that is associated with diabetes. To be able to achieve such a monumental goal, we need to gain a profound understanding of the factors and mechanisms that participate in the generation and maintenance of the beta cell. Furthermore, it is also of equal importance to understand what influences those factors and networks to cause them to become dysregulated and adopt a pathophysiologic state.

In this thesis project, I focused on a transcription factor that had been found to be essential for beta cell differentiation during fetal development (Sussel et al., 1998), and that held great potential for maintaining a vital role during the adult stage of the beta cell as well. I utilized a variety of techniques to explore and understand how Nkx2.2 regulates beta cell function and identity. I discovered that Nkx2.2 is important for the maturation and maintenance of functional identity in beta cells, and it accomplishes this by actively repressing non beta cell endocrine features and simultaneously activating beta cell genes. These findings highlighted important dynamics governing beta cell identity and revealed new questions that point to new avenues of research that should be pursued in the future, both with regards to Nkx2.2 function and more general issues related to beta cell identity and function.

Nkx2.2

During this thesis work I uncovered important roles for Nkx2.2 in the maturation and maintenance of function of the beta cell. Although we have acquired significant knowledge in this area, there is much more to understand about the beta cell and its regulation that will allow us to design effective therapies.

Nkx2.2 regulation of the epigenetic landscape

In my thesis studies, I discovered Nkx2.2 binds to enhancers of genes associated with beta cell function, as well as to genes regulating non beta cell endocrine identity (Figure 2-7C-G). These genes contained chromatin marks that have been found to represent a poised state, as well as activation and repression states. Previous studies have found that such chromatin marks are either acquired sequentially during differentiation of the cell lineage or during the maturation stage (Pasquali et al., 2014; Thorrez et al., 2011; van Arensbergen et al., 2010; Wang et al., 2015). Furthermore, some of these marks seem to become more stable overtime, whereas others require active maintenance instead. It would be important to understand how is Nkx2.2 involved in the acquisition of such marks. To pursue this, I would knockdown Nkx2.2 in mice at different stages through the use of the MIPCreER inducible system. ChIP-Seq of these chromatin marks would be performed in isolated islets of these mice. This would allow for the determination of the time window when Nkx2.2 is needed to impart such regulation. Additionally, it would reveal the chromatin marks that Nkx2.2 is responsible to produce. Furthermore, it would shed light on the states in which Nkx2.2 is required to actively maintain beta cell function, and where Nkx2.2 function becomes redundant once beta cell identity has been established.

Finally, it has been found that genes important to maintain beta cell function and repress other endocrine identities are bivalently marked (H3k4me3+/H3k27me3+) indicating they are in a poised state. This bivalency has been suggested to explain why beta cells become reprogrammed to endocrine identities rather than taking on the identities of unrelated tissues such as heart, liver or intestine (Bramswig et al., 2013). The information gained from this study would provide important information on the association of such poised enhancers and the reprogramming of beta cells.

Binding partners of Nkx2.2

It is recognized that Nkx2.2 is expressed in pancreatic progenitors during development and that it becomes restricted to the beta, alpha and PP cell lineage as differentiation proceeds (Arnes et al., 2012; Sussel et al., 1998). These cells, although close in proximity have very different identities and functions. The mechanism by which Nkx2.2 is able to impart differential regulation in these cell types is likely to be related to the co-factors that it binds. In support of this, Papizan and colleagues (Papizan et al., 2011) have shown that Nkx2.2 assembles a repressor complex in beta cells to repress the Arx promoter and therefore maintain the beta cell versus the alpha cell identity. In contrast, that same repressive complex is not found in the alpha cells, even though Nkx2.2 is present at that same region.

