SYNAPTOTAGMIN IV AND MYT FACTORS PROMOTE β-CELL FUNCTIONAL

MATURATION AND MAINTENANCE

By

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Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

In

Cell and Developmental Biology

May, 2017

Nashville, Tennessee

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ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere appreciation and gratitude to my advisor, Dr. Guoqiang Gu, for all his support during my thesis research. My first thank to him is for allowing me to work in his lab and consistently supporting me with his grants. During more than six years of mentorship, he not only guided me in the critical thinking and research design, but also taught me detailed experimental techniques and polished my oral and written presentation skills. I will always remember all those times when he shared his research experience to me with kindness and patience. He influenced me with his smartness in analyzing scientific problems and hard-working spirit, the two invaluable merits that I shall always keep in mind in my future career development.

I am also full of gratitude to my thesis committee members, Dr. Chin Chiang, Dr. Maureen Gannon, Dr. David Bader and Dr. Tony Weil. They have invested lots of time and energy in my thesis research. I am grateful for their constructive inputs to my thesis projects, their efforts towards my academic improvement, and their attention to my career development. I will never forget those times when they encouraged me to carry out research projects independently, inspired me to think deeply about experiments, and pushed me to improve my academic writing. Their motivation and enthusiasm in research exemplified the values of outstanding scientists, and I was blessed to have them mentor me during my PhD study.

I would like to thank Gu lab members, both current and past, for their help in my research. Yanwen and Ruiying constantly provided me with useful technical support for my experiments. Jingbo and Jing gave me insightful suggestions in analyzing data and planning experiments. I would also like to acknowledge other labs in the Department of Cell and Developmental Biology and β-cell research community, for all their intellectual

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

1.1 Abstract

This thesis aims to understand the maturation and functional maintenance of insulin-secreting β-cells in the pancreas. Here in the introduction, I will first present the overall anatomy and physiology of the pancreas, as pancreas construction and islet architecture are both critical contexts of β-cell research. I will then introduce the fundamental mechanism of β-cell function, i.e. glucose-stimulated insulin secretion (GSIS). Herein I will discuss physiological processes involved in the GSIS pipeline, focusing on the Ca^{2+} signaling-coupled vesicle exocytosis, which is most relevant to my research. After introducing β-cell function, I will present our current understanding of how mature β-cells featuring robust GSIS are formed. Next, I will discuss long-term maintenance of β-cell function and stability during postnatal stages. In particular, I will highlight the various types of cell stress that β-cells are likely to face and the intrinsic key factors required to handle them. To underscore the significance of postnatal β-cell stability, I will present the relationship between β-cell stress and the development of diabetes. In the last part of this introduction, I will focus on the most recent advances that scientists have made in generating β-cells as a restorative cell therapy for the treatment of diabetes, and the major knowledge gap that needs to be addressed in this field. Finally, I will discuss how this thesis research will likely broaden our perspectives of β-cell maturation and functional maintenance and further contribute to generating of long-lived functional β-cells to improve human health.

1.2 Overview of pancreas and islet physiology

Pancreas structure and islet composition

The mammalian pancreas is a multifunctional organ composed of two distinct units: exocrine pancreas and endocrine pancreas. In the exocrine pancreas, acinar cells generate digestive enzymes that help break down carbohydrates, proteins and lipids. These enzymes are transported to the duodenum by pancreatic ducts. The exocrine pancreas accounts for the most organ volume (>95% in mouse and human). On the other hand, endocrine cells are clustered into islets of Langerhans, which are scattered throughout the organ. There are five types of endocrine cells that produce distinct hormones: glucagon-producing α-cells, insulin-producing β-cells, somatostatin-producing δ-cells, ghrelin-producing ξ-cells, and finally pancreatic polypeptide-producing PP-cells. Compared to exocrine secreted enzymes, these hormones from endocrine cells are released directly into the blood circulation.

The composition ratio of islet cell types varies depending on different species and age (Figure 1.1). In general, β-cells are the most abundant. In mice, for which the islet biology has been the most extensively studied, β-cells account for more than 80% of all islet cell mass in adult stages. The majority of β-cells are located in the islet core, surrounded by other islet cell types. In adult human islets, the percentage of β-cells is around 50%, and the proportion of α-cells is much higher (35-40%) than that of rodents (10-15%) (Rorsman and Braun, 2013). Moreover, endocrine cells in the adult human islet are randomly distributed, without a dominant islet core formed by any islet cell types (Steiner et al., 2010). These unique islet compositions between rodents and humans may reflect the difference in nutritional and metabolic features between these two species (Steiner et al., 2010). Nonetheless, studies based on murine models still provide
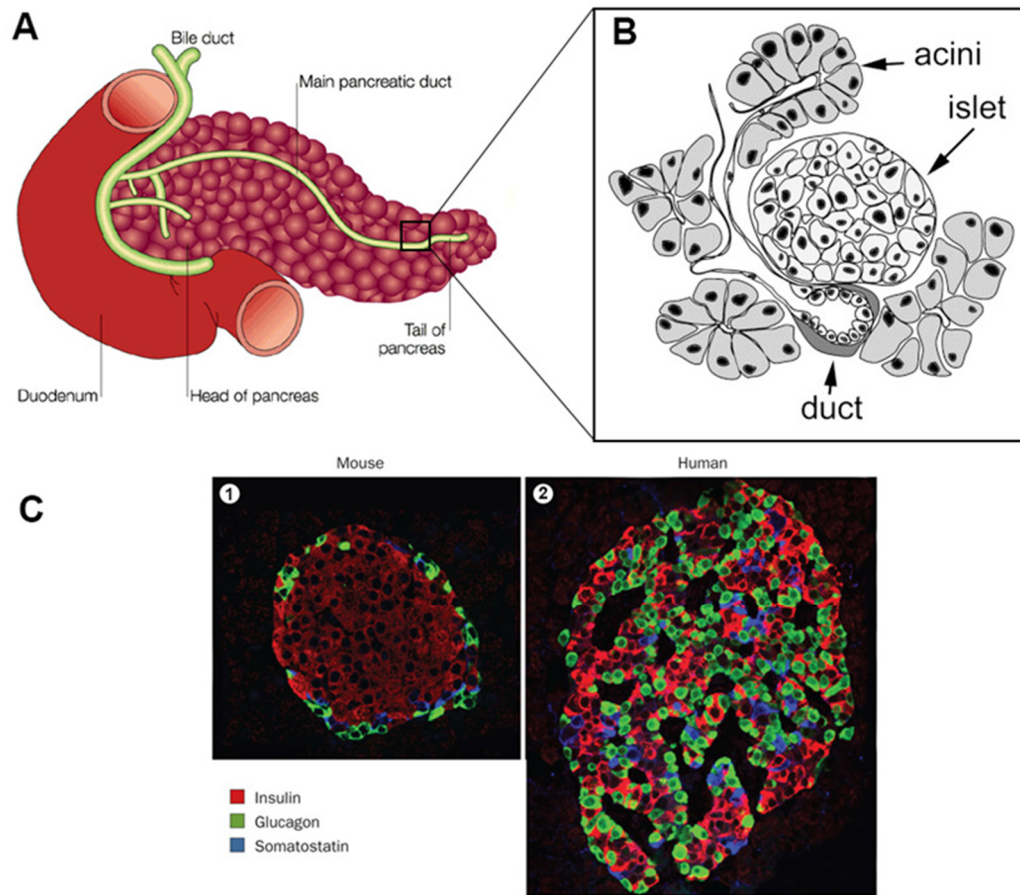
Figure 1.1 Anatomy of the mammalian pancreas and islets. (A) Relative localization of the pancreas, duodenum and bile duct. Digestive enzymes generated by the pancreas are transported by the pancreatic duct into the duodenum. Figure A is adapted from (Edlund, 2002). (B) The pancreas is composed of three morphologically distinct units - acini, ducts, and islets. Each of them has unique functions as described in the text. Figure B is adapted from (Gu et al., 2003). (C) Different compositions of mouse and human islets. In the mouse islet, β-cells are located in the core, and other islet cell types are located in the periphery. Also, β-cells account for the most volume of the mouse islet. In contrast, the human islet has a higher ratio of α-cells. Different islet cell types are distributed randomly throughout the human islet. Figure C is adapted from (Wang et al., 2015).

the most comprehensive understanding in various aspects of β-cell biology, such as β-cell development, maturation, function, proliferation, stress response and survival.

The primary purpose of hormones secreted from endocrine cells is nutritional homeostasis, and their functions tend to interact with one another. Insulin is circulated to target organs or tissues, such as liver, skeletal muscle, and adipose tissue, where it mediates the uptake of glucose by activating the insulin receptor and the related downstream signaling pathway. The physiological outputs of insulin include glucose metabolism, glycogen synthesis and glucose to triglycerides conversion. In contrast, glucagon secreted by α-cells raises blood glucose by promoting liver glycogenolysis, ketogenesis, ureagenesis and lipolysis (Heppner et al., 2010). Glucagon also promotes insulin secretion via the β-cell-expressed glucagon receptor (Gelling et al., 2009; Kawai et al., 1995), reflecting the requirement of insulin in glucose uptake and energy generation after blood glucose is raised by glucagon. Conversely, insulin inhibits α-cell function to secrete glucagon (Franklin et al., 2005; Ishihara et al., 2003). The interaction between these two hormones contributes to blood glucose homeostasis in variable physiological conditions.

On the other hand, somatostatin, secreted by δ-cells, is generally considered to be an inhibitory hormone. In the pancreas, it plays a negative role in both glucagon and insulin secretion by activating K^+ channels to induce membrane hyperpolarization (Hsu et al., 1991; Strowski et al., 2000). Similarly, one major role of pancreatic polypeptide from PP-cells is to suppress insulin secretion, although its functional mechanism has not been comprehensively investigated (Degano et al., 1992; Lundquist et al., 1979). Besides the regulatory function on islet cells, somatostatin and pancreatic polypeptide have been found to act in a wide range of biological events, including the regulation of exocrine secretion (Park et al., 1993), mobility of gastric-intestinal (GI) tract (Holzer et al., 2012), insulin sensitivity and appetite regulation (Wynne et al., 2005). Finally, ghrelin

from ξ-cells is primarily involved in food uptake and energy homeostasis. Unlike glucagon or insulin, which is exclusively generated from pancreatic islets, ghrelin is produced largely by other organs and tissues, such as the stomach and duodenum. Interestingly, pancreatic ξ-cells are phenotypically unstable and have been proposed as a group of progenitor cells instead of a terminally differentiated cell type (Arnes et al., 2012).

Besides pancreatic endocrine cells, there are other cell types within the islet. These non-endocrine cells interact with endocrine cells and form an islet microenvironment that is crucial for various islet activities. First, the islet is highly vascularized. Intra-islet capillaries not only relay blood nutritional information to the islet and transport away secreted hormones, but they also provide crucial cellular and molecular mechanisms for islet cell development, proliferation and function (Eberhard et al., 2010; Lammert et al., 2001). For instance, perturbation of VEGF signaling between islets and endothelial cells affects glucose-stimulated insulin secretion (GSIS) and β-cell mass expansion (Lammert et al., 2003; Li et al., 2006). Secondly, the islet is also extensively innervated. Both sympathetic and parasympathetic neural cells have been demonstrated to be essential for islet development and cell function (Borden et al., 2013; Rossi et al., 2005). For instance, Borden, et al. showed that the β-adrenergic signaling from sympathetic neurons is essential for islet architecture formation and functional maturation (Borden et al., 2013). Interestingly, VEGF from islet vascularization also facilitates islet innervation (Reinert et al., 2014), suggesting that there are functional cross-interactions between these two regulatory systems. Identifying regulatory factors and signal pathways from the islet microenvironment will provide more clues for therapeutic β-cell regeneration to treat diabetes.

Another significant type of interaction within islets is the one between endocrine cells and immune cells. Under normal physiology, immune cells not only play a

protective role, but they also facilitate islet development and function. For instance, Homo-Delarche et al. showed that macrophages secrete cytokines, metalloproteinases and growth factors within islets to remodel the composition of extra-cellular matrix (ECM), which is an essential regulator for islet clustering and activity (Homo-Delarche and Drexhage, 2004). Banaei-Bouchareb et al. identified a diminished β-cell mass at embryonic day 18.5 (E18.5) in a macrophage-depleted mouse model. Notably, the loss of β-cell mass in this mouse model could not be recovered in adult stage, underscoring the critical role of the macrophage in β-cell mass expansion (Banaei-Bouchareb et al., 2004). Another study conducted in adult rats showed that the total depletion of immune cells by thymectomy affects islet volume, β-cell number and proliferation, and insulin granule morphology (Zafirova et al., 1992).

Immune cells also exert an important influence on islet activity under specific physiological impacts. For instance, Xiao et al. found that immune cells can provide stimulatory signals for adult β-cell proliferation upon inflammation (Xiao et al., 2014). Furthermore, Baeyens et al. showed that cytokines from immune cells can work with other growth factors to convert exocrine cells into β-like cells (Baeyens et al., 2014). Most importantly, immune cells in islets are closely related with β-cell stress and apoptosis during the pathogenesis of type I (T1D) and type II diabetes (T2D), which will be detailed in further sections of this chapter.

Pancreas development and β-cell differentiation

Our understanding of pancreas development mostly comes from studies based on mouse models (Figure 1.2). In mice, pancreas development starts at E8.5, when two specific groups of cells (dorsal and ventral) in the posterior foregut region are committed to pancreas progenitor cells by expressing transcription factors Pdx1 and Ptf1a. Lineage tracing experiments showed that these progenitor cells are common ancestors of all

three types of mature pancreatic cells (i.e. acinar, duct and islet cells) (Gu et al., 2002; Kawaguchi et al., 2002). During the following period (E9.5-E12.5), pancreatic buds are expanded by cell proliferation. The dorsal and ventral pancreas subsequently fuse together after a gut rotation. In this stage (termed as "primary transition"), only a small number of endocrine cells are generated, and most of them are glucagon-positive.

Starting from E12.5, the pancreas undergoes the "secondary transition", which is the major endocrine cell differentiation phase. The basic helix-loop-helix (bHLH) transcription factor Ngn3 directs multipotent pancreatic progenitor cells to adopt the endocrine fate and initiates the endocrine differentiation program (Gu et al., 2002; Jenny et al., 2002). The developing pancreas at this stage can be spatially discriminated as two distinct domains: the "trunk domain" that contains bipotent endocrine/duct progenitor cells and newly differentiated endocrine cells, and the "tip domain" that contains acinar and multipotent progenitor cells (Zhou et al., 2007). Plexus remodeling – a dynamic tissue morphogenesis in which pancreatic epithelial tubules are gradually transformed to the mature branched ductal arbor – is tightly associated with endocrine cell differentiation (Bankaitis et al., 2015). Certain regulatory factors act downstream of Ngn3 to direct the differentiation of distinct islet cell types, such as Arx for α-cells and Pax4 for β-cells (Collombat et al., 2003). Although the functional mechanisms of these factors are unclear, it is generally believed that they trigger the cascade of gene expression networks and finally activate the expression of hormones (Pan and Wright, 2011). This is also the time when the majority of exocrine cells are committed from pancreatic progenitor cells.

After E16.5, endocrine cells gradually aggregate into islet clusters and become mature islet cells. After birth, the mass expansion for both exocrine glands and islets is
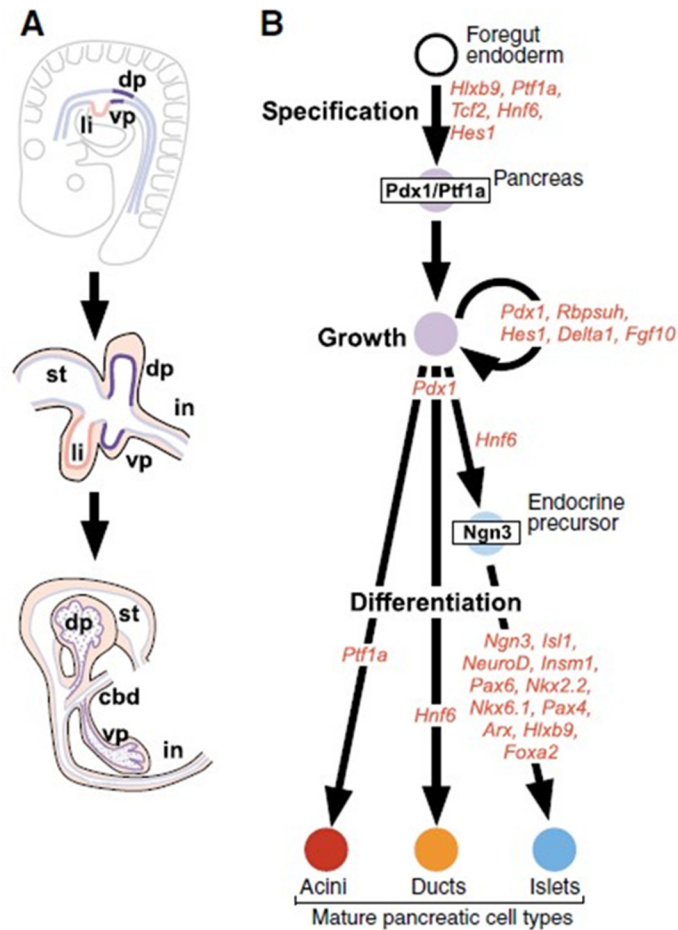
Figure 1.2 The development of mouse pancreas and related gene regulatory networks. (A) Two groups of pancreatic progenitor cells (ventral and dorsal) are first derived from the foregut endoderm at embryonic day 9.5 (E9.5). The two pancreatic primordia are further thickened due to cell proliferation. Subsequently, they fused into a single tissue unit after a gut rotation. li: liver; dp: dosal pancreas; vp: ventral pancreas; st: stomach; in: intestine; cdb: common bile duct. (B) The pancreas development and endocrine cell differentiation are regulated by a set of key transcription factors. See text for more details. Figure is adapted from (Murtaugh, 2007).

mostly due to cell proliferation under normal physiological conditions, and the proliferation rate gradually decreases from juvenile to adult (Pan and Wright, 2011).

1.3 β-cell functional maturation

The GSIS pipeline

The physiological importance of the β-cell cannot be overstated: it is the only source to produce and secrete insulin for glucose homeostasis in vertebrates. The principal mechanism for β-cell function is glucose-stimulated insulin secretion (GSIS), a process involving a tightly regulated cascade of biological events (Figure 1.3). In brief, glucose is first transported into the β-cell by certain glucose transporters. In the β-cell, glucose undergoes glycolysis and further metabolism in the mitochondria. The glucose metabolism generates a large amount of ATP, which leads to the closure of ATP-sensitive potassium (K_{ATP}) channels. Subsequently, β-cell membrane is depolarized, resulting in the opening of the voltage-dependent Ca^{2+} channels (VDCCs). Finally, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is increased by Ca^{2+} influx, and the high $[Ca^{2+}]_i$ triggers the exocytosis of insulin vesicles that are pre-docked to the plasma membrane (De Leon and Stanley, 2007). It should be noted that our understanding of GSIS is mostly based on rodent models, and the difference between rodent and human models have not yet been fully explored. For instance, human β-cells use Glut1 and Glut3 as glucose transporters, rather than Glut2, which is predominantly used in mouse β-cells (Dai et al., 2012; McCulloch et al., 2011). The inconsistency may be due to the distinct nutrient assimilation patterns between these two species, as Glut2 and Glut1/3 have different K_m values to glucose (De Vos et al., 1995). This type of findings raises the caveat that knowledge obtained from mouse studies cannot always be applied directly to the human physiology.
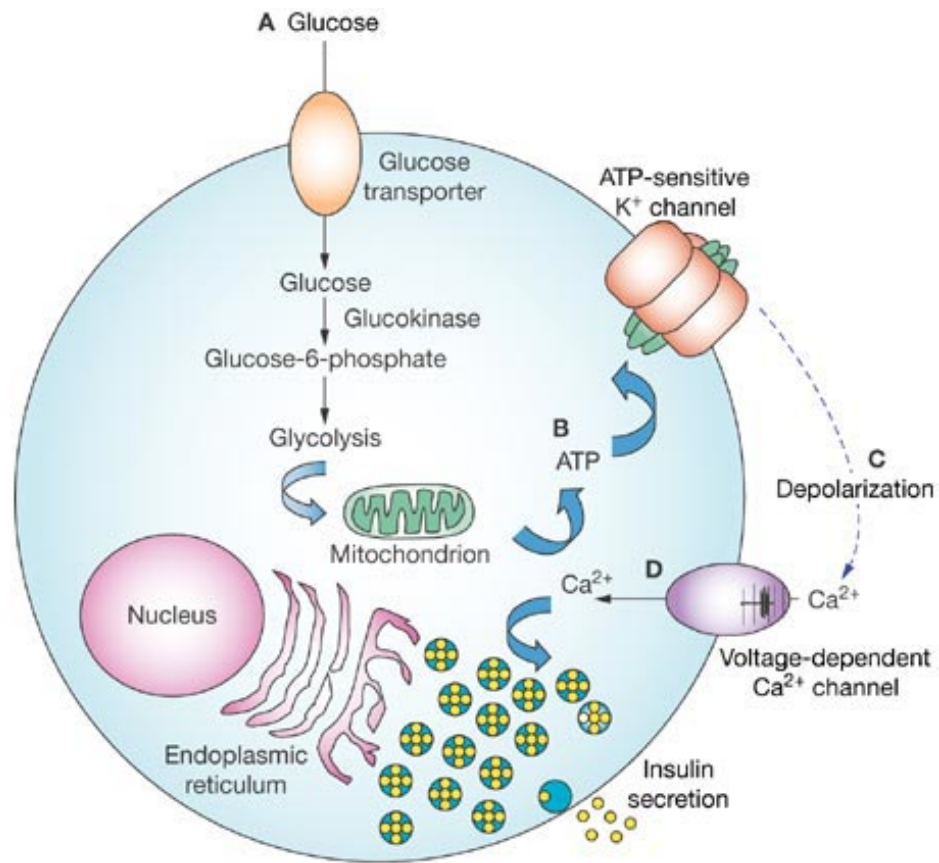
Figure 1.3 Glucose-stimulated insulin secretion (GSIS). GSIS is the fundamental mechanism of insulin secretion. During this process, glucose is transported into the β-cell by the glucose transporter Glut2 (A). In the β-cell, glucose undergoes glycolysis and further metabolism in the mitochondria. The glucose metabolism generates a large amount of ATP (B), which leads to the closure of ATP-sensitive potassium (K_{ATP}) channels. Subsequently, the β-cell plasma membrane is depolarized, resulting in the opening of the voltage-dependent Ca^{2+} channels (C). Finally, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is increased by Ca^{2+} influx, and the high $[Ca^{2+}]_i$ triggers the exocytosis of insulin vesicles that are pre-docked to the plasma membrane (D). Figure is adapted from (De Leon and Stanley, 2007).

There are two distinct phases in insulin secretion. The first phase of secretion is robust and ephemeral (several minutes), and it is followed by a slower but more sustained second phase secretion (up to several hours depending on the glucose level and the duration of the hyperglycemia) (Seino et al., 2011). Correspondingly, it has been proposed that there are two different insulin granule pools within a single β-cell: a readily releasable pool (RRP) that is closely associated with the plasma membrane and accounts for a small percentage of total insulin granules, and a reserve pool (RP) that accounts for the vast majority of granules but cannot be quickly released (Daniel et al., 1999; O'Connor et al., 1980). Previously it was conceived that in the first phase, RRP insulin granules are pre-docked at the plasma membrane so that they can quickly undergo membrane fusion upon Ca^{2+} influx, while RP insulin granules are located deeper inside β-cells and need to be translocated to the plasma membrane before being docked and fused (Barg et al., 2002; Renstrom et al., 1996). New studies based on real-time imaging, however, have found that in both phases of secretion, insulin granules are freshly recruited to the plasma membrane and become immediately fused to the plasma membrane upon stimulation (Kasai et al., 2008; Shibasaki et al., 2007). Other factors, such as the remodeling of F-actin barriers that regulate the granule mobility, may instead account for the difference in the two secretion phases (Wang and Thurmond, 2009, 2010). Despite the disparity, it is well accepted that the insulin secretion is a dynamic biphasic process in which two distinct yet related secretion events are mediated by interactions between numerous membrane-associated molecules and are regulated by a set of signaling pathways (Seino et al., 2011).

Molecular components involved in insulin vesicle exocytosis

One of the most extensively studied groups of membrane proteins during GSIS belongs to the SNARE complex that mediates granule fusion (Gauthier and Wollheim,

2008b; Iezzi et al., 2004; Jahn and Scheller, 2006). The exocytosis of insulin granules requires the pairing of a vesicle-associated membrane protein (VAMP, also known as v-SNARE) with a binary cognate receptor complex that comprises SNAP25 and a syntaxin protein (t-SNARE) to form the heterotrimeric SNARE core complex. In addition, there are a group of accessory factors, such as Munc18 secretory proteins, to regulate the interaction between SNARE complex components (Rizo and Sudhof, 2002).

It has been a major knowledge gap regarding the mechanism by which Ca^{2+} influx triggers the final insulin granule exocytosis. Studies from neural system provide strong evidence that synaptotagmins, a group of vesicle-associated membrane proteins, play the role as Ca^{2+} sensors that connect Ca^{2+} signaling and membrane fusion (Chapman, 2002). In brief, in the absence of Ca^{2+}, the C-terminals of synaptotagmins, known as C2A and C2B, only weakly interact with phosphatidylinostitol 4,5-bisphosphate ($PtdIns-4,5-P_2$) of the plasma membrane. By contrast, upon binding with Ca^{2+}, the C2B domain changes its electrostatic property and penetrates into $PtdIns-4,5-P_2$ rafts in the plasma membrane (Chapman and Davis, 1998). This tight interaction in turn brings the secretory vesicle into a close proximity to the exocytotic site and also promotes the formation of the SNARE complex (Figure 1.4). Subsequently, the binding force within the SNARE complex, combined with synaptotagmin-membrane interactions, overcomes the hydration barrier between the vesicle and the plasma membrane. Finally, upon contact with the plasma membrane, the exocytotic vesicle open the pore for the fusion and release its contents outside the cell (Chapman, 2002).

Several members in the synaptotagmin family have been shown to have little to no Ca^{2+} affinity due to certain amino acid substitutions in the C2A and/or C2B domains, such as Syt4, Syt11 and Syt13 (Sudhof, 2002). The roles of these Ca^{2+}-insensitive synaptotagmins remain controversial, but several studies have shown that they also have important regulatory roles in vesicle exocytosis (Johnson et al., 2010b; Zhang et
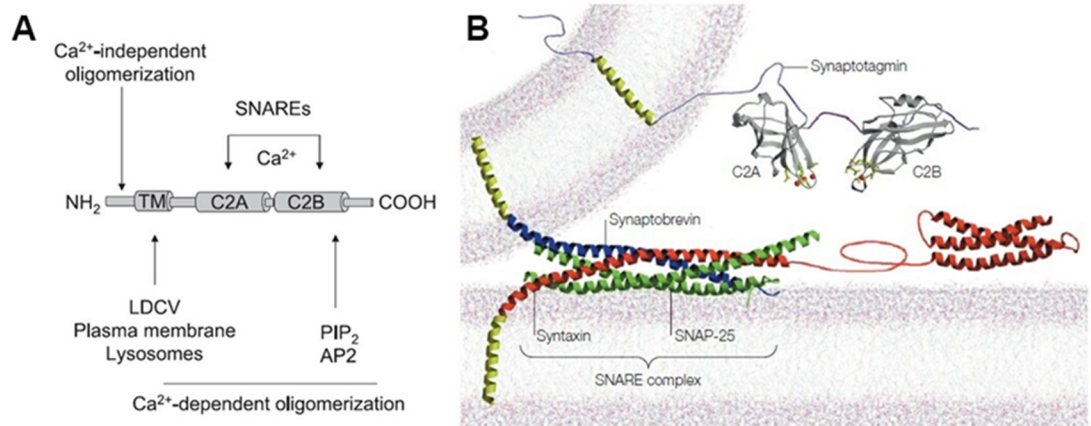
Figure 1.4 Typical synaptotagmins act as Ca^{2+} sensors during the vesicle exocytosis. (A) Schematic diagram showing the domain structures of a typical synaptotagmin. The N-terminal transmembrane domain interacts with the exocytotic vesicle, and two C-terminal domains (C2A and C2B) interact with Ca^{2+}. Upon binding with Ca^{2+}, C2A and C2B domains interact with phospholipids in the plasma membrane and promote formation of SNARE complex. Synaptotamins can form dimers via the N-terminal domain or the C-terminal domains, in a Ca^{2+}-independent or a Ca^{2+}-dependent way, respectively. In addition, synaptotagmins are also involved in regulating endocytosis by interacting with the AP2 complex. LDCV, large dense core vesicle; PIP2, phosphatidylinositol 4,5-bisphosphate; AP2, adaptor protein 2. Figure A is adapted from (Gauthier and Wollheim, 2008a). (B) Relative position of the synaptotamin and the SNARE complex. See the text for more details. Figure B is adapted from (Chapman, 2002).

al., 2010). One possible functional mechanism for these atypical synaptotagmins is to interact with the above-mentioned Ca^{2+}-binding synaptotagmins and modulate the function of the latter, as synaptotagmins commonly form homodimers and heterodimers (Chapman et al., 1996; Fukuda et al., 1999; Osborne et al., 1999).

The synaptotagmin-mediated vesicle fusion has been demonstrated in β-cells (Gauthier and Wollheim, 2008b). Several synaptotagmin family members have been shown to be essential in insulin secretion and glucose homeostasis (Gauthier and Wollheim, 2008b; Gustavsson et al., 2008b; Iezzi et al., 2004). For instance, Gustavsson et al. found that *Syt7* null mutation impairs mouse insulin secretion without affecting other GSIS events such as glucose metabolism, ultrastructural insulin granule organization and Ca^{2+} response (Gustavsson et al., 2008b). In another study based on rat islets and the INS-1 β-cell line, Iezzi et al. found that Syt5 and Syt9 colocalize on insulin granules and that a RNAi-mediated downregulation of their expression impaired the β-cell responsiveness to high glucose stimulation (Iezzi et al., 2004).

It remains largely unexplored how the function of synaptotagmins is regulated. Recent studies suggested that they might be downstream effectors of the cAMP signaling pathway, which is an important regulatory mechanism to potentiate GSIS (Wan et al., 2004; Wu et al., 2015). For instance, Wu et al. showed that cAMP plays the role of "second messenger" to activate PKA, which in turn phosphorylates Syt7 and sensitizes the secretory machinery upon Ca^{2+} influx (Wu et al., 2015). Of significance, the cAMP signaling pathway is the major cellular machinery accounting for the pro-GSIS role of intestine-derived incretins, such as glucagon-like peptide 1 (GLP-1) and gastric inhibitory peptide (GIP) (Seino et al., 2010). Further efforts can be geared towards modulating this signaling pathway and/or the activity of synaptotagmin to improve the glucose response of *in vitro* generated β-cells.

GSIS from the intact islet

β-cells are clustered into the islet, and consequently they secrete insulin as a coordinated cell group. It has been long recognized that the intact islet architecture is essential for robust GSIS. Dispersed β-cells, which lack cell-cell contact, secrete less insulin in response to elevated glucose than intact islets (Halban et al., 1982).

The interaction between physically connected β-cells is necessary in coordinating their heterogeneity. Dispersed β-cells show asynchronous glucose transport, Ca^{2+} influx and insulin secretion (Bennett et al., 1996; Van Schravendijk et al., 1992). In contrast, their behaviors within the islet are mostly synchronous. Previous studies have shown that the gap junction, an intercellular channel mediating the shuttle of small molecules (such as ions) between adjacent cells, is responsible for the functional coordination between β-cells within the same islet (Farnsworth and Benninger, 2014). Loss of *connexin-36* (*Cx36*), a key gap junction component, results in asynchronous Ca^{2+} oscillations in different β-cells and compromises the overall islet Ca^{2+} wave (Benninger et al., 2008). Consistently, *Cx36*^{-/-} islets exhibit a disrupted insulin pulsatility (i.e. reduced insulin peak levels), despite an extended releasing time (Benninger et al., 2008; Speier et al., 2007). As a result, although mice lacking functional β-cell gap junctions have similar average insulin levels compared to their wild-type counterparts, they are glucose intolerant due to aberrant pulsatile insulin secretion. These findings suggest that, in addition to the β-cell function, the coordination of insulin secretions from individual β-cells is essential in generating high insulin level peaks to counter high glucose levels (Benninger and Piston, 2014).

Notably, although the overall islet GSIS is largely synchronized, accumulating evidence have shown that β-cells within an intact islet are still heterogeneous (Roscioni et al., 2016). For instance, the expression levels of β-cell identity genes (such as *insulin*, *glut2*, *Pdx1*, *glucokinase*, *MafA*, etc.) are variable across β-cells within an islet (Beamish

et al., 2016; Jetton and Magnuson, 1992; Johnston et al., 2016). The inconsistency in gene expression may reflect these β-cells have different capabilities in GSIS and cell proliferation (Roscioni et al., 2016).

Interestingly, β-cell heterogeneity may be specifically designed to orchestrate the GSIS within an intact islet. By using live-cell imaging and optogenetics, a recent study showed that a minor population of β-cells within an intact islet can play the role of "pace-maker", i.e. their electrical activity precedes and outlasts that of the rest of β-cells (Johnston et al., 2016). This group of β-cells, termed as "hub cells" for their super connectivity, are important in coordinating Ca^{2+} signaling and insulin secretion across the islet. Notably, compared to the rest of β-cells (i.e. "follower cells"), "hub cells" exhibit higher glucose metabolism but lower insulin content. These properties make "hub cells" adaptive to the activate role in synchronizing glucose-stimulated Ca^{2+} influx throughout the islet. This finding suggests that the specific groups of β-cells in the heterogeneous population may be assigned to subtly different physiological roles involved in GSIS.

Our understanding of β-cell heterogeneity and its relationship with the islet GSIS is far from complete (Roscioni et al., 2016). Further research in this field needs to be conducted to determine how this heterogeneity is established during the pancreas development and how it is maintained postnatally. Furthermore, the investigation of molecular mechanisms involved in GSIS should take account of the whole islet microenvironment, rather than only focusing on the processes happened within a single β-cell.

GSIS and β-cell maturation

GSIS is not only the principal function of β-cells, but also a hallmark of their maturity. The newly differentiated β-cells at embryonic stages, despite expressing

insulin, do not respond to glucose stimulation. Islets isolated from newborn rodents and human fetal tissues secrete nearly equal amounts of insulin under both low (≤ 5.6 mM) and high (≥ 11.1 mM) glucose conditions, suggesting that these young β-cells have not established the functional molecular machinery to process signals from glucose stimulation (Adam et al., 1969; Beattie et al., 1994; Hellerstrom and Swenne, 1991). A recent study found that newborn β-cells are actually glucose-sensitive, but the glucose threshold required to stimulate insulin secretion is much lower than that of adult β-cells (Blum et al., 2012). Therefore, β-cells not only need the robust response with insulin secretion upon high glucose stimulation, but also need the capability to inhibit insulin basal secretion under low glucose levels, in order to be qualified as functional and mature. In mice, temporal insulin secretion assays using isolated islets revealed that β-cells do not acquire maturity until postnatal day 9 (Blum et al., 2012). Due to the lack of temporally continuous human islet samples, the precise time point when human β-cells become mature has not been determined (Pan and Brissova, 2014).

β-cell maturation is a complicated process in which the expression of a set of genes is activated to regulate biological events associated with GSIS. One key transcription factor governing the maturation is MafA. Studies in the mouse model have found that loss of function of *MafA* does not affect β-cell differentiation, but rather compromises β-cell mass and function at postnatal stages (Hang et al., 2014; Zhang et al., 2005). MafA regulates numerous genes involved in GSIS, including the glucose transporter *Glut2*, the zinc transporter *Slc30a8*, the initiator of glucose metabolism *glucokinase*, *insulin*, and many others. The function of *MafA* is less studied in human β-cells due to the lack of sophisticated techniques for manipulating gene expression in human tissues. However, the observation that expression of *MafA* is low in the neonatal stage but substantially increased in the adult stage suggests that this molecule plays a similar role in regulating human β-cell maturation (Dai et al., 2012; Jeon et al., 2009).

Besides transcription factors, the role of microRNAs in tuning gene expression during β-cell maturation has also been identified (Jacovetti et al., 2015; Kanji et al., 2013). Downregulation of certain microRNAs, including miR-17-5p and miR-181b-5p, is required for the expression of genes involved in glucose metabolism and the cell cycle.

GSIS-related physiological processes need to temporally evolve to promote β-cell maturity. Among these, the switch of glucose metabolism was proposed to be a key factor for coupling glucose level and insulin secretion (Dhawan et al., 2015; Pullen et al., 2010; Pullen and Rutter, 2013; Quintens et al., 2008). Compared to the newly differentiated β-cells, mature β-cells have reduced levels of low-K_m hexokinases and instead produce high-K_m glucokinases for the first step of glycolysis. In addition, the expression of *lactate dehydrogenase* and the lactate pyruvate transporter *Slc16a1* is very low in mature β-cells. In this way, mature β-cells not only prevent anaerobic glycolysis, but they also ensure that only high levels of glucose can trigger the amount of ATP required for insulin secretion. Overexpression of neonatal-specific glucose metabolic genes in mature β-cells will disrupt glucose homeostasis and GSIS (Schuit et al., 2012). Dhawan et al. have shown that DNMT3A, a de novo DNA methyltransferase, is responsible for silencing these "disallowed genes" during β-cell maturation (Dhawan et al., 2015). However, the temporal change of other GSIS processes during β-cell maturation, such as glucose transport, insulin synthesis, vesicle transport and Ca^{2+} signaling, remained to be fully investigated.

It should be noted however, that β-cell or islet maturation is a broader concept than the establishment of GSIS *per se*. For instance, the formation of proper islet architecture and the separation of islets from the ductal epithelium are both important morphological changes during neonatal mouse pancreas development. It has been shown that several factors that interfere with β-cell maturation also disrupt these two events, such as persistent expression of *Hnf6* (Gannon et al., 2000) and constitutively

activated Cdc42 (Kesavan et al., 2014) in β-cells. Another significant characteristic of β-cell maturity is reduced cell proliferation. It has been suggested that β-cell division and GSIS are two mutually exclusive events. For example, aged β-cells have reduced proliferation capability but higher insulin production (Arda et al., 2016; Avrahami et al., 2015). There is a need for future research to study how these developmental processes, including reduced cell proliferation, islet cell delamination from pancreatic ducts and islet clustering are orchestrated during β-cell maturation and, more importantly, whether they are required for the establishment of GSIS.

1.4 β-cell long-term functional maintenance

β-cell stress and diabetes

Both T1D and T2D are directly related to β-cell defects. T1D is caused by absolute insulin deficiency due to autoimmune destruction of β-cells, and it usually occurs in juvenile life. In contrast, T2D is more commonly seen in adult or aged individuals. T2D starts with an insulin resistance state that is usually related to prolonged nutrition overload. As a compensational mechanism for insulin resistance, both β-cell mass and insulin secretion per β-cell are increased to produce more insulin (Lin and Sun, 2010). However, this functional compensation usually does not persist, as elevated insulin secretion in a prolonged period can cause β-cell stress, which is detrimental to β-cell function and survival (Donath et al., 2005). Later, a break of this balance results in hyperglycemia, and β-cells display a deteriorative status called "β-cell failure", which is characterized by continuous β-cell loss and significant functional deficiency (Donath et al., 2005; Lin and Sun, 2010). Compared to T1D that makes up 10% of all diabetic cases, T2D is more prevalent. This introduction will focus on the relationship between β-

cell functional maintenance and T2D, as this thesis study is more related to β-cell failure found in T2D.

Under normal physiological conditions, β-cells are categorized as a long-lived cell group. While undergoing high levels of proliferation during immature stages to expand β-cell mass, β-cells are mostly quiescent in the adult stage. A continuous long-term BrdU labeling found that only 1 in ~1,400 adult mouse β-cells undergo replication per day (Teta et al., 2005). In human, β-cell proliferation is even lower (~98% of neonatal human β-cells are quiescent (Wang et al., 2015)). As stated above, β-cell division and function have been proposed to be two mutually exclusive processes. The fact of extreme slow turnover of β-cells highlights the importance of long-term β-cell vitality and function maintenance in mediating normal glucose homeostasis.

Massive insulin biosynthesis presents the β-cell with a heavy load of protein translation and folding. Proinsulin production accounts for half of all proteins translated in β-cells (Schuit et al., 1988), and glucose stimulation results in an up to 20-fold increase in the biosynthesis of insulin and other proteins required for GSIS (Fonseca et al., 2011). The accumulation of unfolded or misfolded proteins can disrupt the ER homeostasis and cause ER stress. As a result, β-cells develop a highly refined mechanism to deal with potential ER stress in order to stay consistently functional (Figure 1.5).

An immediate barrier to counter ER stress caused by misfolded protein accumulation is the endoplasmic reticulum-associated protein degradation (ERAD). The first step of the ERAD pathway is to recognize misfolded or mutated proteins based on their specific biochemical features, such as the exposure of hydrophobic regions. Then, these labeled ERAD substrates are transported from the ER lumen to the cytosol and ubiquitylated by certain E3 ubiquitination ligases. Finally, they are eliminated by the proteome degradation machinery (Ruggiano et al., 2014).
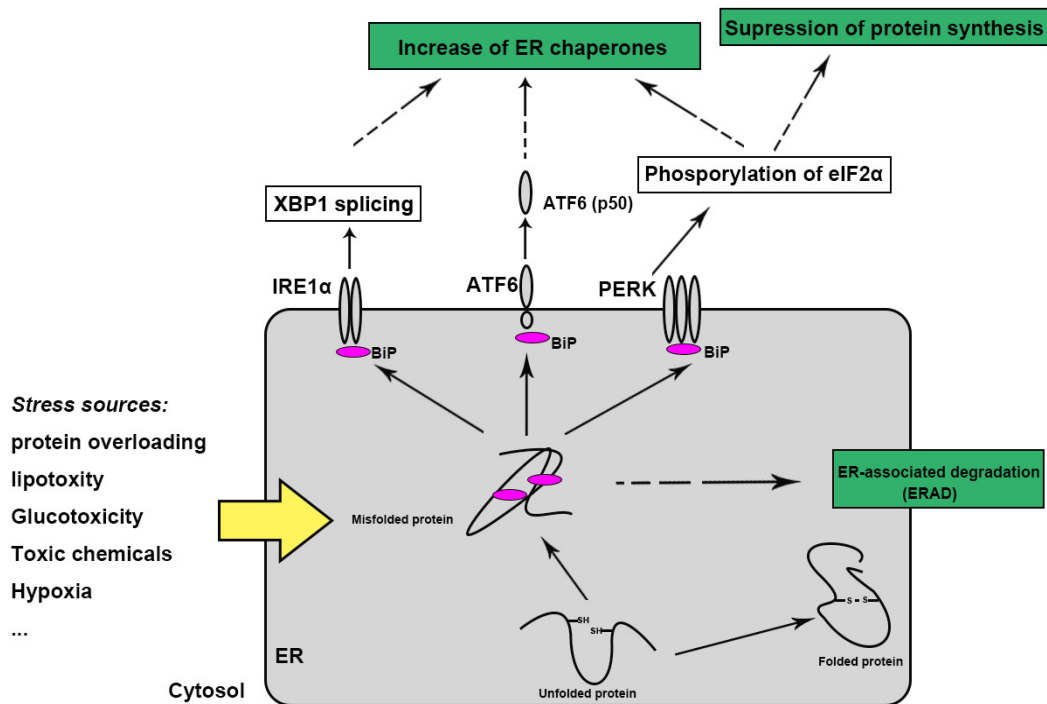
Figure 1.5 The cellular response to ER stress. While various intracellular/extracellular stress sources can contribute to ER stress, the direct factor causing ER stress is the accumulation of misfolded proteins within the ER lumen. The first cellular response to ER stress is to clear the misfolded proteins by the ERAD. In additions, ER stress activates three distinct signaling pathways, which are initiated by IRE1α, ATF6 and PERK, respectively. Following these signaling pathways, the level of ER chaperones is increased to aid protein folding. Also, the protein synthesis is reduced to ameliorate the ER burden of protein folding. BiP, also known as GRP78, is a central regulator of ER stress to activate these three signaling pathways. See the text for more details. Figure is adapted from (Omura et al., 2013).

In addition, ER stress is handled by the unfolded protein response (UPR). There are three major UPR pathways in β-cells, with distinct ER transmembrane proteins to sense unfolded/misfolded proteins in the ER lumen. The first pathway utilizes the endoribonuclease IRE1, which is activated by phosphorylation and oligomerization in response to ER stress. One IRE1 splicing target is the *X-box protein binding 1* (*XBP1*) mRNA, whose spliced form is translated into a functional transcription factor to regulate the expression chaperone and ERAD proteins. As a result, the unfolded/misfolded proteins undergo either refolding or degradation (Fonseca et al., 2011). The second UPR pathway in sensing ER stress is mediated by the transmembrane kinase PERK. When activated, PERK phosphorylates and inhibits eukaryotic initiation factor 2 (eIF2α). This in turn downregulates translation, thereby alleviating the protein folding burden within the ER lumen (Fonseca et al., 2010). The third pathway uses transcription factor ATF6 as the ER stress sensor. When activated, ATF6 is shuttled into the nucleus to increase the expression of genes involved in protein folding, processing and degradation. It should be noted that these UPR pathways are not solely designed to restore the ER homeostasis and promote cell survival; their hyperactivation in response to excessive ER stress can also trigger β-cell dysfunction and cell apoptosis (Oslowski and Urano, 2011; Szegezdi et al., 2006). Indeed, the UPR hyperactivation or malfunction has been proposed as a major cause of β-cell failure in T2D (Prentki and Nolan, 2006).

Many physiological and environmental factors can cause overwhelming ER stress that cannot be overcome and, as a matter of fact, they are directly related to diabetes. First, high glucose is the most common pathological stress to the β-cell. Under chronic high glucose conditions, increased insulin biosynthesis overwhelms ER folding capacity, leading to an imbalance in proinsulin translation and folding. In addition, continuous metabolism of high glucose increases the level of reactive oxygen species (ROS) and induces oxidative stress, which is detrimental to the function of mitochondria

(Lenzen, 2008; Poitout and Robertson, 2008). It has been proposed that chronically elevated glucose, referred to as glucose toxicity, accounts mostly for the β-cell failure in late stages of T2D. Secondly, free fatty acids (FFAs), whose levels are also commonly elevated in T2D patients, contributes to ER stress by depleting the ER Ca^{2+} pool (Gwiazda et al., 2009) and by inhibiting the ER-Golgi trafficking (Preston et al., 2009). Thirdly, hypoxia is also a source for ER stress. The ER lumen maintains an oxidizing environment to promote disulfide bond formation in proinsulin. Hypoxia negatively affects ER redox potential and thus proinsulin assembly (Zheng et al., 2012). In addition, hypoxia downregulates the ATP concentration in the ER lumen and further impairs the function of ATP-dependent chaperone proteins (Laybutt et al., 2007). Last but not least, inflammatory cytokines can also contribute to the ER stress. While it is well-known that cytokines are involved in autoimmune attack in T1D, their roles in causing β-cell stress and apoptosis in T2D have also been identified (Cnop et al., 2005). For instance, interleukin-1β (IL-1β) and interferon-γ (IFN-γ) have both been shown to induce nitric oxide (NO), which in turn depletes the Ca^{2+} pool in the ER lumen by inhibiting the sarco/endoplasmic reticulum pump Ca^{2+} ATPase 2b (SERCA2b) (Cardozo et al., 2005). In a worse scenario, IL-1β can also promote Fas-triggered apoptosis by activating NF-κB (Darville and Eizirik, 2001; Darville et al., 2001).

Intrinsic master factors governing β-cell long-term maintenance

As described earlier, β-cells are subject to detrimental impacts from various extrinsic factors that can cause ER dysfunction and severe cell stress postnatally. In response to these challenges, β-cells have developed a set of cellular mechanisms, such as the above-mentioned UPR, to process potential cell stress. However, it remains largely unknown how the expression of genes in β-cell is organized to counteract potential physiological insults and maintain fully functional in the postnatal stage.

There are several genes that, once mutated, can alone cause serious β-cell

defects and diabetes (Froguel and Velho, 1999; Prentki and Nolan, 2006). Most of these

genes encode transcription factors, whose primary function in controlling gene

expression determines their multifaceted regulatory roles in β-cell differentiation, function

and survival. Therefore, they are possible candidates for orchestrating gene expression

programs involved in the postnatal β-cell function. Below I will discuss the representative

transcription factors in this functional category.

Pdx1

One of most extensively studied genes involved in β-cell function is the

pancreatic duodenal homeobox 1 (*Pdx1*), also known as *Ipf1* (*MODY4*). Mouse studies

found that *Pdx1*, together with *Ptf1A*, determines the earliest pancreatic lineage from the

gut endoderm during pancreas organogenesis (Gu et al., 2002; Jenny et al., 2002). In

the early embryonic development, *Pdx1* is expressed in pancreatic multipotent

progenitor cells, whereas high expression level of *Pdx1* is restricted to β-cells after the

secondary transition.

In postnatal β-cells, Pdx1 regulates the expression of genes involved in various

aspects of β-cell function, including mitochondrial metabolism, ER stress, insulin

secretion, and cell survival (Sachdeva et al., 2009). In many cases, Pdx1 binds to the

promoters of target genes and directly controls their expression. These Pdx1

transcriptional targets include *insulin* (Francis et al., 2005), *glucokinase* (involved in

glucose metabolism) (Watada et al., 1996), *Atf4* (involved in the ER stress pathway)

(Sachdeva et al., 2009), *Sytl4* (involved in vesicle exocytosis) (Benner et al., 2014),

Ucn3 (involved in insulin secretion), *Puma* (involved in cell death) (Benner et al., 2014)

and many others. In addition, Pdx1 is essential to inhibit the expression of *glucagon* and

maintain the differentiated β-cell identity (Gao et al., 2014). A β-cell-specific deletion of

Pdx1 results in β-cell mass reduction, impaired β-cell function and increased

susceptibility to ER stress (Ahlgren et al., 1998). In humans, *Pdx1* heterozygous

mutations cause maturity-onset diabetes of young (MODY), an early-onset T2D

characterized by typical β-cell failure (Froguel and Velho, 1999). These combined

findings suggest that the regulatory role of *Pdx1* in β-cell function is conserved between

rodent and human.

NeuroD1

In the embryonic pancreas, NeuroD1, a bHLH transcription factor, acts

downstream of Ngn3 to regulate pancreatic endocrine lineage differentiation (Naya et al.,

1997). *NeuroD1$^{-/-}$* mice are born with islet hypoplasia and die shortly after birth with

hyperglycemia (Naya et al., 1997).

In the adult pancreas, *NeuroD1* is expressed in all islet cells and is required for

multiple biological events involved in β-cell function. Like Pdx1, NeuroD1 also directly

drives the expression of *insulin* by binding an E-box in the insulin promoter (Naya et al.,

1995). Furthermore, Kim et al. found that NeuroD1 controls the expression of *Sur1* (Kim

et al., 2002), which encodes a component of the K$_{ATP}$ channel, highlighting the regulatory

role of NeuroD1 in GSIS. Interestingly, Gu et al. found that β-cells in adult mice without

NeuroD1 displayed a metabolic profile similar to that of immature β-cells (Gu et al.,

2010a), suggesting that NeuroD1 is required for long-time β-cell maturity maintenance.

Moreover, loss-of-function mutations of *NeuroD1* in humans also cause MODY (Halban

et al., 1982; Naya et al., 1997).

Hnf-1α

Hnf-1α (also known as TCF-1) was first identified as a liver-specific transcription

factor. Later studies from mice and human, however, found that this gene also plays

essential roles in β-cell function. Loss of *Hnf-1α* function in mouse models disrupts islet architecture, reduces β-cell number and impairs insulin secretion (Hagenfeldt-Johansson et al., 2001; Yamagata et al., 2002).

By overexpressing an *Hnf-1α* dominant-negative mutant in β-cells, Yamagata et al. identified an islet dysfunction phenotype that was not apparent until 3 weeks of age (weaning age), highlighting the role of this gene in postnatal islet function (Yamagata et al., 2002). Further investigations found that Hnf-1α directly controls the expression of genes involved in GSIS, including *insulin*, *Glut2* and *L-type pyruvate kinase*, suggesting that Hnf-1α is essential for organizing the gene expression network required for postnatal β-cell maturity (Servitja et al., 2009). In addition, Hnf-1α is essential for the expression of *IGF1,* which encodes a growth factor for postnatal β-cell growth and mass expansion (Speier et al., 2007).