Exploring the different transcription factors and chromatin remodeling complexes that partner with Nkx2.2 will be of great importance to further understand its regulatory functions in these different cell types. Results from the motif analysis that was performed from the Nkx2.2 ChIP-Seq experiment showed a significantly larger percentage of the Nkx2.2 targets to be bound by

Neurod1, Rfx4/5/6, FoxO1 and Nkx6.1 (Figure 2-8C). Therefore, a detailed analysis of the overlapping in binding of these factors would offer important information as to which of them interact to assemble such complexes; as well as whether particular combinations of factors are specifically associated with activation or repression of gene targets. Furthermore, simultaneous or sequential binding of these different factors could be assessed using the novel re-ChIP technology. Finally, interaction confirmation of these factors could be accomplished by co-immunoprecipitation analysis.

Chromatin remodeler complexes are also critical in regulating the epigenetic landscape of the cell (Campbell and Hoffman, 2016). Nkx2.2 has already been found to bind histone modifying factors such as DNA methyltransferases (Dnmt1 and Dnmt3a) as well as to the histone deacetylase 1 (HDAC1) (Levine, Manuscript in preparation; Papizan et al., 2011). Furthermore, a mass spectrometry analysis has already been performed in the laboratory, finding components of the NurD and B-Wich complexes to be bound to this factor as well (data not shown). Interestingly, a large proportion of the genes being repressed by Nkx2.2 contained the H3k27me3 mark (Figure 2-7F-G), which has been found to be conferred mostly by the PRC2 complex (Simon and Kingston, 2013). Therefore, it is likely that this factor also interacts with this chromatin-remodeling complex as well. Correlation of the binding of the aforementioned complexes with the chromatin marks associated with Nkx2.2 targets warrants further examination. Of clinical importance, many of these epigenetic regulator components such as HDAC inhibitors are already being used in the clinical setting (Chan et al., 2016; Petrich and Nabhan, 2016). For this reason, analysis of the beta cell specific epigenetic regulatory mechanisms could offer viable options to more easily modify specific gene programs.

Post-translational regulation of Nkx2.2

It has been convincingly demonstrated that Nkx2.2 is essential for beta cell differentiation (Sussel et al., 1998). In this thesis work I have provided evidence for its critical role in the functional maintenance of such differentiated state. A very important question that remains is what happens to this factor prior or during disease progression. Studies in diabetes mouse models such as the db/db mice show a significant decrease in the expression of Nkx2.2 (Dominguez, Romer and Sussel, data not shown).

In humans, variable expression levels of Nkx2.2 are seen in samples from donors with T2D (data not shown). This variability is likely due to the small sample size used, which cannot account for disease duration and types of treatments applied. Nevertheless, another very likely possibility apart from expression dysregulation is the acquisition of post-translational modifications as a response to environmental signals. Such type of regulation has been found to affect the function of other important beta cell transcription factors (Maganti et al., 2015; Rena et al., 2002).

A former graduate student in the laboratory, James Papizan, has identified potential sites for such types of modifications in Nkx2.2. To further explore this possibility, a mass spectrometry analysis could first be peformed in vitro using mouse and human beta cell lines during normal and challenging conditions; to identity the differential regulation of such residues. Once the most promising modifications were found, mutations in those sites could be introduced in order to assess their implication in co-factor and DNA binding as well as in epigenetic regulation. Identification of the enzymes and signaling pathways involved in the post translational regulation of Nkx2.2 could hold great promise for future therapies.

Loss of functional identity has been confirmed in patients with T1D and T2D (Piran et al., 2014; White et al., 2013; Yoneda et al., 2013). Furthermore, beta cell differentiation from iPS cells is still not quite able to mature those cells properly. We know that Nkx2.2 plays an important role in the maturation of the beta cells as well as in keeping them locked in that state. Overexpressing this factor in the last maturation step within the iPS beta cell differentiation protocol or in the clinical setting could be done trough viral infection. However, the potential dangerous effects render this approach only useful for its mechanistic study. Additionally, overexpressing this factor might not be enough to bring those cells to a functional mature state if it is not receiving the proper signals to guide it. To better approach these issues, finding the enzymes or compounds that could influence this factor in the proper manner could represent a more viable clinical option.