Human epidemiological studies also identified *Hnf-1α* as another MODY gene. Gordart et al. found that a point mutation in the *Hnf-1α* promoter causes MODY by affecting *Hnf-1α* expression (Godart et al., 2000). Another point mutation in the *Hnf-1α* coding region (G319S) leads to a strong genetic predisposition for T2D (Hegele et al., 1999; Triggs-Raine et al., 2002).

MafA

MafA, a member of the Maf transcription factor family, appears to be a unique factor in regulating postnatal β-cell function. Many β-cell- or islet-specific transcription factors (such as the above-mentioned *Pdx1*, *NeuroD1* and *Hnf1α*) are expressed both in the embryonic pancreatic epithelium and in the adult islets/β-cells. Accordingly, inactivation of these genes in the embryonic pancreas either disrupts pancreas development, or compromises endocrine lineage differentiation, whereas their ablation in postnatal β-cells leads to the loss of β-cell maturity and/or β-cell functional failure (Pan

and Wright, 2011). Compared to these factors, the expression is *MafA* is only restricted

to β-cells in both embryonic and postnatal stages (Nishimura et al., 2006a). Moreover,

inactivation of *MafA* does not affect pancreas development but mainly compromises β-

cell function and proliferation (Hang and Stein, 2011). These findings suggest that *MafA*

is specifically involved in β-cell maturation and functional maturation, but not endocrine

cell differentiation.

　　　　As described above, in mouse β-cells, MafA regulates a set of genes involved in

GSIS. (Hang et al., 2014; Zhang et al., 2005). Moreover, the MafA activity is correlated

with mouse β-cell proliferation and function (Guo et al., 2013). In human β-cells, *MafA*

expression pattern and function are both less investigated. However, MafA is an

important gene marker to evaluate the maturity of β-like cells resulting from the *in vitro*

directed differentiation of human embryonic stem cells (ESCs) or induced-pluripotent

stem cells (iPSCs). MafA-positive β-like cells are usually monohormonal and more

glucose responsive (Pagliuca and Melton, 2013). These findings suggest that similar to

its mouse counterpart, the human MafA is also essential for postnatal β-cell function.

Myt factors

　　　　Myelin transcription factors (Myt factors) are zinc-finger proteins that include

three conserved paralogs: Myt1 (NZF-2), Myt1L (Myt1-like or NZF-1) and Myt3 (St18 or

NZF-3). Previously studies in neural development showed that *Myt1* and *Myt1L* are both

involved in nerve cell differentiation. In both *Xenopus* and mice, *Myt1* acts downstream

of certain bHLH transcription factors (e.g. *Neurog1* and *Ascl1*) and counteracts the

Notch-mediated lateral inhibition to allow neuronal differentiation (Bellefroid et al., 1996)

(Vasconcelos et al., 2016). Notably, *Myt3* has the similar roles to work downstream of

Neurog1 and promote the neuronal differentiation of P19 embryonal carcinoma cells,

suggesting that the molecular function is well conserved among Myt family members (Kameyama et al., 2011).

As pancreatic endocrine lineage differentiation shares similar transcription factors and signal pathways with that of neuronal differentiation, our lab has picked Myt factors as possible candidates for regulating the islet cell differentiation (Gu et al., 2004). Previously, our lab found that *Myt1* is the only Myt family member displaying high expression levels in the embryonic developing pancreas (Wang et al., 2007). The expression of *Myt1* is activated by the bHLH transcription factor Ngn3 (Neurog3) and has a feed-forward effect to enhance the Ngn3 level (Wang et al., 2008). A pancreas-specific deletion of *Myt1* in mice resulted in a moderate phenotype of endocrine cell differentiation, which is characterized by the presence of polyhormonal islet cells (Wang et al., 2007). However, inactivation of *Myt1* increases the expression levels of *Myt1L* and *Myt3* in islets, suggesting that possible functional compensation among Myt factors might rescue the islet deficiency in the *Myt1* mutant mice. Therefore, the comprehensive roles of Myt factors needed to be further elucidated. Interestingly, a recent study in cultured β-cells showed that *Myt3* overexpression has protective effects for β-cells against cytokine-induced apoptosis (Tennant et al., 2012). This finding raises a possibility that Myt factor may be essential for β-cell survival and/or identity maintenance. In chapter III, I used a Myt triple knockout mouse line to investigate the roles of Myt factors in β-cell differentiation and functional maintenance.

Studies based on human epidemiology and mouse genetics have identified other important genes involved in postnatal β-cell function, including but not limited to *glucokinase*, *MafB*, *Hnf-1β*, *Hnf-4α*, *Nkx6.1*, *Gata4* and *Pax4* (Plengvidhya et al., 2007; Shaw-Smith et al., 2014; Sujjitjoon et al., 2016). It should be noted that there are common functional interplays between these factors. For instance, MafA and Hnf-1α were both found to regulate the expression of *Pdx1* in adult β-cells. Pdx1, NeuroD1 and

MafA act synergistically to activate *insulin* expression (Cerf, 2006). In summary, β-cell function and its long-term maintenance are governed by a complex gene expression network, which is regulated by a set of key transcription factors.

1.5 Overview of β-cell regeneration

Decades of efforts have been geared towards β-cell regeneration as a restorative therapy to treat diabetes. Several approaches have been proposed and tested for this purpose, such as activating the proliferation of pre-existing β-cells, differentiating β-cells from ESCs or iPSCs, and transdifferentiating β-cells from other terminal cell types (Figure 1.6). Each of these methods has its advantages and disadvantages, and below I will detail both the current progress and knowledge gaps that need to be resolved for each method.

Proliferation from pre-existing β-cells

As stated above, most β-cells are quiescent (i.e. at G_0 phase of the cell cycle) in adult stages. However, under certain physiological conditions, such as pregnancy and insulin resistance in which insulin demand is elevated, β-cells can re-enter the cell cycle to expand β-cell mass. Investigating the molecular mechanisms driving β-cell replication will provide clues that can be further exploited to increase β-cell proliferation and reverse the progression of diabetes.

The transition from G_0 phase to G_1/S phase has been shown to be the major rate-limiting step in β-cell cycle progression. In rodent β-cells, G_1/S phase is initiated by the interaction between a cyclin-dependent kinase Cdk4 and its functional partner cyclin D1 (Ackermann and Gannon, 2007). The Cdk4-cyclin D1 complex phosphorylates and deactivates the key cell cycle inhibitor retinoblastoma protein (Rb), thereby allowing the release of Rb-suppressed targets. One of these targets includes the E2F transcription
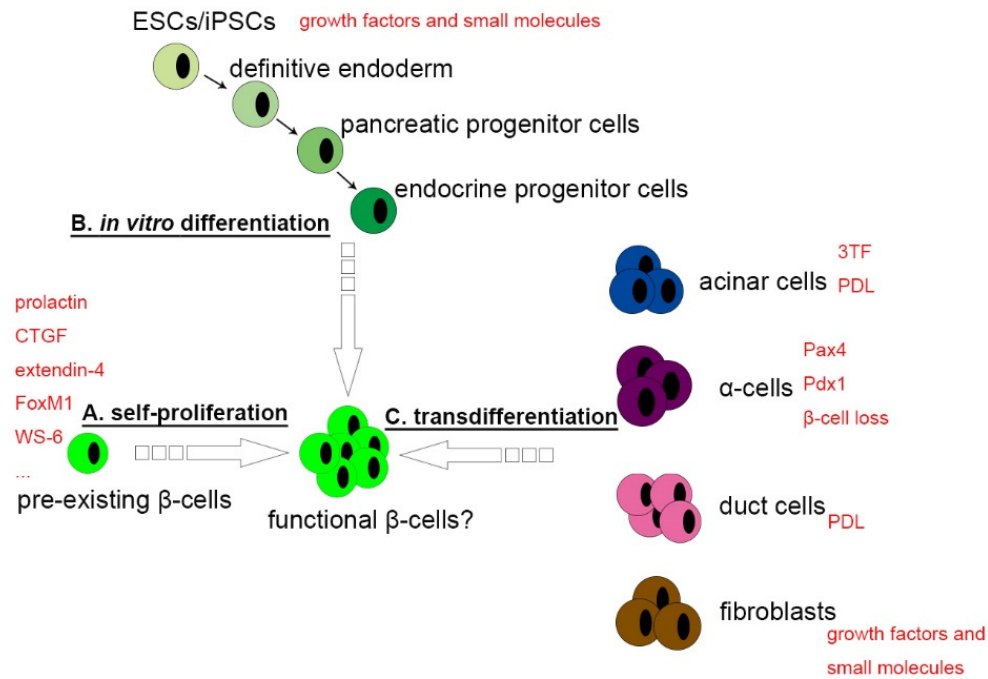
Figure 1.6 Sources of β-cell regeneration. Three β-cell regeneration approaches have been proposed and tested. (A) Self-proliferation from pre-existing β-cells. (B) Directed differentiation *in vitro* from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). (C) Transdifferentiation (also known as reprogramming) from other mature cell types. Growth factors, small molecules and physiological impacts that can promote β-cell regeneration are highlighted in red. It should be noted that newly generated β-cells from these approaches are not fully functional compared their counterparts that are derived from the *in vivo* development.

factors, which are responsible for the expression of genes involved in DNA replication and cell cycle checkpoints.

Due to the importance of the G_1/S phase transition in β-cell proliferation, its regulators are mostly related to β-cell mass dynamics. For instance, p14 (also known as INK4A), a direct inhibitor of the Cdk4/Cyclin D complex, is transcriptionally inhibited by EZH2-mediated H3K27 trimethylation to allow β-cell mass expansion in the young pancreas (Chen et al., 2009a). By contrast, the expression of *p14* is substantially increased in adult β-cells, correlating with the low proliferation rate. In another example, a H3K4 trimethylation mediated by the transcriptional coactivator Menin, increases the expression of *p18 (INK4C)* and *p27 (KIP1)*, both of which act as cell cycle repressors in maintaining β-cell quiescence. The level of Menin is downregulated in pregnancy and obesity to adapt to β-cell mass expansion (Karnik et al., 2007).

Proliferation from preexisting β-cells is considered as an important source for replenishing β-cell mass. As such, different approaches have been utilized to identify growth factors and small molecules that can act as mitogenic stimulators. First, mouse models provide a convenient platform for this type of studies. Investigating gene expression in certain physiological conditions related to β-cell mass dynamics, such as obesity, pregnancy and aging is likely to discover novel cellular targets that can be manipulated to promote β-cell proliferation. For instance, the finding that glucokinase is required for high-fat food-induced β-cell mass expansion has led to the usage of a glucokinase agonist in stimulating β-cell replication (Nakamura et al., 2009). Other factors identified in a similar way includes exendin-4 (GLP-1 receptor receptor) (Xu et al., 1999), CTGF (Guney et al., 2011; Riley et al., 2015), prolactin (Brelje et al., 1994) and so on. Secondly, high-throughput screening (HTS) approaches have been developed to identify pro-proliferative small molecules and peptide based on *in vitro* β-cell or islet culture model. For instance, WS6, a rat β-cell mitogen identified by HTS

assays, was found to be able to stimulate replication for both rodent and human β-cells (Shen et al., 2013).

There are two caveats in using this strategy. The first one is that the newly generated β-cells by replication may not be functional. As stated above, insulin secretion and β-cell division are likely to be mutually exclusive, and it should be determined whether the upregulation of cell proliferation would compromise β-cell function. The second one is to prevent tumor generation. One of the few β-cell tumors, insulinoma, can result from the disruption of the cell cycle, such as the loss of function of the cell cycle inhibitor Menin (Zhuang et al., 1997). It is therefore important to conduct controllable cell proliferation and prevent potential tumorigenesis.

Differentiation of β-cells from ESCs or iPSCs

The understanding of pancreas development and β-cell differentiation, which provides invaluable clues to directed differentiation *in vitro*, and the emergence of iPSCs, which makes stem cells much more available, together boost the generation of β-cells from their undifferentiated and pluripotent progenitors. There are two main options in doing this: by overexpressing key transcription factors involved in lineage specification and β-cell maturation, or by applying key growth factors or small molecules to activate signal pathways for β-cell differentiation. In both ways, ESCs or iPSCs undergo a cascade of intermediate differentiation states that largely recapitulate the *in vivo* development: definitive endoderm, pancreatic endoderm, endocrine progenitor cells, β-like cells and mature β-cells (Pagliuca and Melton, 2013).

Thanks to decades of basic research in developmental biology using mouse models, key transcription factors and signal pathways involved in each stage of β-cell differentiation have been identified (Pan and Wright, 2011). It is generally considered that human pancreas development shares common lineage specification and gene

usage with the ones in mice (Jennings et al., 2015; Pan and Brissova, 2014). Indeed, several groups have utilized immuohistological methods to demonstrate that the expression of key transcription factors during human pancreas development matches their mouse counterparts both temporally and spatially (Jennings et al., 2013; Jeon et al., 2009; Lyttle et al., 2008; Piper et al., 2004). As the investigation of human development by direct genetic manipulation is out of reach, a very recent study took advantage of *in vitro* human ESC differentiation models combined with sophisticated genome editing tools (TALEN and CRISPR/Cas9) to investigate human β-cell differentiation (Zhu et al., 2016b). This study showed that the regulatory roles of key transcription factors, such as Pdx1, Ngn3, PTF1A, MNX1, HES1, are well conserved between mouse and human β-cell development. During *in vitro* β-cell differentiation, these transcription factors, if not directly transduced into pluripotent cells, should be endogenously activated by growth factors or small molecules in order for the directed differentiation to proceed. Therefore, they often serve as biomarkers for successful differentiation in each stage, such as Sox17 and FoxA2 for the definitive endoderm, Pdx1 and Ptf1A for the pancreatic endoderm, Ngn3 for endocrine progenitor cells, Nkx6.1 and Pdx1 for β-like cells, and MafA for mature β-cells.

In addition to growth factors that are used to manipulate specific signal pathways and transcription factors for directed β-cell differentiation, many small molecules have been identified through HTS assays for the same purpose. For instance, Chen et al. found that indolactam V, a PKC activator, can trigger the differentiation of human ESCs into *Pdx1*-expressing cells (Chen et al., 2009b). Indolactam V can be combined with IDE1, another small molecule targeting TGF-β signaling pathway, to further facilitate the induction from ESCs to pancreatic progenitors. Remarkably, recent studies in epigenetics have identified genome-wide epigenetic dynamics during ESCs and/or iPSCs to β-cell differentiation (Xie et al., 2013). This type of knowledge further promotes

the discovery of potential small molecules targeting epigenetic modifiers for directed β-cell differentiation. In particular, the recently developed SAHA-PIP, a sequence-specific DNA-binding hairpin pyrrole–imidazole polyamides (PIPs) combined with a pan-HDAC inhibitor SAHA, can be used to manipulate epigenetics in a site-specific manner (Anandhakumar et al., 2014; Pandian et al., 2012; Syed et al., 2014). Compared to conventional small molecules, this type of techniques can manipulate a broader range of genes in a more specific manner, and therefore they are anticipated to improve gene targeting efficiency for *in vitro* β-cell differentiation.

By optimizing stepwise application of growth factors and/or small molecules, *in vitro* differentiation of β-like cells is readily achievable. However, these β-like cells, despite being monohormonal, usually do not respond to glucose unless they are transplanted into animal models to undergo further maturation. It is still a major challenge to generate authentic β-cells *in vitro* that are functionally equivalent to their *in vivo* differentiated counterparts. Two recent studies by the Melton group and the Kieffer group made a significant progress in addressing this issue (Pagliuca et al., 2014; Rezania et al., 2014). They both optimized the scalable differentiation protocol and successfully differentiated human ESCs to glucose-responsive β-like cells. However, the intrinsic cellular mechanism by which β-like cells gain GSIS profile has not been determined. In addition, how long these *in vitro* generated β-cells can survive while maintaining functional competency remains an open question. These two critical issues need to be addressed before the *in vitro* generated β-cells can be applied in clinical trials.

Transdifferentiation of β-cells from other mature cell types

Similar to directed differentiation from stem cells, transdifferentiating other mature cell types to β-cells relies on transducing β-cell-specific transcription factors or applying growth factors/small molecules to the starting cell types.

Due to genetic similarities, most attempts in this field focus on mature pancreatic cells that share a common developmental origin with β-cells, such as α-cells, pancreatic duct cells and acinar cells. The transcription factors used in transdifferentiation are required not only to establish β-cell-specific gene expression profiles, but also to repress the expression of genes that are specific for starting cell types. For instance, Collombat et al. found that ectopic expression of *Pax4* in α-cells reprograms α-cells into β-cells without affecting β-cell proliferation (Collombat et al., 2009). In this case, Pax4 plays a dual role in both promoting β-cell fate and repressing α-cell fate. In another study, Yang et al. found that ectopic expression of *Pdx1* in immature α-cells represses the expression of *glucagon* and *Arx* and further reprograms these cells into β-cells via a glucagon$^+$insulin$^+$ intermediate bihormonal state (Yang et al., 2011). Interestingly, the reprogramming effect of Pdx1 is context-dependent: its ectopic expression in later mature glucagon-positive cells does not cause a complete α-to-β cell conversation. This finding suggests that the establishment of a new cell identity is affected by the reprogramming factor types and the intrinsic plasticity of starting cell types. While these two studies both took advantage of transgene tools for the overexpression, Zhou et al. utilized an adenovirus system to deliver the desired genes, which is more achievable in clinical trials (Zhou et al., 2008). In this study, the overexpression of three β-cell-specific transcription factors, Pdx1, Ngn3 and MafA (also known as "3TF") transdifferentiates acinar cells into β-cells and successfully ameliorates the streptozotocin-induced hyperglycemia.

Furthermore, various physiological impacts can promote *in vivo* β-cell

neogenesis by transdifferentiation. Among this, the pancreatic duct ligation (PDL), in

which the main pancreatic duct draining the pancreatic tail is surgically ligated, has been

exploited to induce pancreatic duct or acinar cells to β-cell transdifferentiation (Pan et al.,

2013; Xu et al., 2008). However, the outcome of PDL varies among different labs, with

many labs claiming there are very little, if any, β-cell neogenesis events contributing to

β-cell mass expansion (Cavelti-Weder et al., 2013; Kopp et al., 2011; Solar et al., 2009).

In addition, although it was proposed that inflammation might be the key factor for PDL

to promote β-cell regeneration, the underlying mechanism remains largely unknown. In

another example, Herrera and colleagues found that α-to-β cell conversion was triggered

in mice after a near-total β-cell ablation, which was achieved by a β-cell-specific

diphtheria-toxin transgene (Thorel et al., 2010). In this study, when the diabetic mice

were given insulin to maintain euglycemia, the β-cell regeneration from this source

significantly recovered β-cell mass and reversed the progression of diabetes. A later

study from the same group showed that in the juvenile mice with a similar β-cell ablation,

the neogenesis of β-cells was mainly from δ-to-β cell conversion, suggesting that cell

reprogrammable plasticity was different among different stages (Chera et al., 2014).

These studies are particularly intriguing in that their mouse models highly resembled the

T1D scenario, which is also characterized by a near-total loss of β-cell mass. However, it

remains unknown whether these newly generated β-cells can survive and be maintained

in the T1D islet microenvironment, which is enriched with detrimental autoimmune

infiltration.

Finally, small molecules have also been commonly used to aid the conversion of

other mature cell types to β-cells *in vitro*. In a recent study, Ding and colleagues

successfully transdifferentiate human fibroblasts to β-like cells via intermediate

endodermal progenitor cells and pancreatic progenitor cells (Zhu et al., 2016a). In their

approach, the application of small molecules, including CHIR99021 (a GSK-3 inhibitor), RG108 (a DNMT inhibitor), parnate (a LSD1 inhibitor) and sodium butyrate (a HDAC inhibitor) substantially enhanced endodermal reprogramming efficiency. Similar to the scenarios in targeted differentiation, the small molecules used here manipulate cell-specific gene expression profiles by modulating signaling pathways and epigenetic dynamics.

1.6 Significance of the thesis study

Patients with T1D and late stage of T2D are both characterized by severe defects in β-cells. Traditionally, they rely on exogenous insulin administration to aid glucose homeostasis. However, it is difficult to control the precise dosage corresponding to continuously fluctuating blood glucose, and this type of therapy can easily result in hypoglycemia, ketosis and deteriorated insulin resistance (Nathan et al., 2009). As β-cells have the most refined molecular machinery to sense high glucose and secrete insulin at the correct time, targeting β-cells has long been considered as a promising restorative therapy for diabetes (Vetere et al., 2014).

The past decade has seen a remarkable progress in β-cell regeneration, as stated above. However, at least two issues need to be addressed before this technology can be readily applied to clinical treatment. First, the newly generated β-like cells often lack glucose responsiveness as compared to their *in vivo* differentiated counterparts. In most cases, *in vitro* derived β-like cells resemble immature β-cells in fetal or newborn stages (Hrvatin et al., 2014). Secondly, how long these newly derived β-like cells can be maintained as fully functional remains unknown. Indeed, several recent studies have reported that lineage-converted cells are phenotypically instable or prone to apoptosis, highlighting the concern for improving their long-term durability (Chen et al., 2012; Lee et

al., 2013). As β-cells can face various stresses during their long-term life cycle, their robustness and resilience in handling physiological stress are critical for their survival and function. These two bottlenecks necessitate further understanding of β-cell maturation and functional maintenance.

In the first part of this thesis research, I sought to identify master regulatory genes governing β-cell maturation by investigating the acquisition of β-cell maturity in neonatal mice. By dissecting individual biological processes in the GSIS pipeline, our lab has previously discovered that the establishment of proper response to Ca^{2+} influx is a rate-limiting step for β-cell functional maturation (Gu et al, unpublished observations). By a potassium-stimulated insulin secretion assay, here I found that insulin vesicles in immature β-cells are more sensitive to Ca^{2+} simulation than those in mature β-cells. I then focused on genes with regulatory roles in Ca^{2+}-coupled vesicle exocytosis, particularly these exhibiting differential expression during β-cell maturation. These criteria led to my candidate gene, synaptotagmin IV (Syt4), which belongs to the synaptotagmin family with established roles in regulating synaptic vesicle exocytosis in nerve cells. My quantitative gene expression assay showed the expression level of *Syt4* is not only increased during β-cell maturation, but also is among the highest of all synaptotagmins in adult β-cells. Subsequently, mouse studies found that *Syt4* ablation in mature β-cells resulted in elevated basal insulin secretion, while *Syt4* overexpression in immature β-cells repressed basal insulin secretion. These findings suggest that Syt4 is not only necessary but also sufficient to promote GSIS during β-cell maturation. Mechanistically, although Syt4 itself does not act as a Ca^{2+} sensor, I found that it can interact with Syt7, a Ca^{2+}-binding synaptotagmin. Furthermore, *Syt4* overexpression phenocopies *Syt7* ablation in GSIS and insulin vesicle docking, suggesting that Syt4 may interfere with the Ca^{2+} sensing function of Syt7. Overall, this part of my thesis research showed that Syt4 plays an inhibitory role in modulating Ca^{2+} sensitivity of

insulin vesicles, which is necessary to reduce basal insulin secretion and augment GSIS during β-cell maturation. Intriguingly, the research from our collaborators showed that *Syt4* overexpression repressed basal insulin secretion in human pluripotent cell derived β-like cells, suggesting that this molecule could be further exploited to improve β-cell regeneration for clinical usages.

In the second part, I focused on the *Myt* gene family. As described above, previous studies in our lab suggest that Myt factors might play significant roles in β-cell differentiation and/or β-cell function. However, due to the possible functional redundancy, the previous loss-of-function study regarding Myt1 might not reflect the comprehensive function of Myt factors. Here, based on a *Myt* triple-knockout mouse line derived by our lab, I sought to investigate regulatory roles of Myt factors in pancreas development and β-cell generation. To our surprise, the overall β-cell number and insulin expression remained unaffected in fetal and neonatal stages upon deletion of Myt factors. Instead, *Myt* mutant β-cells displayed functional deficiency, cell apoptosis and dedifferentiation in postnatal stages. Moreover, I identified severe cell stress in *Myt* mutant β-cells, which could explain the observed β-cell functional and survival deficiency. In agreement with these defective phenotypes, gene expression analysis found that the expression of genes involved in GSIS and cell survival was compromised upon deletion of Myt factors. Meanwhile, a subset of cell stress and apoptotic genes were activated. Interestingly, I observed the aberrant gene expression of *Myt* mutant β-cells at the neonatal stage, when β-cell identity and subcellular morphology both appeared normal. These combined findings reveal a role of Myt factors in setting up the gene expression network in immature β-cells, which is required for further β-cell maturation and functional maintenance. Overall, my thesis research in this part establishes Myt family members as key factors for generating functional β-cells that not

only display robust GSIS, but also have the capability to maintain a long-term function in postnatal stages.

This thesis research are anticipated to provide valuable insights into optimizing β-cell regeneration. First, the proper adjustment of Ca^{2+} sensing is an important step during β-cell maturation. Therefore, further efforts need to be directed toward understanding the regulatory mechanism by which Ca^{2+} influx triggers insulin vesicle release. Second, Syt4 plays an inhibitory role in basal insulin secretion by reducing Ca^{2+} sensitivity of insulin vesicles. Our finding that *Syt4* overexpression inhibits basal insulin secretion of immature β-cells would promote the usage of this molecule in generating β-cells with better glucose response. Further research can be conducted to identify growth factors or small molecules that can increase Syt4 level in β-like cells derived from *in vitro* differentiation. Third, Myt factors are required for immature β-cells to grow into the fully functional state. Like that of Syt4, the expression levels of Myt factors not only can be used to assess the maturity and functionality of *in vitro* differentiated β-cells, but also can be manually increased to facilitate functional β-cell regeneration. Finally, our finding regarding the temporal expression of *Myt1* in mice provides a novel idea in conducting *in vitro* stepwise differentiation. The expression of *Myt1* is turned on as early as E9.5 in the multipotent pancreatic progenitor cells (Gu et al., 2004), while its major function is exerted only after the abundant appearance of β-cells (i.e. after E15.5). Therefore, it may be worth trying to place the pro-maturation factors in the early steps during the directed differentiation (such as the transition from pancreatic progenitor cells to endocrine progenitor cells).

CHAPTER II

SYNAPTOTAGMIN IV REGULATES PANCREATIC β-CELL MATURATION BY MODULATING THE SENSITIVITY OF INSULIN VESICLES TO CALCIUM STIMULATION

2.1 Abstract

At neonatal stages, immature β-cells reduce basal insulin secretion while enhancing high glucose-stimulated insulin secretion (GSIS) to become functionally mature. Investigating the mechanisms by which the mature insulin secretion profile is established will provide novel insights into functional β-cell regeneration for clinical usage. In this study, we find that insulin vesicle hypersensitivity to Ca^{2+} stimulation is a significant factor contributing to the high basal insulin secretion of immature β-cells. Syt4, a vesicular synaptotagmin with low Ca^{2+} affinity, is responsible for repressing the Ca^{2+} sensitivity of insulin vesicles during β-cell maturation. In mice, inactivating *Syt4* in mature β-cells increases basal insulin secretion and compromises high GSIS, while precocious *Syt4* overexpression in immature β-cells reduces basal insulin secretion. Mechanistically, Syt4 physically interacts with the Ca^{2+} sensor Syt7 and attenuates Syt7-dependent insulin vesicle docking to the plasma membrane. Furthermore, *Syt4* overexpression in human pluripotent cell-derived β-like cells reduces their basal insulin secretion, while *Syt4* knockdown in a human β-cell line impairs the glucose response. Our findings therefore reveal that Syt4-mediated fine-tuning of insulin vesicle Ca^{2+} sensitivity is a key mechanism for β-cell functional maturation.

2.2 Introduction

Mature β-cells have a tightly-regulated insulin secretion profile. They secrete little insulin under basal glucose levels (~5.6 mM in rodents and ~3.3 mM in humans) and increase secretion by several folds at high glucose levels (>10mM) (Rutter et al., 2015). These properties are necessary to prevent both hypoglycemia under fasting state and hyperglycemia after the increase of the postprandial blood glucose. Newly differentiated β-cells, also known as immature β-cells, have the opposite properties: they secrete high levels of insulin at basal glucose levels but lack high GSIS (Grasso et al., 1968; Pildes et al., 1969). Immature β-cells must undergo a maturation process to fine-tune their gene expression, cellular structures, and metabolic profiles to become mature β-cells. The detailed molecular pathways that drive this maturation process, during which insulin secretion becomes glucose responsive, are largely unknown.

Decades of studies have shown that modulating the metabolic profiles of β-cells is a major factor driving β-cell maturation [(Lemaire et al., 2016) and references therein]. Immature β-cells produce high levels of low-K_m hexokinases (HK1 and HK2) and lactate dehydrogenase (LDHA) (Blodgett et al., 2015; Dhawan et al., 2015; Hrvatin et al., 2014; Jermendy et al., 2011). The presence of these enzymes enables the basal flux of glucose to glycolysis, which in turn causes elevated basal insulin secretion (Lemaire et al., 2016). Moreover, immature β-cells lack the cellular machineries for oxidative phosphorylation and membrane excitability. As a result, immature β-cells cannot generate large amounts of ATP needed for high GSIS. (Gu et al., 2010b; Hrvatin et al., 2014; Jermendy et al., 2011; Rorsman et al., 1989). In contrast, mature β-cells produce low levels of HK1/HK2/LDHA; instead, they use the high-K_m glucokinase for the first step of glycolysis. In addition, mature β-cells possess the full complement of gene products necessary for oxidative phosphorylation (Hrvatin et al., 2014; Jermendy et al., 2011). Based on these

metabolic properties, mature β-cells have limited basal glucose metabolism and basal insulin secretion, but they can produce large amounts of ATP for high insulin secretion upon high glucose stimulation (Dhawan et al., 2015).

The transition of metabolic profile, however, is unlikely the sole factor driving β-cell maturation. In mice, although postnatal day 2 (P2) immature β-cells have similar electrical activity and Ca^{2+} influx compared to adult β-cells (Rozzo et al., 2009), the proper β-cell GSIS is not established until after P9 (Blum et al., 2012; Nishimura et al., 2006b). Therefore, Ca^{2+}-secretion coupling may be defective in immature β-cells. Moreover, Blum et al. showed that immature β-cells, but not mature β-cells, increase insulin secretion in response to a weak glucose stimulation (i.e. glucose concentration is elevated from 0.5 mM to 2.8 mM) (Blum et al., 2012). This finding suggests that mature β-cells have a higher glucose threshold for insulin secretion than that of immature β-cells. The factors that determine this threshold remain unknown; however the efficiency of both glucose metabolism and Ca^{2+}-secretion coupling can contribute to this threshold regulation.

Synaptotagmins (Syts) are vesicular proteins that regulate Ca^{2+}-secretion coupling in nerve cells (Craxton, 2004; Sudhof, 2012). Upon Ca^{2+} binding, the C2A and C2B domains of Syts insert into the phospholipids of the plasma membrane to shorten the distance between the synaptic vesicle and the plasma membrane. The close proximity between the vesicle and the plasma membrane facilitates the assembly of SNARE complex, which in turn promote the vesicle-plasma membrane fusion (Sudhof, 2012). In β-cells, several Syts (Syt1, 3, and 7) have been shown to perform similar functions in promoting insulin vesicle exocytosis (Brown et al., 2000; Gao et al., 2000; Gauthier et al., 2008; Gauthier and Wollheim, 2008a; Gustavsson et al., 2008a; Gut et al., 2001; Iezzi et al., 2005; Iezzi et al., 2004; Nakajima-Nagata et al., 2004; Wu et al., 2015). However, compared to the neuronal system, the molecular mechanism for Syts-mediated Ca^{2+}-secretion coupling is much less studied in β-cells.

There are 17 Syts in mammals (Berton et al., 2000; Dean et al., 2009; Fukuda et al., 2003). Not all Syts act equally in promoting secretion: eight of them (Syt1-3, 5-7, and 9-10) have high affinity for Ca^{2+} and they were reported to potentiate secretion (Bhalla et al., 2008). The other family members (Syt4, 8, and 11-17) lack high Ca^{2+} affinity due to certain amino acid substitutions in the C2A and/or C2B domains (Littleton et al., 1999; Thomas et al., 1999), and they appear to repress secretion (Bhalla et al., 2008). Among this latter group, Syt4 is the best studied isoform. It has been shown that Syt4 represses basal neural transmission in cochlear inner ear hair cell synapses and PC12 cells (Johnson et al., 2010a; Machado et al., 2004; Moore-Dotson et al., 2010). Moreover, Syt4 directly interacts with Syt1 to inhibit the interaction between Syt1 and phospholipids, thereby reducing the efficiency of Syt1-mediated vesicle-plasma membrane fusion (Littleton et al., 1999).

Here we provide evidence that insulin vesicles in immature β-cells have higher sensitivity to cytoplasmic Ca^{2+} stimulation than those in mature β-cells, resulting in elevated basal insulin secretion. During the neonatal stage, increased expression of *Syt4* contributes to the downregulation of this Ca^{2+} sensitivity and further promotes β-cell functional maturation. Mechanistically, Syt4 binds to Syt7 and interferes with the Ca^{2+} sensor function of Syt7. Of significance, *Syt4* can be overexpressed to inhibit the basal insulin secretion in human pluripotent cell derived β-like cells, highlighting its potential application in improving β-cell regeneration for clinical usage.

2.3 Results

Immature β-cells secrete more insulin than mature β-cells upon equivalent membrane depolarization and Ca^{2+} influx

Ca^{2+} influx triggers insulin granules to fuse with the plasma membrane to release insulin. Thus, both the level of free intercellular Ca^{2+} ($[Ca^{2+}]_i$) and the

competence of insulin vesicles in responding to Ca^{2+} affect the extent of GSIS. We

here propose a model that the regulation of insulin vesicle Ca^{2+} sensitivity is a key

step in establishing proper GSIS profile (Figure 2.1A). In this model, insulin vesicles

in immature β-cells are highly sensitive to Ca^{2+}. Therefore, low $[Ca^{2+}]_i$ under basal

glucose levels can stimulate the release of insulin vesicles, resulting in elevated

basal insulin secretion in immature β-cells. Moreover, the sustained basal insulin

secretion depletes releasable insulin vesicles and thus impairs the secretion under

high glucose levels. In contrast, insulin vesicles in mature β-cells are less sensitive to

Ca^{2+} so that only high Ca^{2+} influx under high glucose levels is able to initiate vesicle

release. In this way, mature β-cells have low basal insulin secretion and maintain a

substantial releasable insulin vesicle pool for the high glucose-stimulated insulin

secretion (Figure 2.1A).

We tested this model by assaying KCl-stimulated insulin secretion (KSIS) of

isolated mouse islets. KCl directly depolarizes the β-cell plasma membrane to

activate the Ca^{2+} influx. KSIS therefore reveals the insulin vesicle sensitivity to Ca^{2+}

without the complication of glucose metabolism.

Without glucose, KCl can promote insulin secretion in both immature (P1 and

P4) and mature β-cells (P12 or adults) (Blum et al., 2012). Upon 25 mM KCl

stimulation, we found that immature β-cells secreted ~4-fold more insulin than mature

β-cells (secretion was normalized against total insulin content, Figure 2.1B). The

different KSIS was observed during both 10 minutes and 45 minutes of KCl

stimulation (Figure 2.1B), which correspond to the first and second phase of insulin

secretion, respectively (Rorsman and Renstrom, 2003). Furthermore, although

including 2.8 mM basal glucose in the KSIS assay induced higher secretion over

using KCl alone, immature β-cells still secreted over 4-fold more insulin than mature

β-cells (Figure 2.1B). This latter finding suggests that the difference in KSIS between

immature and mature β-cells is not a result of lacking glucose metabolism. For this

analysis, we did not utilize islet-equivalent (IEQ) or DNA content per-islet to

normalize the insulin secretion. As neonatal islets have a lower number of β-cells than mature islets, insulin secretion over IEQ/DNA content cannot be reliably used to compare secretion between neonatal and mature β-cells.

Insulin granules in immature β-cells have higher sensitivity to Ca^{2+}-mediated exocytosis

In order to understand why the KSIS is higher in immature β-cells, we first examined insulin content per β-cell. If the immature β-cell contains less total insulin than the mature β-cell, the percentage of secreted insulin from the immature β-cell can appear higher due to the smaller denominator. To test this possibility, we quantitatively isolated β-cells from the RIP-mCherry mouse line (Zhu et al., 2015) and assayed the insulin content. After normalizing the insulin content to the number of β-cells, we found that each immature (P1 and P4) and mature (P12 and P60) β-cell contained ~8 pg and ~13 pg insulin, respectively (Figure 2.1C). This 1.6-fold difference could not fully account for the ~4-fold differences in KSIS between immature and mature β-cells (Figure 2.1B).

The higher KSIS in immature β-cells might also due to higher insulin content in the secretory vesicle. To this end, we compared the diameters of insulin vesicle dense cores in immature and mature β-cells via transmission electron microscopy (TEM). The vesicle dense core contains the insulin crystal, and it size reflects the insulin content in the vesicle. We did not detect a significant difference in the size of vesicle dense core, which displayed a variation less than 10% amongst samples from all stages (Figure 2.2). These combined findings suggest that the higher KSIS in immature β-cells is not due to differences in the total insulin content per β-cell or vesicle properties, but should rather be attributed to more secretion events per β-cell.

Since KCl stimulates insulin secretion by directly depolarizing the plasma membrane, we speculated that the high basal insulin secretion in immature β-cells is mostly due to either elevated Ca^{2+} influx or Ca^{2+} hypersensitivity of insulin vesicles.
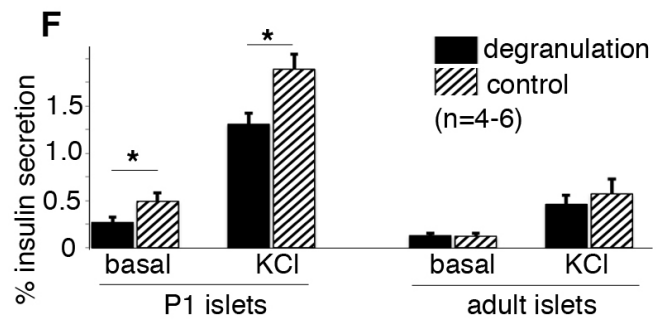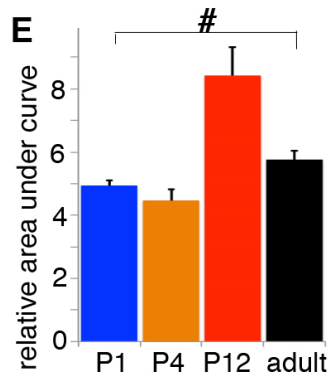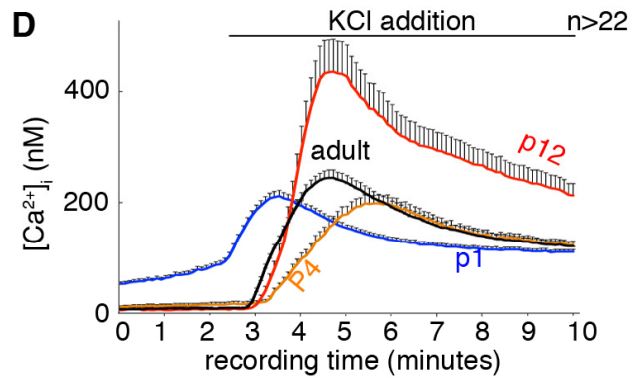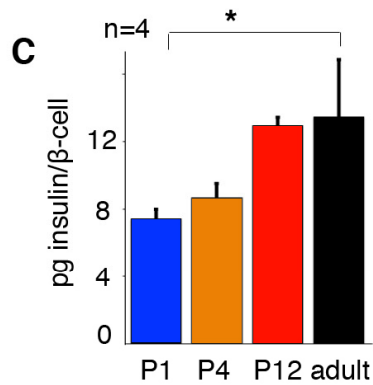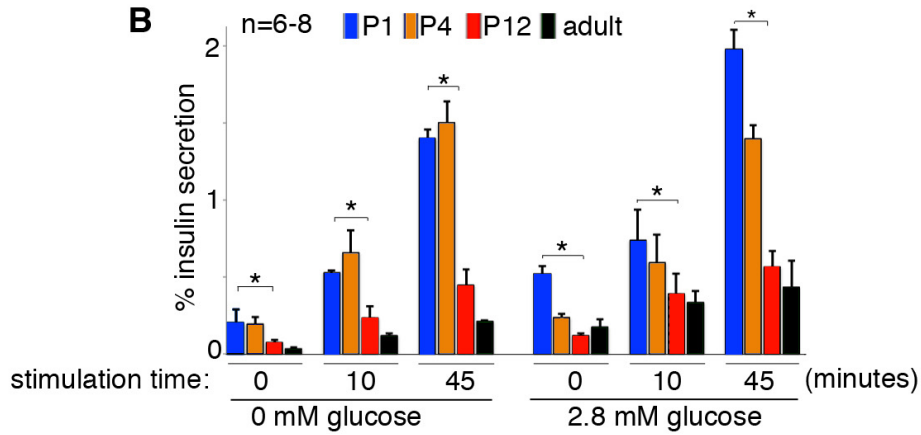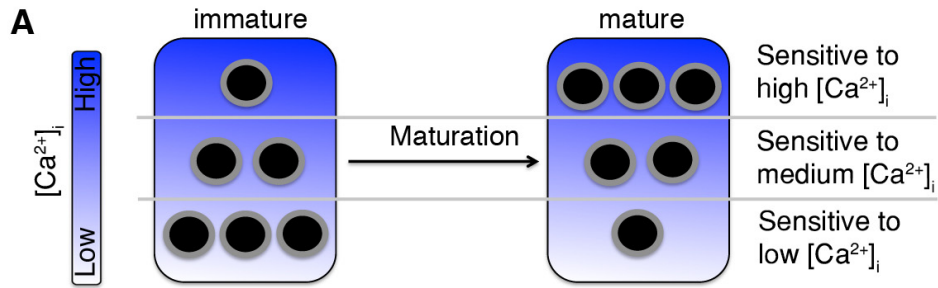
Figure 2.1 Equivalent membrane depolarization results in more insulin secretion in immature than that in mature β-cells. (A) A hypothetic model to explain the insulin secretion profile in immature β-cells. We artificially classified the releasable insulin vesicles into three distinct pools that response to high, medium, and low levels of Ca^{2+}, respectively. Immature β-cells have a higher proportion of low Ca^{2+} responsive vesicles than mature β-cells. (B) The percentage of insulin secretion induced by 25 mM KCl at P1, P4, P12 and adult islets with or without 2.8 mM basal glucose. (C) The comparison of insulin content per β-cell in these postnatal stages. (D) Ca^{2+} influx induced by 25mM KCl, with the presence of 2.8 mM basal glucose. (E) Quantification of the relative Ca^{2+} activities defined with area-under-curve in panel D. Only the areas after KCl addition were included. (F) KSIS of isolated islets with or without glucose-induced degranulation, which was achieved by incubating islets in 5.6 mM glucose for one hour prior to the KSIS assay. (*: $p<0.05$, #: $p=0.43$, student t-test)
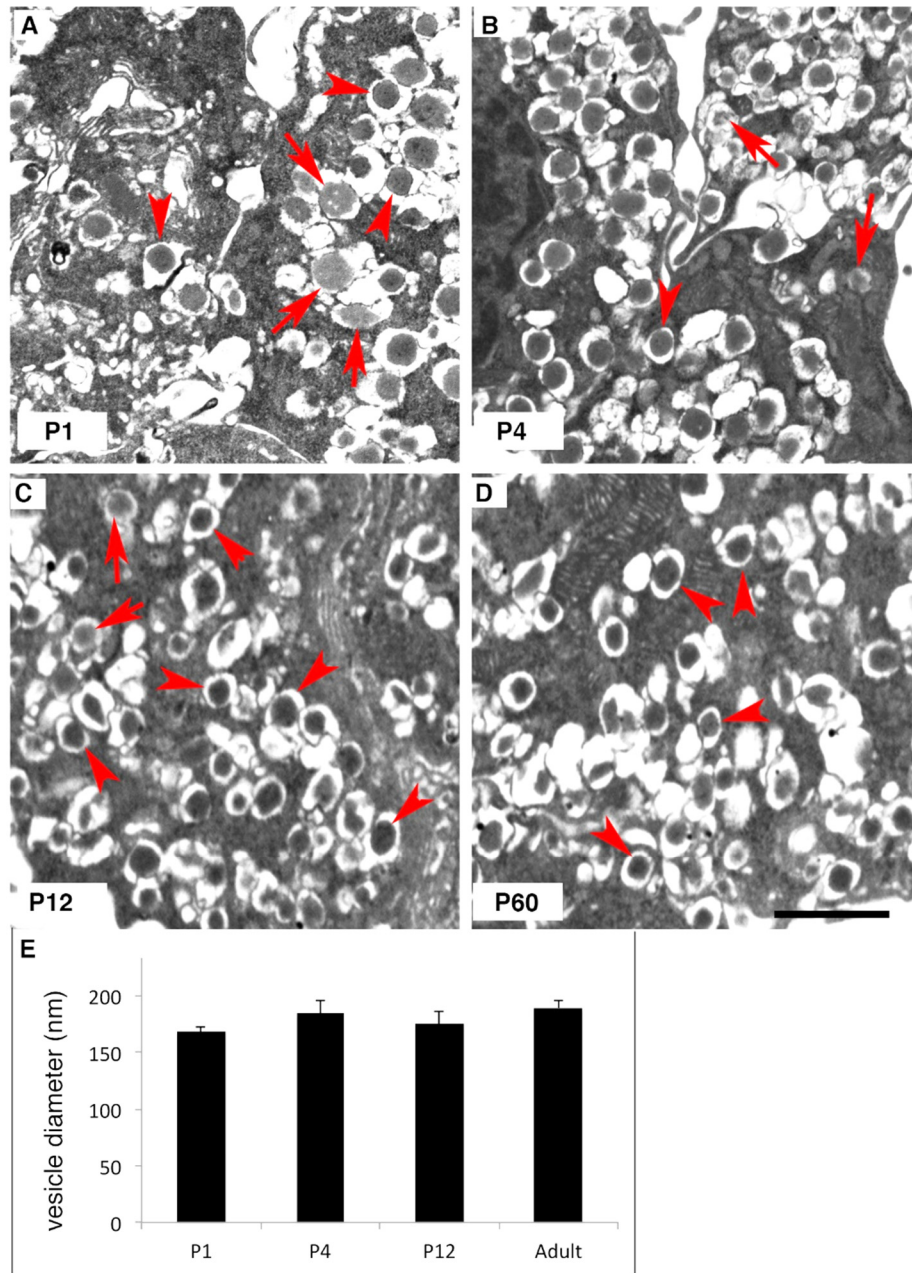
Figure 2.2 The diameter of vesicular dense core does not change from immature to mature β-cells. (A-D) Representative TEM images of β-cells at indicated stages. Arrows point to examples of immature vesicles, recognized by lower electron density and the smaller halo surrounding the dense core. Arrowheads point to mature vesicles, with obvious electron dense core and prominent surrounding halo. Scale bar: 1 μm. (E) Quantifications of dense core diameters at different stages (n = 50-100).

To test the first possibility, we systematically assayed the change of $[Ca^{2+}]_i$ during

KSIS at different postnatal stages. P1 immature β-cells showed considerable basal

$[Ca^{2+}]_i$ (50-70 nM). Upon KCl addition, their $[Ca^{2+}]_i$ quickly reached ~210 nM and

gradually dropped to a plateau around 110 nM within a few minutes (Figure 2.1D). By

comparison, P4 immature β-cells had lower basal $[Ca^{2+}]_i$ (~10 nM), which raised to

~200 nM upon KCl stimulation and dropped to a plateau (~120 nM) within minutes

(Figure 2.1D). Mature β-cells (P12 and adult) also had low $[Ca^{2+}]_i$ (5-10 nM). Their

KCl-induced $[Ca^{2+}]_i$ quickly reached 420 or 240 nM, followed by gradual decreases to

plateaus of 200 or 120 nM, respectively (Figure 2.1D). By comparing the area-under-

curve, an indicator of the overall Ca^{2+} influx, we found that P1, P4, and adult islets

displayed similar magnitude of total Ca^{2+} influx (Figure 2.1E). For unknown reasons,

P12 islets showed higher KCl-induced Ca^{2+} influx than that of other samples

(compare the red line with others in Figure 2.1D), although the KSIS in P12 islets

was similar to that of adult islets. These combined data suggest that the higher KSIS

in immature β-cells is not a consequence of higher KCl-induced Ca^{2+} influx. Instead,

it is more likely that the insulin vesicles in immature β-cells have a higher sensitivity

to Ca^{2+} induction than those in the mature β-cells.

If the above conclusion is true, then a modest glucose-induced Ca^{2+} influx

would reduce the pool of releasable vesicles in immature β-cells and compromise the

subsequent KSIS. Indeed, a one hour pre-incubation with 5.6 mM glucose prior to the

secretion assay substantially reduced the level of KSIS from newborn islets, but not

from adult islets (Figure 2.1F), suggesting that insulin vesicles in immature β-cells are

more prone to release under low glucose-induced Ca^{2+} influx.

Increased Syt4 expression correlates with reduced basal insulin secretion

We next sought to identify intrinsic regulatory factors that contribute to the

fine-tuning of the Ca^{2+} sensitivity of insulin vesicles during β-cell maturation. Notably,

both activators and inhibitors of secretion could be involved: either the increased

expression of inhibitors (Figure 2.3A1) or the decreased expression of activators (Figure 2.3A2) could explain the elevated Ca^{2+} threshold for triggering insulin vesicle release in mature β-cells. In this study, we focused on vesicular proteins, although other cytoplasmic factors could indirectly participate in this modulation. Specifically, we picked synaptotagmins (Syts) as the candidates based on their known roles in coupling Ca^{2+} influx and synaptic vesicle exocytosis in neural transmission. Our previous genome-wide gene expression analysis identified eight *Syt* family members with increased expression levels in mature β-cells, and they are characterized by either high Ca^{2+} affinity (*Syt3, 5, 7,* and *9*) or little to no Ca^{2+} affinity (*Syt4, 11, 13, and 14)* (Hickey et al., 2013).

We examined the expression of these *Syts* in purified β-cells at three postnatal stages: P1 (β-cells are immature), P12 (β-cells have become mature, but the mice have not been exposed to high carbohydrate diets) and P35 (β-cells are fully functional and are characterized by massive insulin synthesis and secretion as the mice are exposed to high carbohydrate diets). Quantitative RT-PCR showed that the expression of four *Syts* (*Syt3*, *Syt4*, *Syt11*, and *Syt14*) displayed a significant increase from P1 to P35 (P≤0.019), whereas the other four did not (P≥0.072) (Figure 2.3B). We also calculated the relative abundance of each Syt by normalizing the amplification efficiency of PCR primers. Among them, *Syt4* displayed the highest expression level (>8 folds higher than *Syt3*, *Syt11*, and *Syt14*) (Figure 2.3B). Moreover, immunofluorescence staining found that the Syt4 protein level substantially increased from immature β-cells (P1 and P4, Figure 2.3C, D) to mature β-cells (P12 and P60, Figure 2.3E, F), corroborating the qRT-PCR results. This led us to focus on the role of *Syt4* in β-cell functional maturation in our subsequence study.
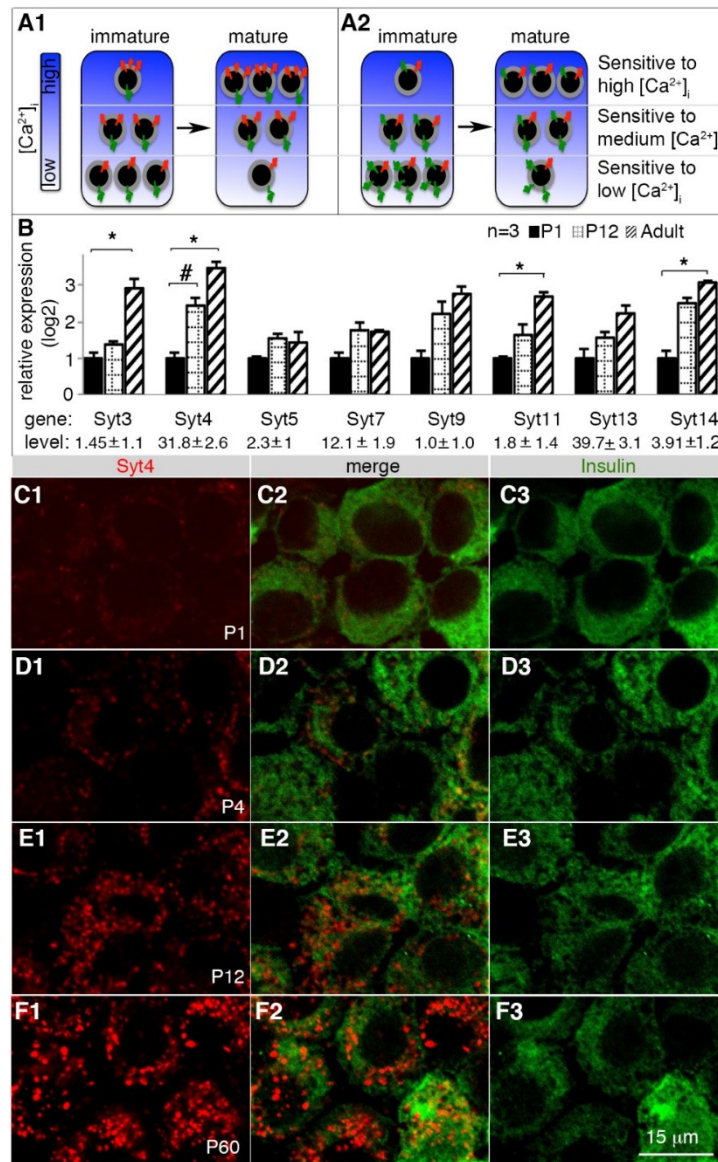
Figure 2.3 Increased *Syt4* expression level coincides with β-cell maturation. (A) A hypothetical model of modulating the Ca²⁺ sensitivity of insulin granules (black circles), with either increased Ca²⁺ sensing inhibitors (A1) or decreased Ca²⁺ sensing activators (A2) to account for the decreased Ca²⁺ sensitivity. The red and green flags on vesicles indicate inhibitors and activators, respectively. (B) The mRNA expression levels of select Syts in β-cells at P1, P12 and adult (P60) stages. β-cells were isolated and purified by flow cytometry from RIP-mCherry mice at indicated stages. (*: p=0.019, Between-subjects ANOVA test. #: p=0.04, student t-test). Note that the relative levels of each Syt mRNA, calculated based on the cycle threshold and the amplification efficacy of oligos used for PCR, are presented as well. (C-F) LSM confocal images of Syt4 (red) immunofluorescence in β-cells at indicated stages.