Beta cell identity and function

The findings of my studies have uncovered interesting features about the beta cell that include an enhanced plasticity, a surprising cellular heterogeneity as well as dynamic epigenetic landscape. Their potential influence in the regulation of beta cell function and identity, as well in the development and progression of diabetes merits their further understanding.

Beta cell re-differentiation

The unexpected cell plasticity found in this study, which was reflected in their partial or complete trans-differentiation into other endocrine identities, opened up exciting windows of opportunity. Due to the fact that beta cells can acquire other identity features, it is likely that they can also recover their identity if the disturbed main pathways are repaired and restored to normal

conditions again. The current treatments for diabetes mainly focus on inducing the beta cell to secrete more insulin to cope with the elevated blood glucose levels. This kind of approach only stresses the cell, and leads it to its own failure and demise. However, there are efforts focusing on the possibility of re-differentiation of the beta cells; they range from insulin therapy to give the dedifferentiated and exhausted beta cells a rest, to the screening of chemical compounds libraries to find candidates that can reinstate mature beta cell identity again(Blum et al., 2014; Wang et al., 2014). It will be important to determine the timing and circumstances in which beta cells can be re-differentiated, as well as if prevention of identity loss is also an alternative.

Importance of enhancer regulatory complexes in diabetes

An interesting finding of this work relates to the regulation of gene targets through enhancer binding. Furthermore, the importance of the complex regulatory networks that assemble at those enhancers is gaining more attention. Pasquali and colleagues (Pasquali et al., 2014) found that such enhancers posses important SNPs that have been associated with T2D. Exploring whether these genetic variations play an important role in the predisposition of beta cells to disease, by making them more vulnerable to the loss of identity and function during challenging conditions warrants further assessment.

Beta cell heterogeneity

Deletion of Nkx2.2 in the beta cell population resulted in a variety of cellular responses that were translated in the development of different populations within this particular cell type. This unexpected outcome pointed to the existence of heterogeneity in the beta cell population, a concept that has become more evident and which is receiving an increase amount of attention

both in the beta cell and the diabetes field (Ellenbroek et al., 2013; Pipeleers, 1992; Speier et al., 2007; Wills et al., 2016). I focused on studying and understanding these heterogeneous populations, specifically how they differed from each other and the influence they had on the phenotypes observed. To do this at the individual cell level, the ideal method was single cell RNA-Seq, a procedure that allows you to sort and assess the expression of individual cells (Tang et al., 2010). Although this technology has made great progress and has become more widely used in other fields, it was not suitable to efficiently undertake this particular challenge at the time. Therefore, I opted for different approaches to answer such questions. However, optimization of such technology will allow us to more specifically study this heterogeneity that seem to play an important role during challenging conditions such as diabetes. Future understanding of beta cell heterogeneity could shed light on the existence of markers or differences that makes some beta cells more prone to the immune attack present in T1D. It could also explore the existence of cells that are more amenable to replication, and understand what makes them different from the rest, so that such pathways could be exploited for future treatments. Lastly, it could also explain why some cells are more amenable to be differentiate during the iPS cell differentiation protocol and others remain in an undifferentiated state (Pagliuca et al., 2014; Rezania et al., 2014).

In conclusion, this thesis work has revealed the importance of the function of Nkx2.2 during the maturation and adult stage of the beta cell. It has deepened our understanding of the mechanisms that regulate beta cell function, and has demonstrated the conservation of Nkx2.2 function in both species, then proceeded to show the common features between mice and humans.

The findings obtained here have revealed the importance of active repression of non beta cell endocrine identities in order to mature and maintain a functional beta cell. Furthermore, they demonstrate that such regulation of plasticity is time and context-dependent and requires specific signaling mechanisms. This information can be applied in several areas of diabetes therapy that may include: the optimization of protocols that aim to differentiate functional beta cells from iPS cells, as well as in therapies that aim at re-differentiate beta cells that have lost their identity. Both of these approaches are among the most promising therapies that offer the much-needed regain of the lost beta cell mass that drives this disease.

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