Syt4 is required for β-cell functional maturation

Based on previous studies regarding the inhibitory roles of Syt4 in neural vesicle exocytosis (Johnson et al., 2010a; Machado et al., 2004; Moore-Dotson et al., 2010), we hypothesized that increased *Syt4* expression during β-cell maturation is not only coincident with, but also required for the downregulation of insulin vesicle Ca^{2+} sensitivity and the repression of basal insulin secretion. To test this possibility, we first characterized the glucose metabolism and insulin secretion of the global *Syt4* knockout mouse line (*Syt4*^{-/-}) that has been generated previously (Ferguson et al., 2004). Although earlier studies showed that the *Syt4*^{-/-} mice were viable with no obvious physical abnormalities (Ferguson et al., 2004; Zhang et al., 2011), we found that they displayed a weak but significant defect in glucose clearance at 8 weeks of age (Figure 2.4A), while their insulin sensitivity remained normal (Figure 2.4B). Furthermore, *Syt4*^{-/-} mice exhibited a clear trend of reduced serum insulin during the glucose tolerance test, suggesting that their β-cell function is impaired (Figure 2.4C).

To test if the β-cell functional defect in *Syt4*^{-/-} mice was caused by improper cell differentiation, we investigated islet morphology, expression of islet cell "identity" genes (including endocrine hormones, *Glut2*, *MafA*, *MafB*, *Pdx1*, and *Nkx6.1*), and β-cell subcellular morphology. In all of these analysis, we could not detect obvious differences compared to the wild-type control, suggesting that *Syt4* is not required for islet differentiation or morphogenesis (Figure 2.5). Instead, this gene appears to be specifically involved in postnatal β-cell function.

Next we isolated islets from *Syt4*^{-/-} mice and their wild-type littermates and performed *in vitro* insulin secretion analysis. Compared to the serum insulin measurement, this method minimizes any physiological influence from the *in vivo* milieu and therefore directly targets the islet function. Intriguingly, islets isolated from two-week old *Syt4*^{-/-} mice displayed significantly higher basal insulin secretion compared to those from wild-type mice. Meanwhile, the response of *Syt4*^{-/-} islets to high glucose remained largely normal (Figure 2.4D1). This finding suggests that Syt4
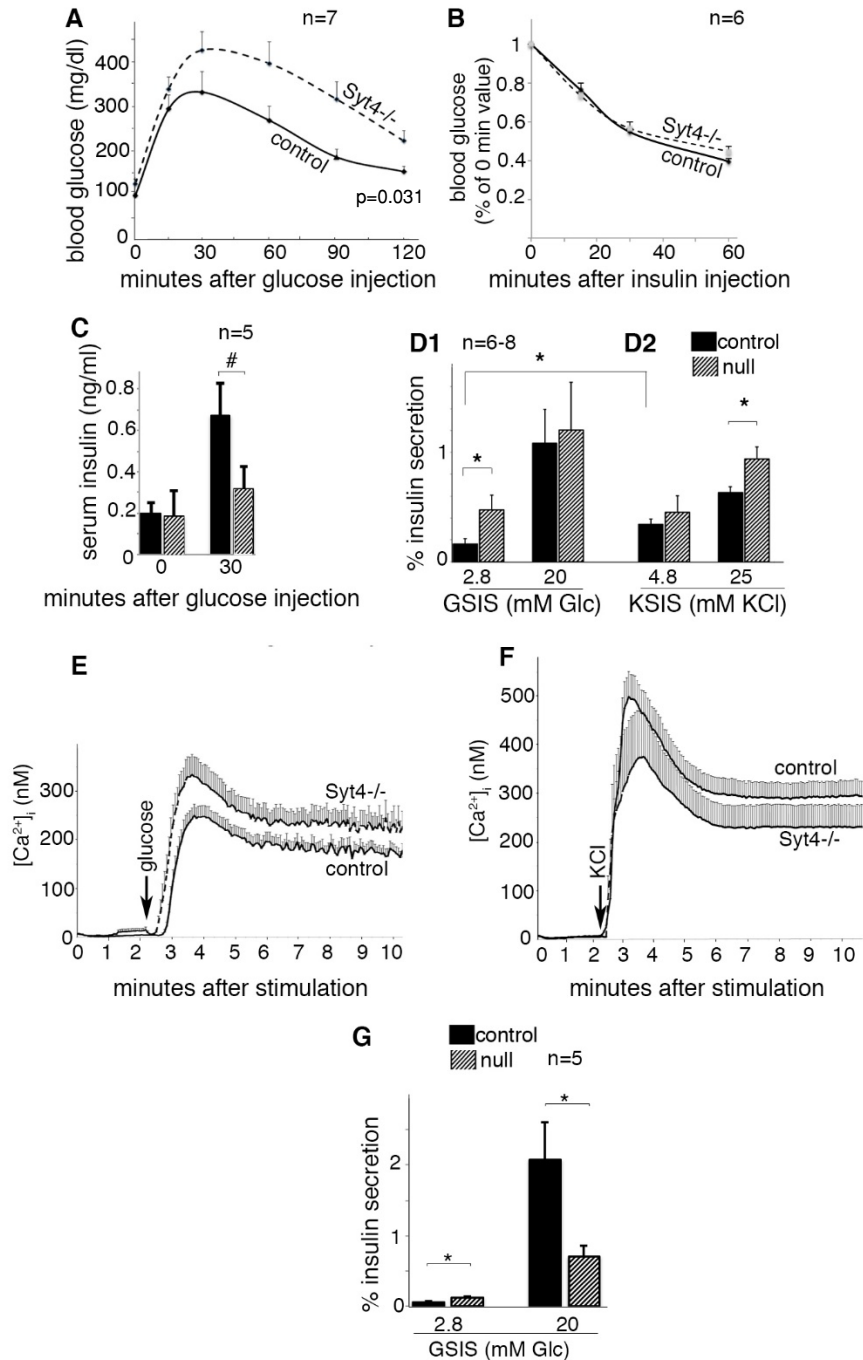
Figure 2.4 Inactivation of *Syt4* potentiates basal insulin secretion. (A) IPGTT in 8-week old control wild-type (WT) and *Syt4* null mice. The p-value was calculated by repeated-measure ANOVA. (B) Insulin sensitivity assay in 8-week old mice. Presented are the percentage of blood glucose levels after insulin injection divided by the glucose level before insulin injection. (C) Serum insulin levels during GTT test. #: p=0.07 (student t-test). (D) GSIS and KSIS of P14 islets. *: p<0.05, student t-test. (E, F) Cytoplasmic Ca^{2+} concentration induced by 20 mM glucose (C) or 25 mM KCl (D), with p>0.1 for both experiments. (G) GSIS of 8-week old *Syt4* null islets.
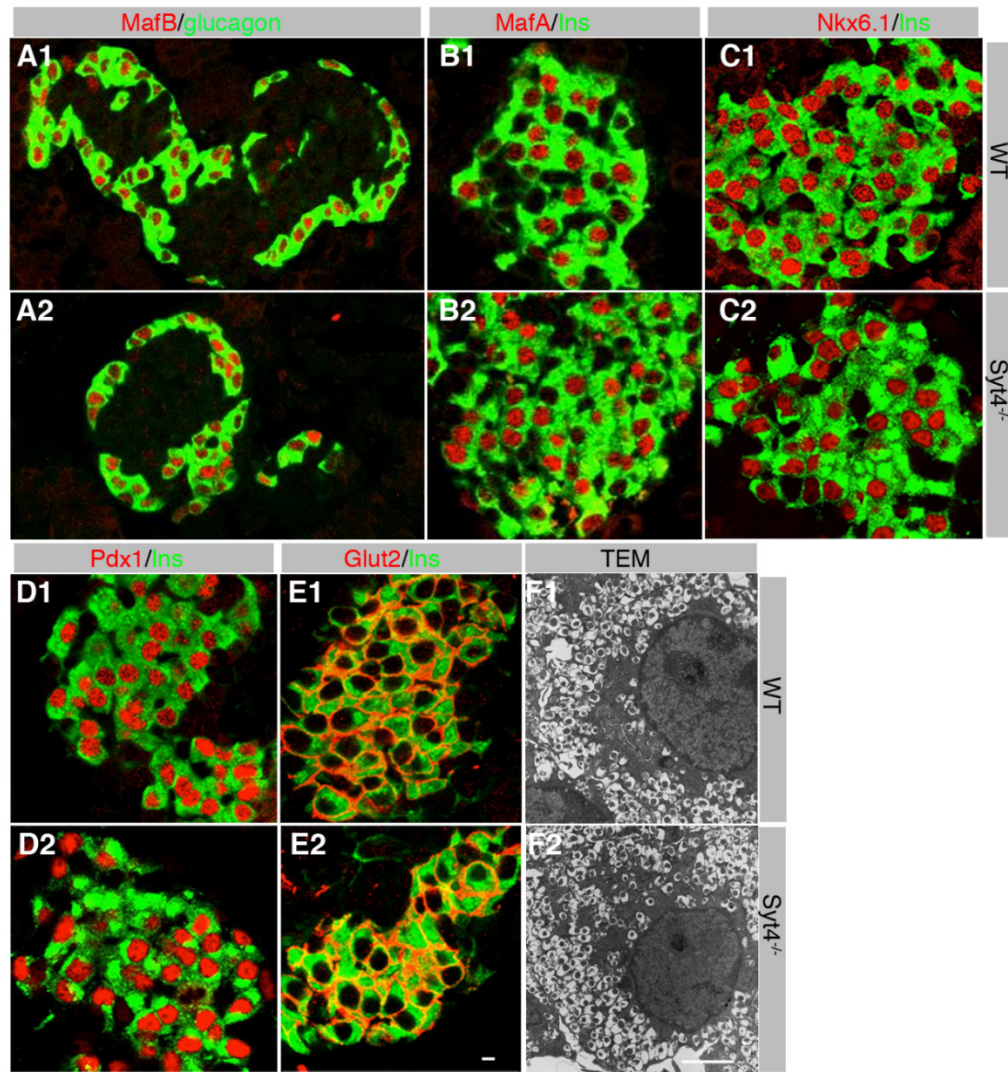
Figure 2.5 Islets in *Syt4*^-/- mice are grossly normal. (A-E) The protein expression of select endocrine genes was detected by immunofluorescence in pancreas sections from 8-week old wild-type control or *Syt4*^-/- mice. Each pair of images was captured with the same settings in order to compare the signal intensity between the wild-type control and mutant samples. (F) The subcellular morphology of β-cells from 8-week old control or *Syt4*^-/- mice was detected by TEM. Scale bar: 4 μm.

is required to repress basal insulin secretion in the newly matured β-cell and further supports the inhibitory role of Syt4 in modulating the Ca^{2+}-sensitivity of insulin vesicles.

If our hypothesis is true, the increased Ca^{2+}-sensitivity of insulin vesicles upon loss of *Syt4* function should also promote KSIS. Indeed, we found that KSIS from *Syt4[^-/-]* islets was significantly higher than that of control islets (Figure 2.4D2). Interestingly, preventing β-cell degranulation before the KSIS assay normalized the insulin secretion at basal KCl level in both control and *Syt4[^-/-]* islets, mainly by increasing the basal insulin secretion of control islets (Figure 2.4D, compare the basal insulin secretion levels in control islets in Figure 2.4D1 and D2). It is likely that the absence of glucose prior to KSIS results in accumulation of more Ca^{2+}-sensitive granules in control β-cells, which are readily releasable at the basal KCl level.

To further confirm our hypothesis, we examined the $[Ca^{2+}]_i$ in *Syt4[^-/-]* islets during GSIS and KSIS. Compared with control islets, the mutant islets showed nearly equal $[Ca^{2+}]_i$ (Figure 2.4E, F) upon both types of stimulation. This finding suggests that the different GSIS/KSIS profiles of *Syt4[^-/-]* β-cells does not result from variations in Ca^{2+} influx or any biological processes that act upstream of Ca^{2+} influx. Instead, the distinct GSIS/KSIS profiles of *Syt4[^-/-]* islets are caused by altered Ca^{2+}-sensitivity of insulin vesicles.

We also examined how loss of *Syt4* function affected islet function at later postnatal stages. Similar to the scenario in young mature islets, loss of *Syt4* also resulted in higher basal insulin secretion at 8 weeks of age, although at a smaller fold (compare Figure 2.4G and Figure 2.1D1). Interestingly, the high glucose-induced insulin secretion in these older *Syt4[^-/-]* islets was significantly reduced (Figure 2.4G), which was distinct from that of young mature islets (Figure 2.4D1). It is possible that the prolonged high level of basal insulin secretion depletes the releasable insulin vesicles, rendering an insufficient amount of insulin vesicles reserved for the secretion at high glucose levels. However, other possibilities such as the adaptive

change of β-cells to altered physiological conditions, including mild glucose intolerance, cannot be excluded without further evidence.

Taken together, our data suggest that loss of *Syt4* does not affect the general β-cell identity but instead compromises β-cell maturity by increasing the Ca^{2+}-sensitivity of insulin vesicles and impairing the repression of basal insulin secretion.

Syt4 overexpression represses basal insulin secretion but impairs islet morphogenesis

We next asked if precocious *Syt4* overexpression could have the opposite effect of inactivating *Syt4*, i.e. if *Syt4* overexpression could expedite β-cell functional maturation. If true, Syt4 would be a promising target for improving the maturity of *in vitro* differentiated β-cells.

For this purpose, we derived three *TetO-Syt4* transgenic mouse lines (#1, #2, and #3), which were further crossed with a *RIP-rtTA* mouse line to generate *TetO-Syt4;RIP-rtTA* (termed *Syt4OE* for simplicity) mouse lines. Doxycycline (Dox) was continuously administrated to the *Syt4OE* mice starting from embryonic day 16.5 (E16.5), when abundant β-cells are differentiated from endocrine progenitor cells (Figure 2.6A). Following Dox treatment, we detected a 6-8 fold increase in the Syt4 level in P1/P2 *Syt4OE* islets*,* as compared to that of *RIP-rtTA* littermates with Dox treatment or age-matched *Syt4OE* mice without Dox treatment (Figure 2.6B and data not shown). The expression level of *Syt4* thus became comparable to that of newly matured β-cells. The mouse lines #1 and #2 were selected to further test the physiological effects of *Syt4* overexpression.

Next, we investigated how the increased expression of *Syt4* affected the GSIS of immature β-cells. For *in vitro* GSIS assays, islets were isolated from *Syt4OE* mice and *RIP-rtTA* control mice (both were treated with Dox) at P4 and P7, the two representative postnatal stages when β-cells are immature and are characterized by high basal insulin secretion. Intriguingly, precocious *Syt4* overexpression significantly

A E16.5 ————————— P10
　　　　DOX+
　　P1　P4　P7　P10　　　P35
　　　date of islet collection

B ΔCT (RT-PCR)
#1
#2
#3

C % insulin secretion
■ con ▨ OE
n=6-11
2.8　20　　2.8　20 (mM)
P4　　　　P7

H % of Ki67+ β-cells
n=4　*
Con　OE

I β-cell mass (mg)
n=5-6　*
Con　OE

J random-fed blood glucose (mg/dl)
n=5-6　*
Con　OE

Gcg/Ins/SS　　Glut2/Ins　　MafB/Ins/Gcg　　Ki67/Ins/Gcg

D1　**E1**　**F1**　**G1** — WT (P10)

D2　**E2**　**F2**　**G2** — Syt4OE (P10)

K1 WT (P35)　**L1** WT (P35) Glut2　**M1** WT (P35)

N % insulin secretion
■ con ▨ OE n=6-8
*
2.8　20 (M)

K2 Syt4OE (P35)　**L2** Syt4OE (P35) Glut2　**M2** Syt4OE (P35)

O blood glucose (mg/dl)
*　*　n≥7
Syt4OE
control
30　60　90　120
Min after glucose injection

58

Figure 2.6 *Syt4* overexpression expedites β-cell functional maturation but impairs islet morphogenesis. (A) The scheme for Dox administration and phenotype characterization. *Syt4OE* mice were continuously fed with Dox from E16.5 until either the day of sacrifice (for analysis at P1, P4, P7 and P10), or P10 (for analysis of P35 adult mice). (B) The increased level of Syt4 in three *Syt4OE* mouse lines. The mRNA level of *Syt4* in P1/P2 islets was detected by quantitative RT-PCR. Islets were isolated from three independent *Syt4OE* mouse lines. The Ct values were normalized to that of control islets at the same stages. (C) *Syt4* overexpression repressed basal insulin secretion in immature β-cells. Islets were isolated from P4 or P7 *Syt4OE* mice for the GSIS assay to examine insulin secretion within a 45-minute window. (D-F) Protein expression of selected endocrine genes in P10 control and *Syt4OE* islets. The expression of each protein was detected by immunofluorescence in 6 μm pancreatic paraffin sections. Gcg: glucagon; Ins: insulin; Ss: somatostain. Images of control and *Syt4OE* samples were captured with identical parameters to show the difference in signal intensity. (G, H) Reduced β-cell proliferation upon *Syt4* overexpression. Proliferative β-cells were detected by Ki67 labeling and quantified. (I) Quantification of relative β-cell mass in P10 control and *Syt4OE* mice. (J) Random-fed blood glucose in P10 control and *Syt4OE* mice. (K-M) Protein expression of select endocrine genes in P35 control and *Syt4OE* islets, which was detected by a similar method to that in panel D-F. In panels L, a single Glut2 channel was used to highlight the signal intensity. (N, O) Transient *Syt4* overexpression compromised GSIS and glucose clearance in adult mice. In panel N, islets were isolated from P35 control and *Syt4OE* mice for the GSIS assay similar to panel C. In panel O, IPGTT assays were conducted in P35 control and *Syt4OE* mice. Scale bar: 20 μm. *: $p < 0.05$, student t-test.
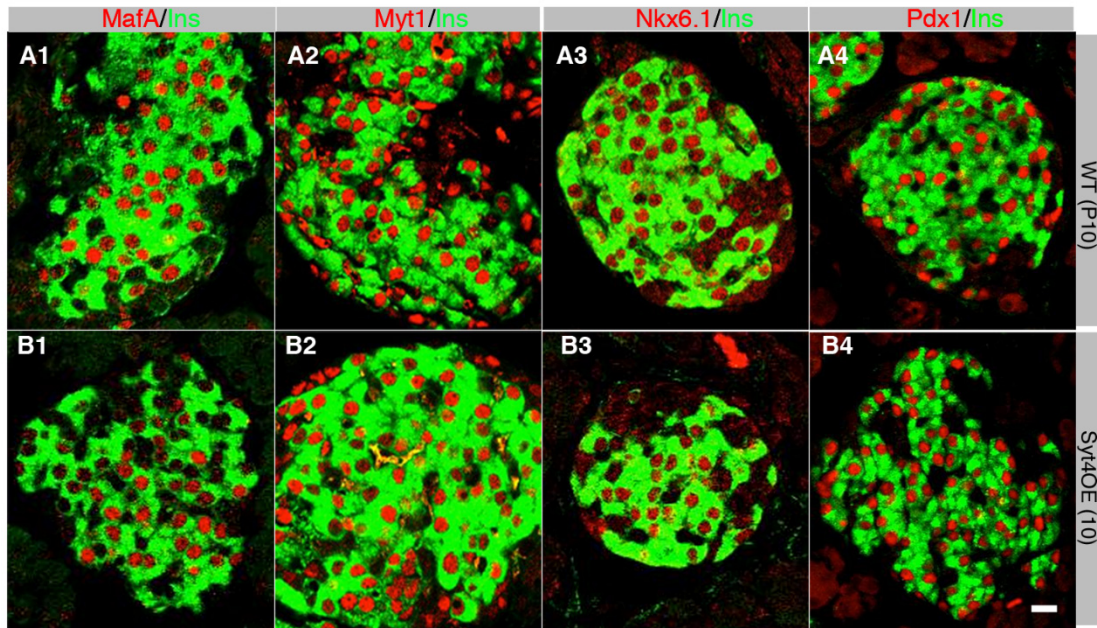
Figure 2.7 Expression of select endocrine-specific factors upon *Syt4* overexpression. The protein expression of each gene was detected by immunofluorescence in 6 μm pancreatic paraffin sections. Images of control (A1-A4) and *Syt4OE* (B1-B4) samples were captured with identical parameters to show the difference in signal intensity. Scale bar: 20 μm.

repressed basal insulin secretion at both of these stages while not affecting insulin secretion under high glucose levels (Figure 2.6C). As elevated basal insulin secretion is a significant hallmark for β-cell immaturity, our results suggest that *Syt4* overexpression promotes β-cell functional maturation.

To assess other consequences of the precocious *Syt4* overexpression on immature islets/β-cells, we examined islet morphology and the expression of islet- and β-cell-specific genes. *Syt4* overexpression did not alter the expression of selected "identity" genes for β-cell development and function, including *insulin*, *MafA*, *Myt1*, *Nkx6.1,* and *Pdx1* (Figure 2.6 and Figure 2.7). However, islets from *Syt4OE* mice had several noticeable abnormalities. At P10, a majority of control islets (76.9±2.3%, with >100 islets from each of the five pancreata examined) exclusively had β-cells in the core (i.e. no other types of endocrine cells are located 2-cell-diameter away from the edge of islets. Figure 2.6D1). In contrast, nearly half of *Syt4OE* islet sections (48.6±3.4%) had non-β-cells in the core (Figure 2.6D2). For unknown reasons, *Syt4* overexpression reduced Glut2 levels and increased MafB levels in β-cells (Figure 2.6E and F). *Syt4* overexpression also compromised β-cell proliferation and reduced β-cell mass (Figure 2.6G-I). This latter result is consistent with previous findings that β-cell function and proliferation are mutually exclusive (Arda et al., 2016; Avrahami et al., 2015) and further suggest that certain β-cell immature properties are required for β-cell proliferation and mass expansion.

In agreement with the reduced basal insulin secretion, *Syt4OE* mice displayed a trend of higher random-fed blood glucose levels (by ~20%, Figure 2.6J). Interestingly, the abnormal islet phenotypes persisted 4 weeks after Dox withdrawal, with non-β-cells constantly being observed in the center of islets (Figure 2.6K) and reduced Glut2 level on the β-cell membrane (Figure 2.6L). In contrast, *MafB* expression in β-cells was reduced to undetectable levels (Figure 2.6M). These gene expression patterns were accompanied by reduced GSIS in isolated islets (Figure 2.6N) and a trend of defective glucose clearance (Figure 2.6O, P=0.06 with repeated

measure ANOVA. p<0.05 for time points 30 and 60 minutes, student t-test). Taken together, these data suggest that precocious *Syt4* overexpression, despite promoting β-cell functional maturation, has long-lasting detrimental effects on islet morphogenesis and function *in vivo*.

Syt4 proteins localize to both vesicles and non-vesicular compartments in β-cells

To explore the molecular mechanism by which Syt4 regulates insulin secretion, we first examined its subcellular localization. We have considered and tested several microscopy techniques for this purpose. First, confocal laser scanning microscopy, which has been used in several previous studies, lacks the necessary resolution to pinpoint Syt4 subcellular localization (Fukuda et al., 2001; Ibata et al., 2000) (Figure 2.3C-F). Second, electron microscopy (EM) combined with immunogold labeling, despite offering superior resolution, did not detect significant immunogold signals in β-cell sections (see Figure 2.8 for the best examples). We reasoned that the technique difficulties of the EM might involve poor antibody qualities and/or inefficient permeabilization of gold particles into intact islets. Finally, we explored super-resolution structured-illumination-microscopy (SIM). In our case, SIM enabled an unequivocal visualization of insulin granular structures and also offered more sample depth than EM, making it an ideal method to detect Syt4 subcellular localization (Figure 2.9).

SIM imaging showed that a small portion of Syt4 proteins colocalized with insulin granules (Figure 2.9). The majority of Syt4 molecules, however, were identified to be either surrounded by an ER marker Protein disulfide-isomerase (or PDI, Figure 2.10A) or colocalized with a cis-Golgi marker GM130 (Figure 2.10B). Without further mechanistic studies, it is not clear whether the fraction of Syt4 proteins in the ER and Golgi play essential roles for β-cell function or if they are "stock pools" to be further transported toward insulin vesicles. Nonetheless, these
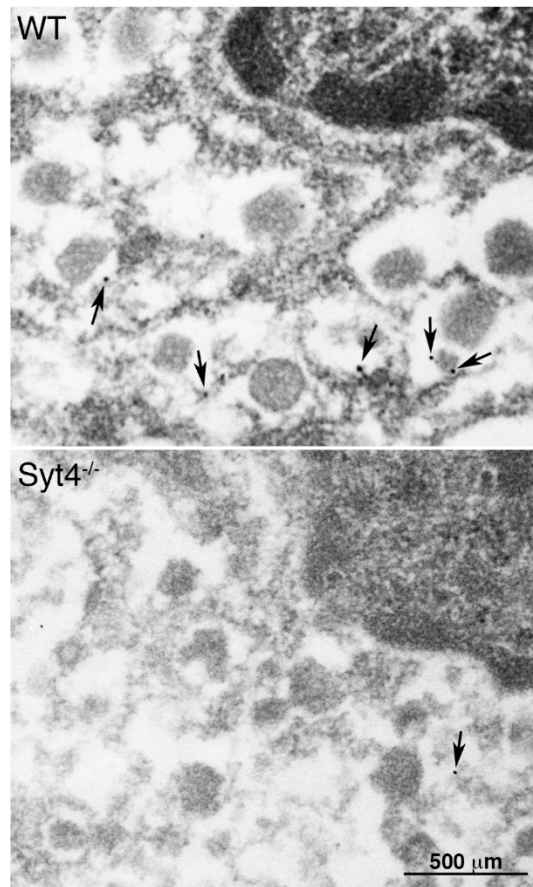
Figure 2.8 ImmunoEM to examine Syt4 subcellular localization.
Examples of anti-Syt4 immunogold EM in P14 β-cells (wild-type, top. *Syt4-/-* null, bottom). Several immunogold particles (arrows) were found in the wild-type β-cell. It is not clear whether the gold particles localize on insulin vesicles or other membranous structures, such as ER or Golgi. An immunogold particle was also detected in the *Syt4-/-* β-cell, likely representing background. Note that these images were from the sections with the largest number of gold particles. The majority of imaged sections contained no gold particles.

data support a conclusion that at least a substantial portion of Syt4 proteins are localized and exert their functions on insulin granules.

Syt4 interacts with the Ca^{2+} sensor Syt7

Since Syt4 does not bind Ca^{2+}, it is unlikely that this protein can directly modulate the Ca^{2+} sensitivity of insulin vesicles. During synaptic vesicle exocytosis in neuronal transmission, Syt4 binds Syt1 and interferes with the function of Syt1 in coupling Ca^{2+} influx and vesicle fusion (Bhalla et al., 2008; Littleton et al., 1999). We therefore proposed a similar working model in β-cells: Syt4 regulates insulin secretion by interacting with certain "Ca^{2+} sensor" Syts and affecting the function of the latter in promoting vesicle exocytosis upon Ca^{2+} stimulation. Compared with the neuronal system, however, the expression level of *Syt1* is very low in β-cells. Instead, *Syt7* is the most highly expressed *Syt1* functional paralog (Figure 2B). Furthermore, Syt7 has well-demonstrated roles in insulin secretion (Dolai et al., 2016; Gustavsson et al., 2008a; Wu et al., 2015). These findings led us to focus on the interplay between Syt4 and Syt7.

SIM imaging showed Syt7 immunofluorescence signals on insulin granules and non-granular compartments (Figure 2.9B). Intriguingly, we observed colocalization of Syt4 and Syt7, suggesting that there is a physical interaction between these two proteins (Figure 2.9C). To confirm this, we co-expressed Syt7 and a HA-tagged Syt4 in heterologous HEK293 cells and examined their physical association by a co-immunoprecipitation assay. Indeed, an anti-HA antibody pulled down both HA-tagged Syt4 and Syt7 (Figure 2.9D).

If our hypothesis was true, then *Syt7* loss of function should have similar effects on insulin secretion with *Syt4* gain of function. Indeed, inactivating *Syt7* in immature β-cells attenuated insulin secretion, an observation similar to that caused by *Syt4* overexpression (Figure 2.9E). These combined data support the model that
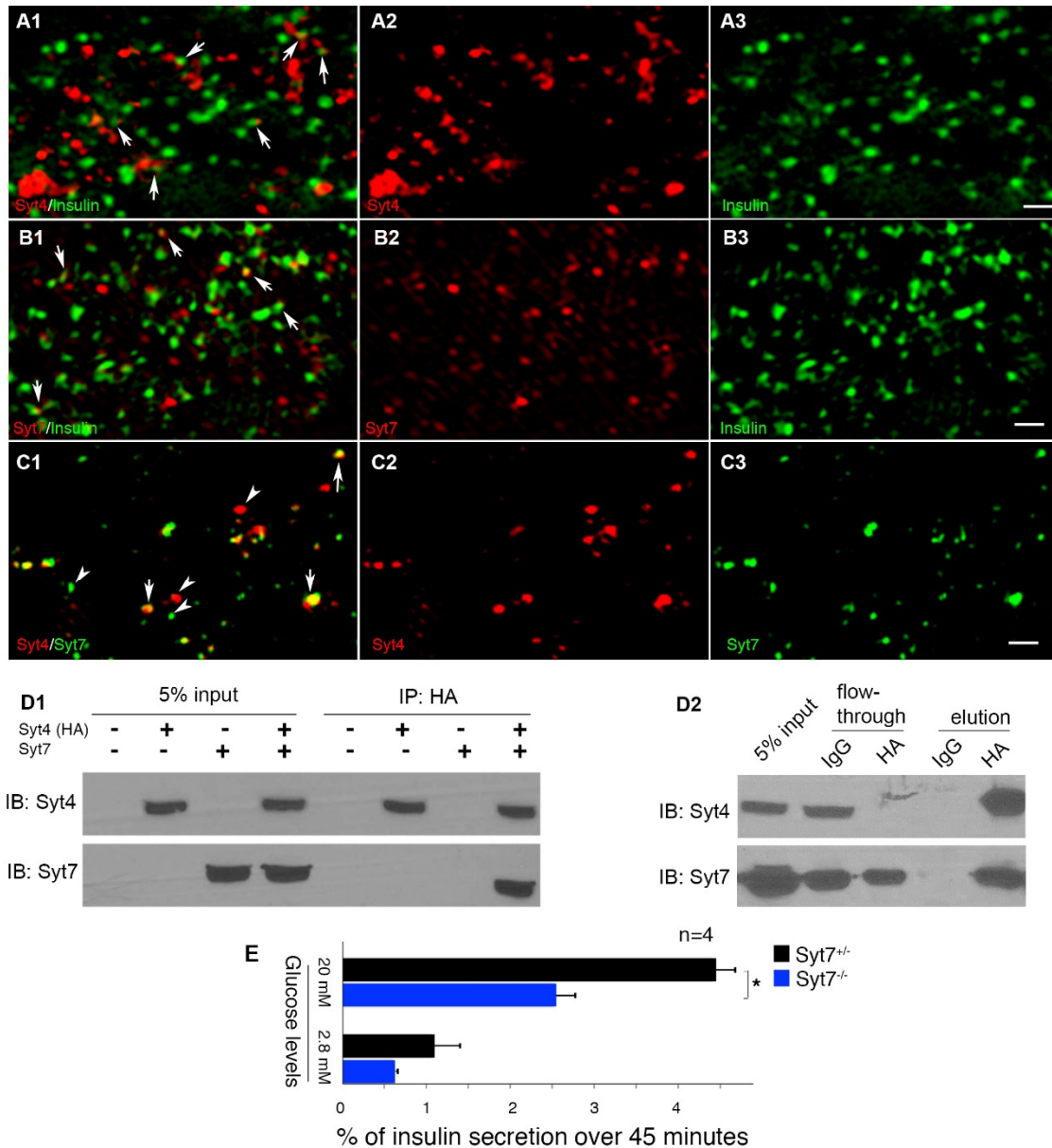
Figure 2.9 The subcellular localization of Syt4 and its interaction with Syt7. (A and B) Representative SIM images showing the subcellular localization of Syt4 and Syt7 in adult wild-type β-cells. Arrows in A1 and B1 pointed to co-localization of between Syt4 and insulin, and between Syt7 with insulin, respectively. (C) Co-localization of Syt4 and Syt7 in β-cells. Arrows point to spots of Syt4 and Syt7 co-localization. Arrowheads point to spots of single Syt4 or Syt7 signal, supporting the specific detection of Syt4 and Syt7, respectively. (D1 and D2) Immunoprecipitation showing Syt4-Syt7 interaction. HEK293T cells were co-transfected with HA-tagged Syt4 and Syt7 and lysed 48 hours later. The whole cell lysates was immunoprecipitated by an anti-HA antibody or an IgG. The flow-through (i.e. unbound) and pull-down components were immuno-blotted with an anti-Syt4 antibody and an anti-Syt7 antibody, respectively. (E) GSIS of *Syt7* null (*Syt7-/-*) and control (*Syt7+/-*) immature islets at P4. Insulin secretion within a 45-minute window is presented. Scale bar: 0.5 μm. *: p=0.02, student t-test.
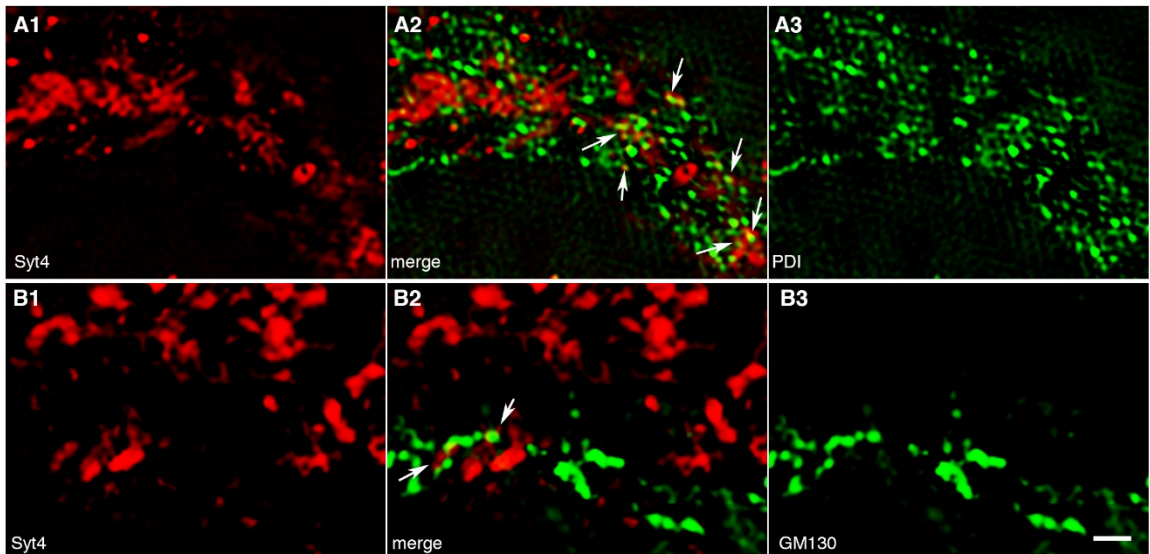
Figure 2.10 Syt4 co-localizes with the ER marker PDI and Golgi marker GM130. Representative SIM images of adult wild-type β-cells co-stained for Syt4 and PDI (A) or GM130 (B). Arrows pointed to the overlap between Syt4 and PDI (A) or GM130 (B), respectively. Scale bar: 1 μm.

Syt4 interacts with Syt7 and disrupts the function of Syt7 in promoting insulin vesicle exocytosis.

Syt4 regulates insulin vesicle docking

Recent reports found that Syt7 promotes insulin granule replenishment to the docked pool that are physically close to the plasma membrane (Dolai et al., 2016; Osterberg et al., 2015). Therefore, we speculated that if Syt4 negatively regulates the function of Syt7, it would play an inhibitory role in insulin vesicle docking.

To test this possibility, we utilized TEM to examine the vesicular docking with *Syt4* gain- and loss-of-function. For the former, we focused on β-cells at P1 and P4, when the endogenous *Syt4* expression has not been abundantly activated. Conversely, for the latter, we examined β-cells at P14 and P24, when Syt4 levels have reached high (Figure 2.2). In addition, β-cells at P7 were examined in both scenarios as they are characterized by a modest expression level of *Syt4*.

Syt4 overexpression did not significantly change the average size of dense core insulin vesicles, which are the major releasable insulin carriers (Figure 2.11A and B). However, *Syt4* overexpression slightly (~20%) but significantly increased the number of these vesicles in β-cells at P4 and P7 (Figure 2.11A, B, E). Meanwhile, *Syt4* overexpression significantly reduced the number of insulin vesicles docked within 20 nm of the plasma membrane (Figure 2.11F).

Accordingly, inactivation of *Syt4* did not change the size or density of insulin vesicles, but resulted in a significant increase in the number of docked vesicles in the P14 newly matured β-cells (Figure 2.11C-F). Surprisingly, unlike P14, *Syt4*^{-/-} β-cells at P24 (several days after weaning) had a significantly reduced number of docked vesicles. This observation was consistent with the reduced GSIS in post-weaning *Syt4*^{-/-} islets (Figure 2.4G). However, it is not clear whether this latter finding results from increased insulin secretion after weaning, which could have depleted the
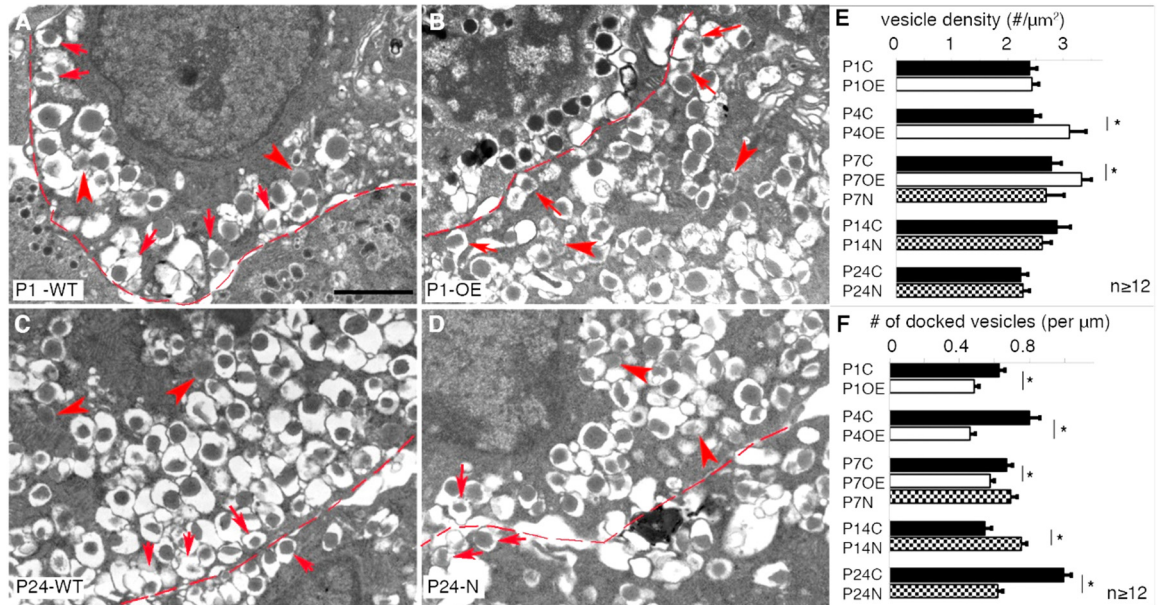
Figure 2.11 Syt4 regulates insulin vesicle docking. (A-D) Representative TEM images of β-cells with *Syt4* overexpression or inactivation at indicated postnatal stages. (A and B) P1 wild-type control and *Syt4OE* β-cells. Note that in the bottom-left of panel A are some pancreatic polypeptide granules in a PP cell. In the top-left corner of panel B are some glucagon granules (the darkest granules). (C and D) P24 wild-type control and *Syt4⁻ᐟ⁻* (P24-N) β-cells. Arrows in (A-D) point to several docked mature vesicles. Arrowheads in (A-D) point to several immature vesicles, which are recognized by the low electron density of the insulin core. Red dash lines delineate the plasma membrane. Scale bar: 1 μm. (E) Quantification of dense vesicles at indicated stages in β-cells with *Syt4* overexpression (OE) or inactivation (null, or N). (F) Quantification of docked vesicles in β-cells. *: $p < 0.05$, student t-test.

docked vesicles, or if Syt4 also affects intracellular insulin granule transport in mature β-cells at this stage.

Syt4 regulates insulin secretion in human β-cells

Lastly, we sought to determine if Syt4 also regulates the insulin secretion in human β-cells. Of significance, published RNA-seq data showed that *Syt4* is not only one of seven *Syts* (*Syt4-6*, *11*, *13*, *16*, and *17*) that are highly expressed in functional human β-cells, but is also one of the three *Syts* (*Syt4*, *13*, and *16*) whose expression is increased during human β-cell maturation (Blodgett et al., 2015). The expression pattern of *Syt4* in human β-cells is therefore highly correlated with their acquisition of functional maturity. Notably, the other two Syts (Syt13 and 16) sharing a similar expression pattern with Syt4 are also characterized by little to no Ca^{2+} affinity, suggesting that these inhibitory Syts might be specifically involved in human β-cell maturation.

We first tested the effect of *Syt4* overexpression in human embryonic stem cell derived β-like cells (Russ et al., 2015). Compared to *bona fide* human functional β-cells, β-like cells exhibit higher basal insulin secretion and less response to glucose stimulation, and are considered to be more like human fetal immature β-cells (Hrvatin et al., 2014). To this end, we generated lentivirus that mildly overexpresses human *Syt4*. Three days after virus infection, the β-like cells exhibited significantly reduced basal insulin secretion compared with controls. This finding suggests that similar to its mouse counterpart, human Syt4 also represses basal insulin secretion and prompts β-cell functional maturation (Figure 2.12A). Interestingly, overexpression of *Syt13* and *Syt16* had a similar effect (Figure 2.12A), suggesting that these Syts with low Ca^{2+} affinity have a common role in repressing basal insulin secretion in human β-cells.

Next, to determine the necessity of *Syt4* in human β-cell function, we decided to perform a loss-of-function study in the human β-cell line EndoC-βH1. Compared to
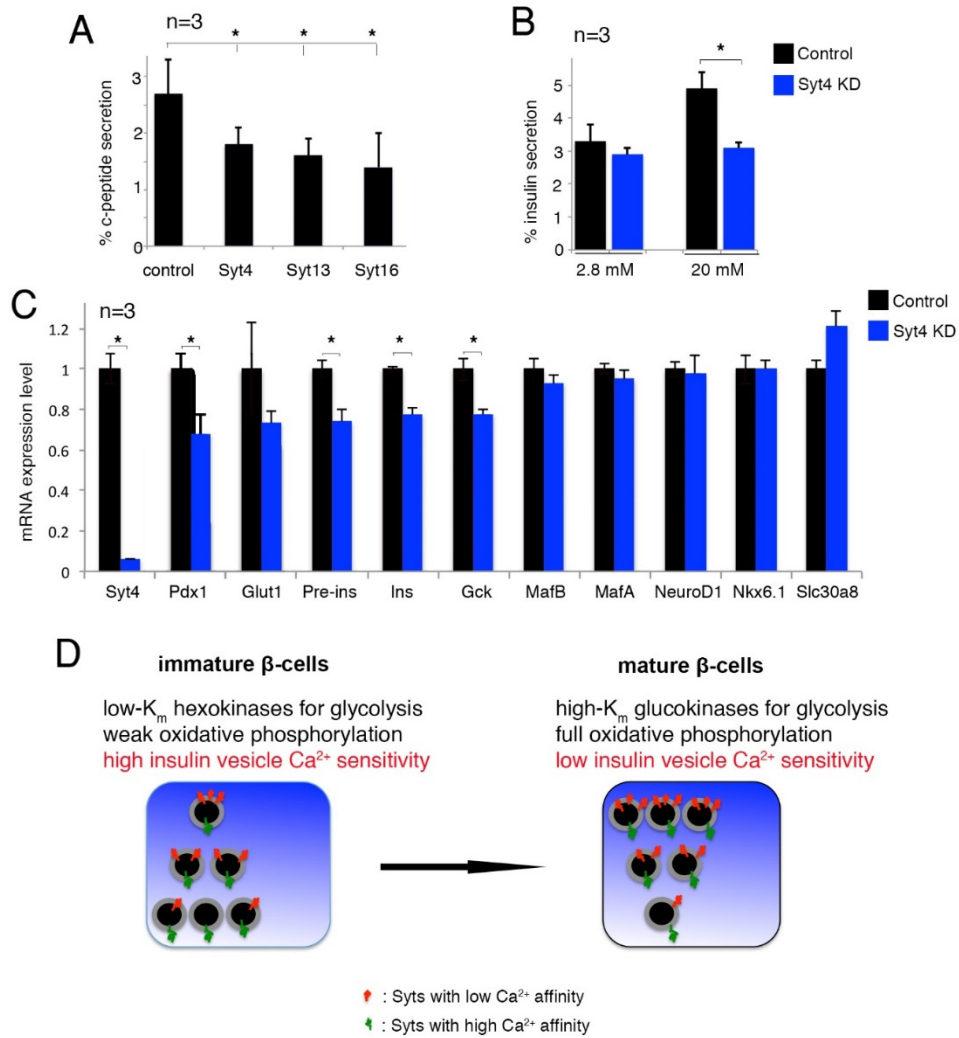
 Figure 2.12 Syt4 regulates insulin secretion of human β-cells (A) The percentage of C-peptide secretion in human ESC-derived β-like cells under 2.8 mM glucose with the overexpression of select *Syts*. (B) GSIS in human EndoC-βH1 cells transfected with scramble siRNA (Control) and *Syt4* siRNA (Syt4 KD). (C) mRNA expression levels of select genes in human EndoC-βH1 cells transfected with scramble siRNA (Control) and Syt4 siRNA (Syt4 KD). (D) A model of mechanisms that converge to regulate β-cell maturation. One aspect includes the fine-tuning of glycolysis and oxidative phosphorylation, which ensures that glycolysis only occurs at high glucose levels and large amounts of ATP are generated for Ca^{2+} entry when necessary. Another aspect includes the Ca^{2+} sensitivity of insulin vesicles. In this pathway, the increased ratio between Syts with low Ca^{2+} affinity and Syts with high Ca^{2+} affinity desensitizes the insulin vesicles so that the insulin vesicle release is only triggered by high $[Ca^{2+}]_i$ under high glucose levels. These combined properties of mature β-cells repress low basal insulin secretion while enhancing the insulin secretion under high glucose stimulation. *: $p<0.05$, student t-test.

other stable β-cell lines, the EndoC-βH1 cell line displays a moderate glucose response and can be readily manipulated for gene expression analysis (Ravassard et al., 2011). In addition, the endogenous expression level of *Syt4* in EndoC-βH1 cells is comparable to that in primary human islets (data not shown). A siRNA-based knockdown successfully reduced the Syt4 level by over 95% in EndoC-βH1 cells (Figure 2.12C). However, we did not detect a significant change in basal insulin secretion (Figure 2.12B). It is not clear whether this observation is due to the artificial nature of the human cell line, or due to the redundancy of *Syt4*, *Syt13*, and *Syt16* in repressing basal insulin secretion. However, similar to the scenario in adult mouse β-cells, *Syt4* knockdown significantly reduced insulin secretion upon high glucose stimulation (Figure 2.12B). These data suggest that *Syt4* is indeed required for maintaining human β-cell function, which is consistent with the previous finding that non-functional human β-cells have low *Syt4* expression (Andersson et al., 2012).

Knockdown of *Syt4* had little to no effect on the expression of select β-cell specific genes, such as *MafA, MafB, NeuroD1, Nkx6.1,* and *Slc30A8* (Figure 2.12C). Interestingly, we observed a slight (15-30%) yet significant reduction in *Pdx1*, *Gck,* and *insulin* mRNA (Figure 2.12C). This finding suggests that *Syt4* knockdown does not affect the general β-cell identity, but can cause some small instability in β-cell functional gene expression, likely via the feedback of the altered GSIS.

2.4 Discussion

Producing functional β-cells from heterologous resources can facilitate transplantation-based diabetes therapy (Schiesser and Wells, 2014; Tabar and Studer, 2014). To this end, several studies have reported the successful production of insulin-secreting β-like cells from human ES or iPS cells *in vitro* (Pagliuca et al., 2014; Rezania et al., 2014; Russ et al., 2015). However, compared to fully functional β-cells from organ donors, the *in vitro* derived β-like cells display a lower dynamic

range of insulin secretion, poorer vesicular packaging, and/or attenuated glucose-induced Ca^{2+} influx (Pagliuca et al., 2014; Rezania et al., 2014; Russ et al., 2015). The suboptimal properties of *in vitro* derived β-cells highlight the importance of their further maturation (Kieffer, 2016; Kushner et al., 2014). Here we show that a previously unrecognized mechanism, reducing the Ca^{2+} sensitivity of insulin vesicles, contributes to β-cell maturation. This mechanism not only lowers basal insulin secretion to avoid hypoglycemia and but also improves the dynamic range of insulin secretion, at a defined β-cell mass, to handle large dose of insulin demand.

Ca²⁺ is the trigger for membrane fusion between secretory granules and the plasma membrane. Biochemical pathways that regulate $[Ca^{2+}]_i$ and/or Ca^{2+} responses are therefore expected to regulate β-cell functional maturation. Indeed, proper glucose sensing and metabolism, which result in Ca^{2+} influx, are key factors for β-cell maturation (Blodgett et al., 2015; Dhawan et al., 2015; Hrvatin et al., 2014; Jermendy et al., 2011). In contrast, because KCl-induced Ca^{2+} influx can induce robust insulin secretion in immature β-cells (Gu et al., 2010b; Hole et al., 1988; Rorsman et al., 1989), modulating the responsiveness of vesicles to Ca^{2+} has not been proposed as a significant mechanism in regulating β-cell maturation.

Our qualitative studies described here support a model (Figure 2.12D) wherein the granules in immature β-cells have higher sensitivity to Ca^{2+}, due to low abundance of Syt4 and/or other low Ca^{2+} affinity Syts. This elevated Ca^{2+} sensitivity enables sustained high insulin secretion at low Ca^{2+} influx under basal glucose conditions (Rozzo et al., 2009). A consequence of high basal insulin release is the depletion of releasable vesicles, which in turn compromises the insulin secretion upon high glucose stimulation. During maturation, increased expression of *Syt4* and/or similar inhibitory *Syts* represses the vesicular sensitivity to Ca^{2+} so that only high Ca^{2+} influx can initiate vesicle release. These properties, combined with the modulation of glucose metabolism, ensure that only high glucose levels can initiate significant insulin release so that neither hypoglycemia nor hyperglycemia will occur.

The known activities of Syts support their proposed roles in our model. Syt4 has been reported to repress vesicle-plasma membrane fusion, in both artificial (Bhalla et al., 2008) and *in vivo* settings (Johnson et al., 2010a; Machado et al., 2004; Moore-Dotson et al., 2010). The activating role of Syt7 in insulin secretion is well established (Dolai et al., 2016; Gustavsson et al., 2008a; Wu et al., 2015). Mechanistically, our studies showed that both Syt4 and Syt7 are found on insulin vesicles and they form a complex with each other in cell culture. It is likely that the presence of Syt4 alters the conformation of Syt7 on vesicles, which results in lowered affinity of Syt7 for the plasma membrane phospholipids. The efficacy of Syt7-mediated membrane fusion is thereby reduced, much like the scenario of Syt4-Syt1 interaction that occurs in nerve cells (Littleton et al., 1999). Our observation that precocious expression of *Syt4* reduced the number of the plasma membrane-docked vesicles is consistent with this possibility.

Our model can explain several previous findings on the GSIS dynamics in β-cells. It is generally accepted that insulin vesicles in β-cells are a heterogeneous population, with newly synthesized vesicles showing higher propensity for release (Hoboth et al., 2015; Howell and Taylor, 1967; Ivanova et al., 2013; Michael et al., 2007; Sando et al., 1972; Wang et al., 1988). Yet the mechanisms that regulate this heterogeneity remain unknown. The transportation of vesicles does not appear to be a restricting factor, because the released insulin vesicles only represent a small portion of all the vesicles localized close to the plasma membrane (McDonald et al., 2010; Varadi et al., 2003; Varadi et al., 2005; Zhu et al., 2015). It is possible that differences in insulin vesicle Ca^{2+} sensitivity, due to various levels of Syt proteins, contribute to the observed heterogeneity. Such a possibility could be explored by real-time imaging of pre-labeled Syts-positive vesicles in the future.

Furthermore, our model can explain why mature β-cells have an increased threshold for GSIS (Blum et al., 2012). In this case, a substantial number of vesicles in immature β-cells are sensitive to low $[Ca^{2+}]_i$ stimulation, which contributes to high

basal insulin secretion. A gradual increase of glucose likely mobilizes different pools of vesicles, resulting in the observed increase in GSIS when glucose is shifted form low (<0.5mM) to medium (2.8-5.6 mM) (Blum et al., 2012). In contrast, vesicles in mature β-cells are mostly responsive only to high Ca^{2+} levels. In this scenario, a glucose shift from 0.5 to 5.6 mM cannot raise $[Ca^{2+}]_i$ enough to break the elevated Ca^{2+} threshold required for vesicle secretion.

Our studies further suggest that properly controlled maturation is essential for long-term β-cell function. Transient *Syt4* overexpression in neonatal β-cells induces precocious β-cell maturation, which has long-lasting detrimental effects on islet GSIS, accompanied by improper islet morphogenesis, β-cell gene expression, and reduced β-cell proliferation. It is possible that high basal secretion of insulin and unidentified growth factors in the immature β-cells modulates islet morphogenesis and epigenetic programming of β-cells for long-term function in paracrine and/or autocrine fashions. For instance, insulin/IGF-based signaling has been shown to be essential for functional β-cell mass maintenance [(Stewart et al., 2015) and references therein]. Interrupting or expediting this type of regulatory factors in the neonatal islets may permanently compromise β-cell function. An implication of our results is that the β-cell maturation needs to occur in a temporally controlled manner in order to produce fully functional and long-lasting islets. Alternatively, it is possible that the transient *Syt4* overexpression exerts unknown effects on β and non-β endocrine cells, which resulted in abnormal gene expression, defective morphogenesis, and dysfunction.

Our findings have raised several questions for future studies on β-cell maturation. First, what other factors are involved in modulating vesicular Ca^{2+} sensitivity? Our overall findings have highlighted Syt proteins as key regulators of Ca^{2+} sensitivity. Alternatively, non-vesicular proteins/pathways, or even glucose metabolites, can indirectly modulate the Ca^{2+} sensitivity of insulin vesicles. Indeed, many enzymes and scaffold proteins, such as calmodulin (Goodyer et al., 2012;

Stevens, 1983), utilize Ca^{2+} as a co-factor. In theory, any of these factors can establish stage-specific Ca^{2+} signaling that affects the Ca^{2+} sensitivity of insulin vesicles in a cell-context dependent fashion.

Second, the biochemical mechanisms of Syt4 function require further study. Our findings suggest that direct Syt4-Syt7 interaction on vesicles can reduce the Ca^{2+} sensitivity of granules. However, only a small portion of Syt4 proteins are detected on insulin vesicles and in the Golgi. The majority of Syt4 proteins localize outside the Golgi and vesicles, likely in the ER compartment. It remains to be determined whether this subcellular localization pattern reflects the trafficking process of Syt4 from the site of production (the ER) to the Golgi and then to the vesicles, or if Syt4 has additional functions in the ER, Golgi, and/or other organelles.

Third, the mechanisms that regulate the expression of *Syts* are not known. To this end, thyroid hormones have been established as inducers of β-cell maturation in rodents by activating the expression of *MafA* (Aguayo-Mazzucato et al., 2013). Yet MafA, despite enhancing GSIS, does not repress basal insulin secretion (Aguayo-Mazzucato et al., 2011). Thus, it would be interesting to test if thyroid hormones and other related hormones/factors related to postnatal β-cell growth can promote the expression of *Syts* as well. Alternatively, a Syt4 reporter cell system (such as cells engineered with a *Syt4* promoter/enhancer-driven luciferase) can be established to identify growth factors and small molecules that can activate *Syt4* expression based on large scale screening.

Fourth, loss of *Syt4* promotes vesicle docking in pre-weaning mature β-cells but attenuates it in post-weaning mature β-cells (Figure 2.11F). The mechanisms accounting for this difference are not known. It is possible that Syt4 has different functions in regulating vesicle transport/docking in pre- and post-weaning β-cells. It is also possible that these observations result from the different pleiotropic effects of the global *Syt4* null mutation between pre- and post-weaning stages. Temporally

controlled, β-cell-specific loss of function studies are required to address these two possibilities.

In summary, our studies reveal a previously unrecognized mechanism that regulates β-cell maturation in both rodents and humans. Manipulating the Ca^{2+} sensitivity of insulin vesicles, along with regulating metabolic profiles, will likely aid in the production of mature human β-cells for transplantation-based diabetes therapy.

2.5 Materials and Methods

Mice derivation and usage

Mouse usage followed protocols approved by the Vanderbilt University IACUC for Dr. Gu. Wild-type CD1 mice, used for routine crosses, were from Charles River Laboratories. *RIP-rtTA* and *Syt4*^{+/-} founder mice were from the Jackson Laboratory. *RIP-mCherry* mice were characterized and reported in (Zhu et al., 2015). The *TetO-Syt4* mice were derived by pronuclear injection, with construct made by ligating a TetO-CMV promoter (PTRE2, Clontech), the *Syt4* coding sequence, and a SV40 polyA signal as described previously (Wang et al., 2008). For *Syt4* over expression, doxycycline (2 mg/ml) was added to the drinking water of pregnant mother mice (before birth) or feeding mother mice (after birth) from E16.5 to the time of tissue collection. Genotyping was performed by standard PCR protocols. Genotyping primers used in this study were listed in Table 1.

Glucose tolerance test, insulin tolerance test and *in vivo* insulin secretion assay

Intraperitoneal glucose tolerance test (IPGTT) was performed as described previously (Wang et al., 2007). Briefly, mice were fasted overnight (16 hours) and were injected intraperitoneally with glucose at 2 mg per g body weight. At a set of time points (0, 15, 30, 45, 60, 90, 120 minutes) after glucose administration, blood

samples were collected by tail blooding, and blood glucose levels were measured using a compact glucometer and test strips (BD/Medtronic MiniMed Blood Glucose Monitor).

For inulin tolerance test (ITT), mice were fasted for 4 hours and were injected intraperitoneally with insulin at 1 IU per g body weight. At a set of time points (0, 20, 40, 60 minutes), the blood sample collection and blood glucose measurement were performed similarly as described in IPGTT.

For *in vivo* insulin secretion assay, blood samples were obtained before or 30 minutes after glucose injection from the mouse saphenous vein. Serum insulin levels were assayed using an ELISA kit (Linco, St. Charles, MO) following manufacturer instructions.

Islet isolation and *in vitro* insulin secretion assay

Islets were isolated from newborn and adult mice as described previously (Huang and Gu, 2017; Zhao et al., 2010). Briefly, the pancreas was dissociated by either collagenase perfusion (for adult mice) or by direct collagenase digestion followed by a histopaque (Sigma) gradient centrifuge (for mice before 2 weeks of age). The dissociated pancreas was washed three times by Hank's buffered salt solution (HBSS) containing 10% FBS to remove collagenase. Islets were then hand-picked under a dissection microscope and were transfer into the RPMI medium (10% FBS) with or without glucose depending on different assay purposes.

For *in vitro* GSIS, isolated islets were cultured in the RPMI medium (10% FBS) containing <5.6 mM glucose to prevent degranulation. Basal glucose and KCl concentrations were 2.8 and 5 mM, respectively. Stimulating glucose and KCl concentrations were 20 and 25 mM, respectively. The secreted insulin was normalized to total insulin contents, which were obtained by lysing islets with ethanol-HCl lysis buffer (Huang and Gu, 2017). Insulin was assayed by an insulin ELISA kit (Alpco) following the manufacturer protocol. Note that islets used for correlating

GSIS/KSIS with Ca^{2+} influx were incubated for ~40 hours in the RPMI medium (10% FBS plus 11 mM glucose) before the secretion assay or Ca^{2+} recording. Islets used for other secretion assays were recovered in the RPMI medium (10% FBS plus 11 mM glucose) for 2 hours.

Immunofluorescence (IF)

Immunofluorescence for endocrine hormones, Ki67, Pdx1, MafA, MafB, Nkx6.1, and Glut2 was based on paraffin sections. For paraffin embedding, sample tissues were fixed by 4% paraformaldehyde for overnight at room temperature. Fixed samples were then dehydrated through an increased ethanol gradient. Subsequently, sample tissues were cleared by xylene and embedded into paraffin blocks. Paraffin blocks were sectioned into 6 μm slides. After sectioning, paraffin slides were dewaxed by xylene and then were rehydrated through a reduced ethanol gradient. After a 95 ºC water bath-based antigen retrieval, sectioned slides were permeabilized by 1% triton X100 for 10 minutes, blocked by 5% donkey serum and 10% BSA for 30 minutes to eliminate nonspecific antigen binding, and finally were applied with primarily and fluorophore-conjugated secondary antibodies. The information of primary antibodies used in this study, including antibody sources, usages, and antigen retrieval methods was listed in table 3.

For IF of Syt4, Syt7, GM130, and PDI, islet cells attached to glass coverslips were used. Briefly, islets were isolated and partially dissociated into small cell clusters (~5-20 cells per cluster) with trypsin. The cells were then cultured on glass coverslips coated with human fibronectin in RPMI 1640 medium (containing 5.6 mM glucose and 10% FBS). After 24 hours, samples were fixed by 4% paraformaldehyde for 15 minutes and processed for routine IF. For co-immunostaining with antibodies from the same host (e.g. antibodies against PDI, Syt4, and Syt7), one round of staining was completed first with proper fluorophore-conjugated secondary antibodies. The samples were blocked by anti-IgG of the host, followed by routine IF

for the second antibody conjugated with another fluorophore type. The information of primary antibodies was listed in table 3.

RNA preparation and Quantitative RT-PCR

RNA was extracted from isolated islets by Trizol (Invitrogen) following the manufacturer instruction. RNA integrity was monitored by an Agilent Bioanalyzer 2100. A minimum RNA Integrity Number (RIN) of 7 was required for subsequent analysis. Quantitative RT-PCR (qRT-PCR) was performed following standard protocols (Wang et al., 2008). cDNA synthesis used the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative RT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad) on an iCycler Thermal Cycler. The abundance of most transcripts was normalized versus the abundance of the internal control (HPRT or 18s rRNA). qRT-PCR primers used in this study were listed in Table 2.

Microscopy

For immunofluorescence imaging, confocal-laser scanning microscopes and SIM were used. When the expression levels of a protein between samples were compared, slides or cells were processed side-by-side, and images were captured utilizing identical optical and electronic settings.

TEM samples were processed following established protocols (Zhao et al., 2010). Whole isolated islets were fixed by the EM fixing buffer (2.5% glutaraldehyde in 100 mM phosphate buffer at pH 7.0). For the immunogold TEM, whole isolated islets were first fixed by 4% paraformaldehyde and stained with primary antibody and gold particle-conjugated secondary antibodies. Immunogold labelled samples were fixed again with the EM fixing buffer for TEM. For unbiased vesicle characterization (density, diameter, and docking), image capture and quantification were performed by different personnel under double-blind settings.

Quantification of β-cell mass and β-cell proliferation

For quantifications of β-cell mass, entire pancreas paraffin blocks were sectioned at 6 μm intervals. One third of all sections across the pancreas were stained with insulin, Ki67 and DAPI and then scanned by a ScanScope FL slide scanner (Aperio Technologies, Inc.). The relative ratio of β-cell area was calculated by dividing the insulin-positive area by the DAPI positive area. The relative β-cell mass was then calculated by multiplying the relative ratio of β-cell area by the pancreas weight. For quantification of β-cell proliferation, a minimum 3,000 insulin-positive cells from randomly picked islets were counted manually. The percentage of proliferating β-cells was calculated by dividing the number of Ki67/insulin double-positive cells by the total number of insulin-positive cells.

Co-immunoprecipitation and Western blot

HEK293T cells were co-transfected with plasmids expressing transmembrane domain deleted Syt4 (tagged by HA) and Syt7. 48 hours after transfection, cells were lysed by the co-IP buffer (150 mM NaCl, 8 mM Na_2HPO_4, 2 mM NaH_2PO4, 1% NP-40, 0.5% sodium deoxycholate, protease inhibitor cocktails). Cell lysates were mixed either with mouse anti-HA crosslinked magnetic beads (Vanderbilt Antibody and Protein Resource) or with normal mouse IgG (Millipore) and protein A conjugated magnetic beads (Millipore) at 4 °C for 4 h. The beads were washed with TBST (20 mM Tris-HCl pH7.6, 150 mM NaCl, 0.1% Tween-20) five times, and beads-bound proteins were analyzed by Western blot with the above-described anti-Syt7 antibody or anti-Syt4 antibody following routine protocols.

Free cytoplasmic Ca^{2+} recording

Calcium recording and analysis followed the published method (Jacobson et al., 2010). Briefly, islets were cultured on ploy-lysine coated glass dishes for 48 hours in the RPMI medium containing 10% FBS and 11 mM glucose. The glass-attached

islets were loaded with 2 mM FURA-2 AM (Invitrogen) for 25 minutes in the culture medium followed by a 20-minute starvation in the REC buffer (119 mM NaCl, 2.5 mM $CaCl_2$, 4.7 mM KCl, 10 mM Hepes, 1.2 mM $MgSO_4$ and 1.2 mM KH_2PO_4) with 2 mM glucose. The islets were then transferred to the REC buffer with 2.8 mM glucose for 3 minutes for the recording prior to stimulation. Then, the islets were transfer to the REC buffer with 20 mM glucose or with 25 mM KCl for Ca^{2+} recording for the recording after stimulation. Buffer changes were achieved by a 2 ml per minute perfusion. Ca^{2+} imaging was performed using a Nikon Eclipse TE2000-U microscope. The ratios of emitted fluorescence intensities at excitation wavelengths of 340 and 380 nm (F340/F380) were recorded every 5 second. The total Ca^{2+} was calculated following published procedures, with EGTA and ionomycin used to calibrate the low and high Ca^{2+} levels, respectively (Grynkiewicz et al., 1985).

EndoC-βH1 cell line usage

EndoC-βH1 cell line was a gift from R. Scharfman. Cell culture followed published protocols (Ravassard et al., 2011). For loss of function assays, siRNA mix from Dharmacon was utilized. Gene expression and GSIS assays were conducted 3 days after siRNA transfection.

Statistical analysis

Unpaired student t-test was used to determine whether one data point was significantly different between two sample groups. Repeated-measure ANOVA was used to determine whether the combined data points were different between two sample groups (e.g. IPGTT between wild-type and *Syt4*$^{-/-}$ mice). Finally, between-subject ANOVA was used to determine whether one data point was different among multiple sample groups (e.g. the expression levels of *Syt4* among P1, P12 and P60 islets). A p-value of 0.05 or lower was considered to be significant. Quantification data were all presented as (mean ± SEM).

CHAPTER III

MYT FACTORS ARE REQUIRED TO GENERATE FUNCTIONAL β-CELLS

3.1 Abstract

Regenerative medicine has made a remarkable progress in deriving β-cells from embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) for cell transplantation-based therapy against diabetes. However, most β-cells derived from *in vitro* directed differentiation using current approaches lack glucose responsiveness and long-term survival capability. Mechanistic studies of β-cell maturation and functional maintenance are therefore crucial in providing novel insights for the generation of *bona fide* β-cells for clinical usages. Here, we have shown that Myelin transcription factors (Myt factors) are necessary to generate functional and long-term stable β-cells. Pancreas-specific knockout of Myt factors has little effect on β-cell fate specification in the embryonic stage, but results in severe postnatal β-cell defects, including declined proliferative capability, increased cell apoptosis, abolished glucose response and prolonged cell stress. Moreover, the β-cell defects become exacerbated with age, and continuous deterioration of β-cell function is accompanied by gradual disappearance of β-cell identity. Notably, *Myt* mutant β-cells have aberrant gene expression at the neonatal stage, prior to the appearance of functional and morphological defects. These findings suggest that newly differentiated β-cells need Myt factors to establish a gene expression network for mass expansion, functional maturation, long-term survival and β-cells identity maintenance.

3.2 Introduction

β-cells residing in the pancreatic islet of Langerhans produce and secrete insulin for glucose homeostasis. Both major types of diabetes are directly connected to the defects in β-cells. Type I diabetes (T1D) is caused by autoimmune destruction of β-cells and thus the absolute lack of insulin. T1D patients have to rely on exogenously delivered insulin for glucose clearance. On the other hand, type II diabetes (T2D) starts with a pre-diabetic state of insulin resistance, which is usually related to chronic nutrient overload. As a compensatory mechanism, β-cell mass and insulin secretion per β-cell are initially upregulated to meet the elevated insulin requirement and sustain relative euglycemia (Kahn et al., 2006). However, this compensatory mechanism usually does not persist, as prolonged overloading of insulin production can cause β-cell stress, which is detrimental to β-cell function and survival (Prentki and Nolan, 2006). At a later stage, T2D is triggered by "β-cell failure", a pathological status characterized by reduced insulin secretion, β-cell dedifferentiation and β-cell apoptosis (Prentki and Nolan, 2006). Because of the physiological importance of β-cells, regenerative medicine has proposed to derive this group of cells from various cell sources to develop a transplantation-based treatment for diabetes (Vetere et al., 2014). Therapeutic β-cell regeneration requires a comprehensive understanding of β-cell differentiation, maturation and long-term functional maintenance (Borowiak and Melton, 2009; Pagliuca and Melton, 2013).

During embryogenesis, β-cells are differentiated from a subgroup of pancreatic epithelial progenitor cells expressing the helix-loop-helix transcription factor *Ngn3*. A set of transcription factors are responsible for β-cell fate specification and further functional maturation. These important transcription factors include, but are not limited to, Pax4, Pdx1, Nkx6.1, Nkx2.2, Pax6, Insm1, Mnx1 and MafA (Pan and Wright, 2011). For instance, the homeodomain transcription factor Pax4 counteracts Arx to favor a β-cell

fate choice at the expense of α-cell fate (Collombat et al., 2003). *Pdx1*, whose

expression is broadly found in early pancreatic multipotent progenitor cells but later

mostly restricted to β-cells, is also required for the establishment and maintenance of β-

cell identity (Gao et al., 2014). MafA is a key factor promoting β-cell functional

maturation and postnatal mass expansion. Loss of *MafA*, despite not affecting β-cell

differentiation, compromises postnatal β-cell proliferation and function (Hang et al.,

2014). Besides possessing crucial regulatory roles in pancreas development and β-cell

generation, these key transcription factors also serve as biomarkers to monitor the

pipeline of the *in vitro* directed differentiation from ESCs or iPSCs to β-cells (Pagliuca

and Melton, 2013).

In the postnatal stage, proliferation accounts for the majority of β-cell mass

expansion, although neogenesis from other islet cell types and non-islet cells can also

be identified under specific physiological impacts (Paris et al., 2004). The proliferative

capability of β-cells is gradually reduced along with aging unless stimulated by certain

physiological impacts, such as pregnancy and obesity. A variety of extracellular growth

cues, as well as intrinsic cellular factors, have been identified to regulate β-cell

proliferation (Ackermann and Gannon, 2007).

Compared to β-cell differentiation and proliferation, the mechanism governing β-

cell long-term stability is less well understood. The β-cell turnover is generally very slow

in healthy animals (Teta et al., 2005). β-cells bear a heavy burden of protein synthesis

as large amounts of insulin are constantly generated during the life cycle. As such, they

have developed a set of highly regulated cellular activities, including glucose

metabolism, protein translation and folding, vesicle transportation and vesicle exocytosis

(Vetere et al., 2014). It is unknown, however, how the gene expression is adapted for β-

cell function during the transition from immature β-cells to mature β-cells. In addition, β-

cells are subject to potential cell stress that may be caused by various factors, including

hyperglycemia, reactive oxygen species, inflammatory cytokines, and free fatty acids. Accordingly, β-cells have also developed a cellular machinery to counteract cell stress (Fonseca et al., 2010). Also unclear is the regulation of β-cell genetic network during the postnatal β-cell preservation in confronting potential cell stress.

Notably, many key transcription factors involved in the embryonic β-cell differentiation are also required for postnatal β-cell functional maintenance. For instance, Sato et al. found that HNF4α activates an ER chaperone co-regulator Anks4b to protect β-cells from ER stress (Sato et al., 2012). Similarly, Sachdeva et al. found that Pdx1 is also required for the maintenance of ER homoeostasis by directly controlling the expression of genes involved in the ER stress pathway (Sachdeva et al., 2009). By using a β-cell-specific transgenic mouse model, Hu He, et al. showed that overexpression of *Pax4* has protective effects for β-cells against cytokine-induced cell-stress and apoptosis (Hu He et al., 2011). In a loss-of-function study, Gu et al. showed that *NeuroD*1 expression in the β-cell is required to establish the mature glucose metabolic profile and maintain functional K_{ATP} channels, both of which are essential for glucose-stimulated insulin secretion (GSIS). It is likely that the role of these transcription factors in the postnatal β-cell functional maintenance is distinct from that in the embryonic β-cell differentiation (Pan and Wright, 2011). However, it remains unclear whether other transcription factors identified in the embryonic pancreas development are also essential for postnatal β-cell function. Furthermore, the temporal-specific regulatory roles of these factors need to be dissected in order to discriminate the part involved in the embryonic β-cell differentiation from the part involved in postnatal β-cell function.

Myelin transcriptional factors (*Myt1*, *Myt1L* and *Myt3*) are zinc-finger proteins that share highly conserved sequence homology. During embryonic pancreas development, the expression of *Myt1* is activated by Ngn3 (Wang et al., 2008). *Myt1* is then broadly expressed in the endocrine lineage, including endocrine progenitor cells and mature islet

cells (Wang et al., 2007). Although Myt1 is the only Myt family member that can be detected in the embryonic pancreas, the deletion of *Myt1* activates the expression of *Myt1L* and *Myt3* (Wang et al., 2007), highlighting the possibility of functional redundancy among these *Myt* paralogs. Our previous studies showed that *Myt1* knockout leads to moderate defects in β-cell differentiation and glucose tolerance (Wang et al., 2007). However, since increased Myt1L and Myt3 levels may have compensatory effects to rescue the defects, the comprehensive physiological roles of Myt factors remains to be fully elucidated. Moreover, whether Myt factors are mostly involved in the embryonic β-cell differentiation or in the postnatal β-cell function has not been decided.

In the present study, we have generated a conditional triple knockout mouse line in which *Myt1*, *Myt1L* and *Myt3* are simultaneously deleted in the pancreas. In this mouse model, we investigated how β-cell differentiation and postnatal function were affected upon deletion of Myt factors. To our surprise, embryonic endocrine cell differentiation and islet formation both appeared to be largely normal. However, the *Myt* mutant mice displayed overt diabetes in postnatal stages, and their β-cell defects resembled "β-cell failure" in the late stage of T2D, as characterized by reduced insulin secretion, β-cell dedifferentiation and β-cell demise. Notably, dysregulated gene expression of *Myt* mutant β-cells was identified at birth, when the β-cell identity and subcellular morphology were normal. Therefore, although Myt factors are dispensable for β-cell fate specification during the endocrine cell differentiation, they help to establish a gene expression network for the newly differentiated β-cells to become fully functional and long-term sustainable in the postnatal stage.

3.3 Results

All three Myt factors are expressed in postnatal islets

We first generated antibodies against Myt1L and Myt3, in addition to the previously reported Myt1 antibody (Wang et al., 2007), to examine the protein levels of Myt factors in the pancreas. The sensitivity and specificity of these antibodies were confirmed in pancreas islets (Figure 3.1). In the embryonic pancreas, we did not detect the protein expression of *Myt1L* and *Myt3* (Figure 3.1A), which was consistent with our previous analysis of mRNA expression levels (Wang et al., 2007). Intriguingly, both Myt1L and Myt3 proteins became detectable in some endocrine cells upon deletion of *Myt1* (Figure 3.1B), reinforcing our previous conclusion regarding the potential compensatory effect within Myt factors (Wang et al., 2007). In comparison, all three *Myt* genes were found to be abundantly expressed in adult islet cells (Figure 3.1C). This observation suggests that Myt factors may have unique function in postnatal pancreatic endocrine cells.

Knockout of Myt factors leads to overt diabetes with reduced insulin secretion

To eliminate possible functional redundancy among Myt factors (Wang et al., 2007), we generated *Myt1*, *Myt1L*, and *Myt3* triple conditional knockout mice ($Myt1^{F/F}$;$Myt1L^{F/F}$;$Myt3^{F/F}$, or *6F* for simplicity) by homologous recombination (See Materials and Methods). To investigate the overall role of Myt factors in the pancreas, we crossed *6F* mice with *Pdx1-Cre* transgenic mice (Gu et al., 2002). In *Myt* conditional knockout mice ($Myt1^{F/F}$;$My1L^{F/F}$;$Myt3^{F/F}$;*Pdx1-Cre*, termed *6F;Cre* for simplicity), we found a very small percentage (<10%, Figure 3.1D) of islet cells with detectable expression of Myt factors, most likely due to an incomplete recombination
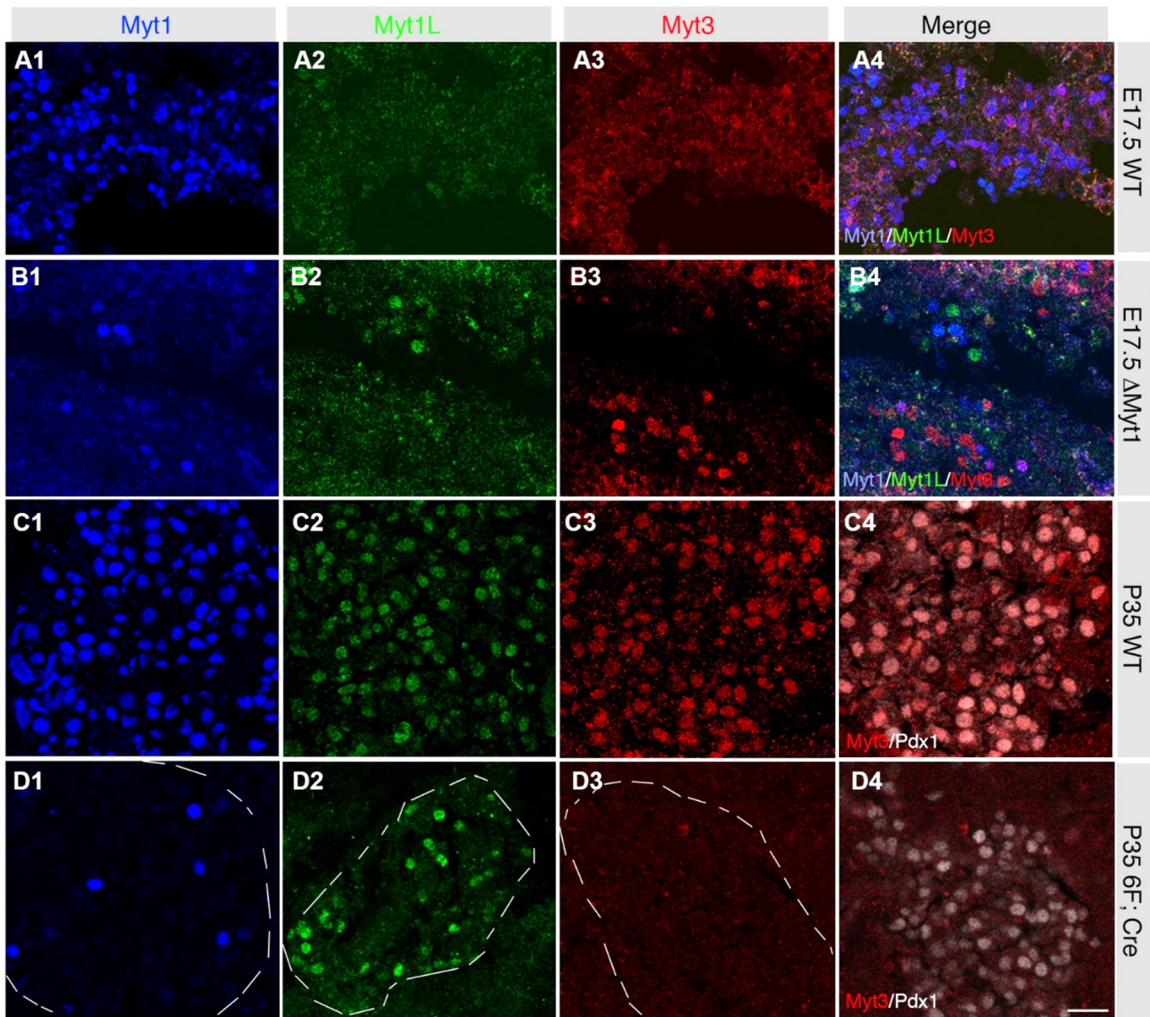
Figure 3.1 Pdx1-Cre-mediated *Myt* deletion substantially reduces Myt protein levels in pancreatic islets cells. (A and B) The expression of Myt factors in the embryonic pancreas of wild-type (WT, A1-A4) or Pdx1-Cre-mediated *Myt1* knockout (*ΔMyt1*, B1-B4) mice. E17.5: embryonic day 17.5. (C and D) The expression of Myt factors in the postnatal pancreas of wild-type (WT, C1-C4) and Pdx1-Cre-mediated Myt triple knockout (*6F;Cre*, D1-D4) mice. Note that due to technique issues, Myt factors cannot be co-stained in the postnatal pancreas. C1, C2 and C3 are different pancreatic sections, and C3 and C4 are from the same pancreatic section, with C4 being labeled with Pdx1 antibody to distinguish the islet. The relationship of images D1-D4 is the same as that of C1-C4. P35: postnatal day 35. Scale bar: 20 μm.
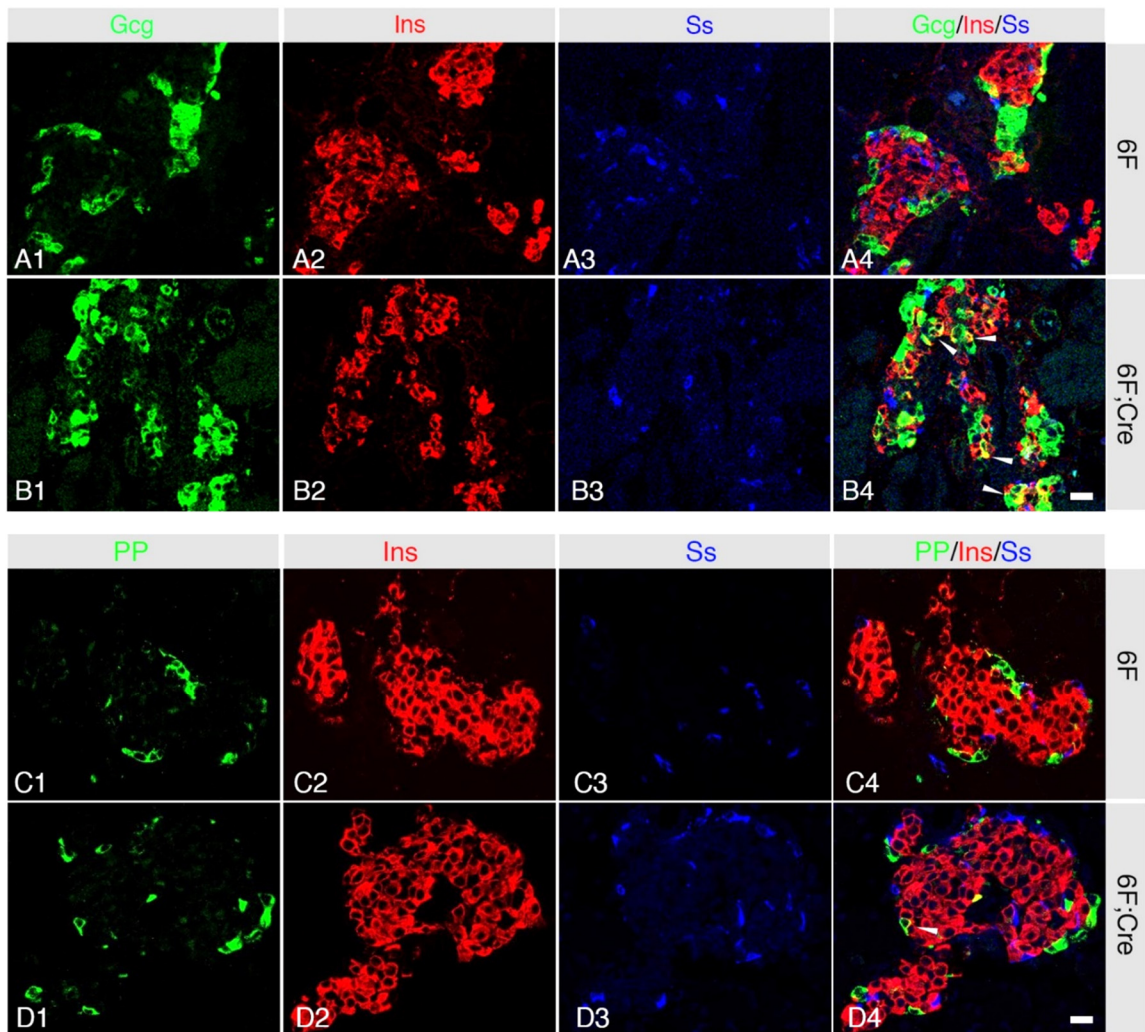
Figure 3.2 Knockout of Myt factors results in bihormonal islet cells in newborn mice. The expression of *glucagon* (Gcg, green), *insulin* (Ins, red), *somatostatin* (Ss, blue) and *pancreatic peptide* (*PP,* green) in control *6F* (A1-A4, C1-C4) and *Myt* mutant *6F;Cre* mouse pancreas (B1-B4, D1-D4). Arrowheads in B4 and D4 point to bihormonal islet cells. Scale bar: 20 μm.

mediated by the Pdx1-driven Cre recombinase. Nonetheless, the overall >90% recombination efficiency allowed us to study the function of Myt factors.

In newborn *6F;Cre* mice, we identified a small percentage (3.1±0.5% of all islet cells, n=5) of bihormonal islet cells that co-express *insulin* and *glucagon*, or co-express *insulin and PP* (Figure 3.2), which have been reported in the *Myt1* single knockout study as incompletely differentiated endocrine cells (Wang et al., 2007). Other morphological aspects of pancreas development, including β-cell mass and the ratio of islet cell types, appeared to be normal (Figure 3.4C and data no shown). These combined findings suggest that Myt factors do not have a prominent function in islet cell fate specification, but they are required for complete differentiation of monohormonal islet cells.

The *6F;Cre* mice did not show any obvious physical defects in the neonatal stage. However, starting from the weaning stage, these mice exhibited substantially reduced body weight and elevated fasting blood glucose levels compared to *6F* littermates or age-matched *Pdx1-Cre* mice (Figure 3.3 and data not shown). These phenotypes reflected defects in glucose homeostasis and biological assimilation. The differences between *Myt* mutant mice and control mice became more prominent with age. By two months of age, over half of the *6F;Cre* mice died with severe growth retardation and extremely high blood glucose (>500 mg/dL). We therefore restricted all of our studies to mice younger than 6 weeks, when most of the mutant mice were still alive.

In agreement of elevated blood glucose, we found that the increase of serum insulin level in responding to glucose stimulation was nearly abolished in *6F;Cre* mice (Figure 3.3D and E). This observation suggests that the abnormality in glucose homeostasis is due to defects in β-cell function. Interestingly, *6F;Cre* mice initially showed lower random-fed blood glucose than control littermates, although this trend was reversed after weaning (Figure 3.3C). This finding suggests that inactivation of Myt
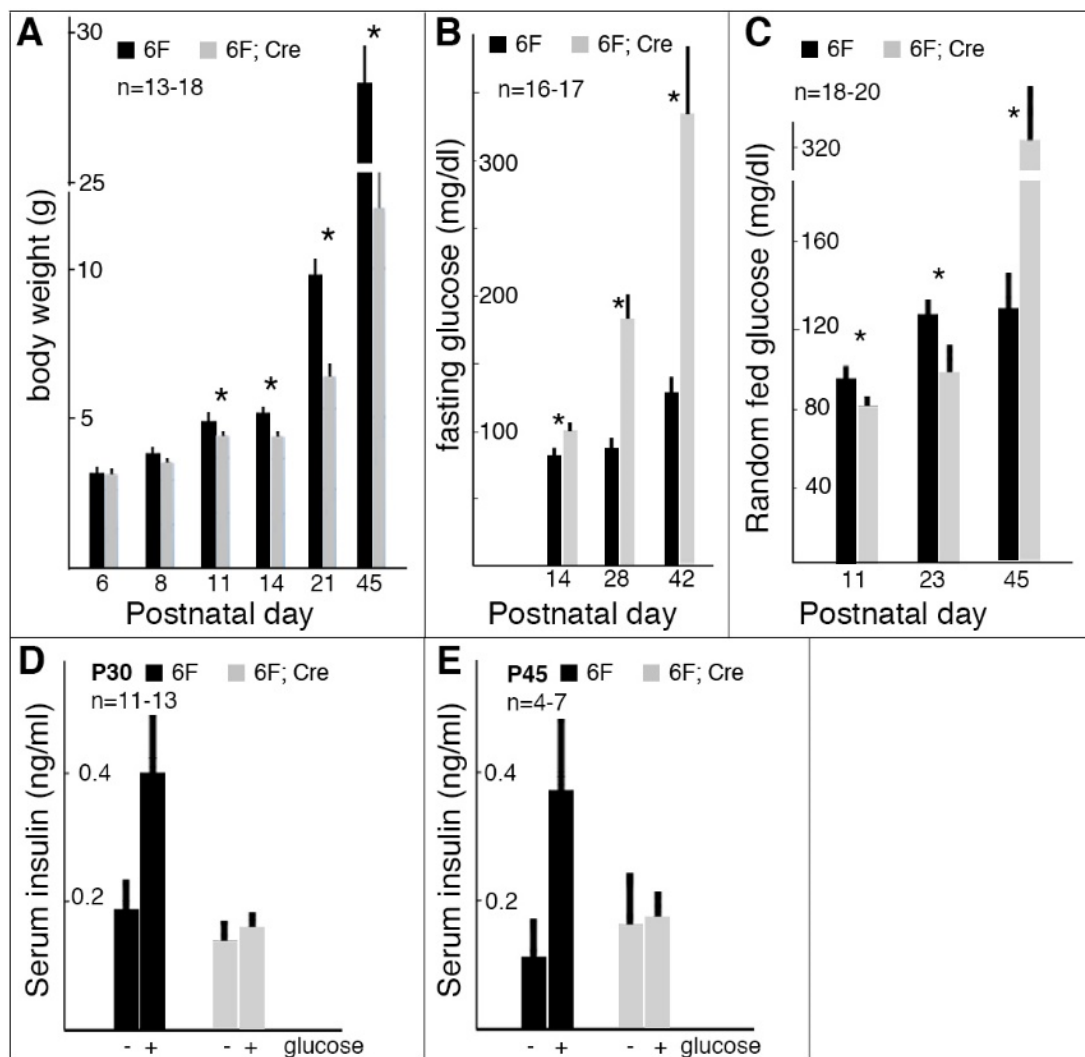
Figure 3.3 *6F;Cre* **mice develop overt diabetes in post-weaning stages.** Body weight (A), fasting glucose (B), random glucose (C), and serum insulin upon glucose challenge (D and E) of control *6F* and *Myt* mutant *6F;Cre* mice were measured at indicated postnatal ages. *p<0.05, student t-test.

factors does not totally eliminate insulin secretion, but rather compromised the β-cell response to glucose stimulation. Taken together, although Myt factors appear to be largely dispensable for β-cell fate specification during embryonic pancreas development, they are required for β-cell function in postnatal stages, when the animals start relying on self-generated insulin for glucose metabolism.

Myt mutant mice exhibit dramatic β-cell mass decline concomitant with insufficient cell proliferation and increased cell apoptosis

Next we sought to examine in detail the β-cell defects caused by deletion of Myt factors. In *6F;Cre* mice, we first noticed dramatic decline of islet number and β-cell mass (Figure 3.4A and B). In our time course β-cell mass analysis, although *6F;Cre* mice were born with normal β-cell mass, their β-cell mass was only half of that in *6F* mice at postnatal day 14 (P14) (Figure 3.4C). Notably, the mutant mice at this stage have not displayed the glucose metabolism defects, suggesting that the loss of β-cell mass is not due to glucotoxicity but is caused by intrinsic β-cell defects. The β-cell mass was further reduced, and there were essentially no detectable islet structures within the mutant pancreas at two months of age (Figure 3.4B and data not shown).

To investigate factors contributing to the decline of β-cell mass, we used Ki67 labeling and BrdU incorporation to examine β-cell proliferation at different postnatal stages. The proliferation index in *6F;Cre* mice was significantly reduced in early life (P7 and P14, Figure 3.4D-F), suggesting that *Myt* mutant β-cells did not possess an equal proliferation capability compared to their wild-type littermates. Interestingly, there was no obvious difference in the ratio of BrdU incorporation at P30, probably due to some proliferation compensation triggered by the reduced β-cell mass.

Meanwhile, we also examined the β-cell death by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and by detecting the apoptosis
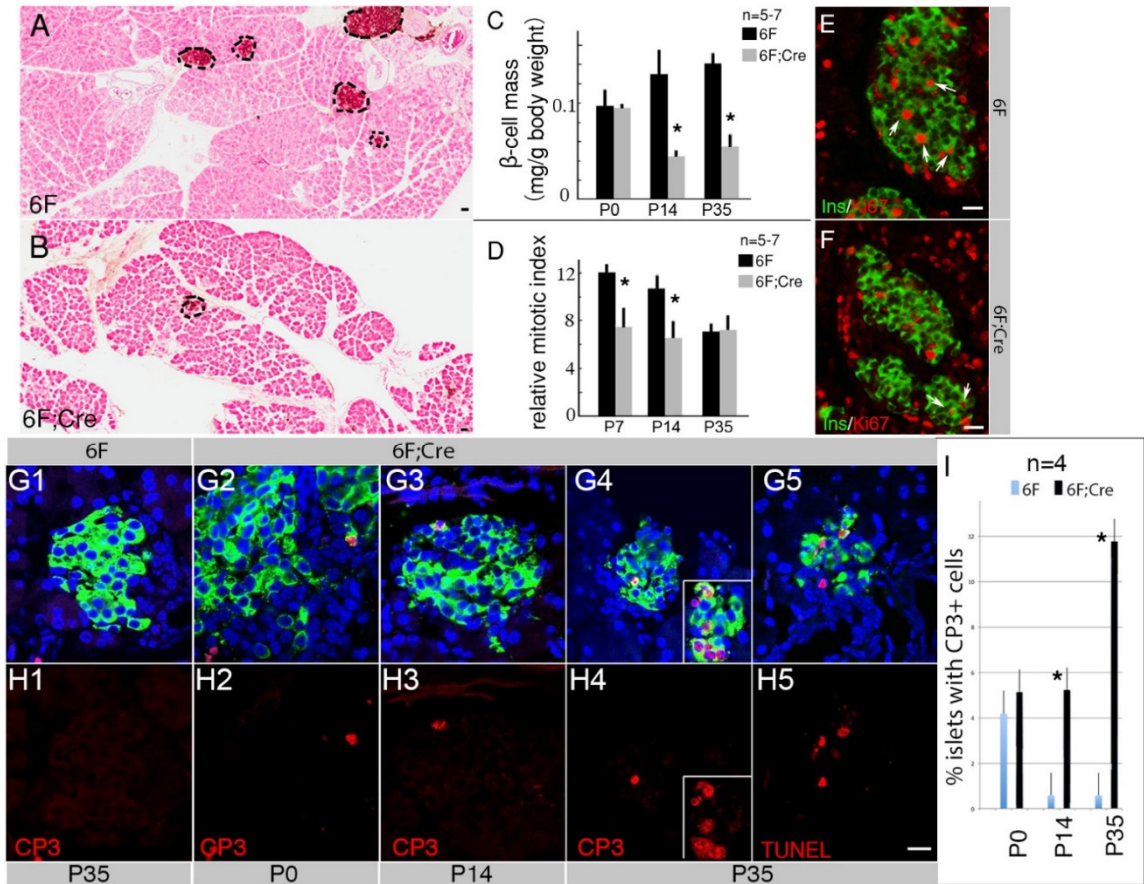
Figure 3.4 Loss of Myt factors results in β-cell mass reduction. (A and B) Representative images of insulin immunohistochemistry in control (*6F*) and *Myt* mutant (*6F;Cre*) pancreata at P14. (C) Quantification of β-cell mass at indicated postnatal ages. (D) Quantification of β-cell proliferation at indicated stages. Mitotic cells were detected by Ki67 labelling (P0 and P4) or BrdU incorporation (P35). Relative mitotic index was calculated by normalizing the number of Ki67 or BrdU positive β-cells to the total β-cell number. (E, F) Representative images showing Ki67-positive β-cells (arrows) in control (*6F*) and *Myt* mutant (*6F;Cre*) islets at P14. (G-H) Representative images showing β-cell apoptosis at indicated stages. Immunofluorescence of activated-caspase 3 (CP3, red) in control (G1) or *Myt* mutant islets (G2-G5) at indicated stages. Insert in G4 shows a CP3-enriched islet. (G5) DNA fragmentation detected by TUNEL assay in *My* mutant islets at P35. β-cells are labeled by insulin immunofluorescence (green). Each image in (G1-G5) has a corresponding image in (H1-H5) showing the apoptotic signal. (I) Quantification of islet clusters with detectable CP3 signal. Scale bar: 20 µm. *p<0.05, student t-test.

marker cleaved caspase 3 at different postnatal stages. Consistent with previous reports

regarding the high cell turnover rate of young β-cells (Scaglia et al., 1997; Teta et al.,

2005), we found that in control *6F* mice, β-cell apoptosis was more readily detectable at

the neonatal stage but very rare at the adult stage. In comparison, both TUNEL and

caspase 3 staining signals could be found in *6F;Cre* islets at different postnatal stages

(Figure 3.4G-I). Taken together, these findings suggested that severe reduction of β-cell

mass in *Myt* mutant mice was caused by defects in both β-cell proliferation and survival.

Therefore, Myt factors play pro-proliferative and anti-apoptotic roles in postnatal β-cells.

β-cell function is compromised in *Myt* mutant mice

In addition to the β-cell mass decline, we also identified abnormal islet structures

in *6F;Cre* mice. Although islet morphology in newborn *6F;Cre* mice appeared to be

normal, the islet integrity was gradually lost in later postnatal stages (compare the β-cell

clustering between *Myt* mutant and control at P0, P7 and P14 in Figure 3.6).

Furthermore, we examined β-cell subcellular structures by transmission

electronic microscope (TEM). Similar to the scenario of islet clustering, there was no

obvious difference between *Myt* mutant β-cells and control β-cells in the insulin vesicle

morphology at P0 (Figure 3.5A1 and B1). Strikingly, however, *Myt* mutant β-cells had

much smaller and less dense insulin vesicles at P14 (Figure 3.5A2 and B2). A further

quantification revealed a dramatic reduction of large dense-core vesicles (LDCVs) in *Myt*

mutant β-cells (Figure 3.5C). As LDCV is the major type of vesicles involved in

packaging and transporting insulin (Kasai et al., 2010), this observation suggests that

loss of Myt factors is detrimental to β-cell function. Indeed, GSIS assay found that *Myt*

mutant islets displayed elevated basal insulin secretion and reduced response to

glucose stimulation (Figure 3.5D). This observed GSIS pattern resembled that of
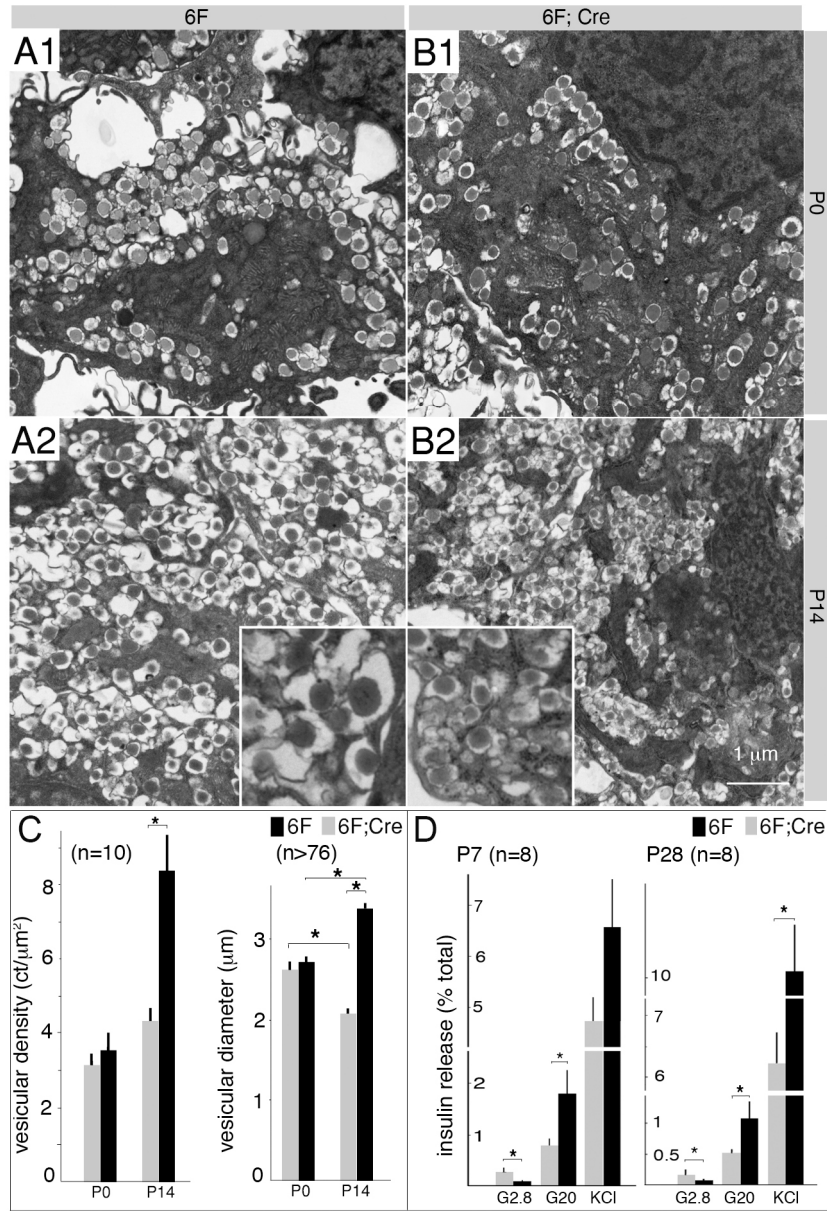
Figure 3.5 Loss of Myt factors compromises β-cell function. (A-B) Representative electron microscopy images of control (*6F*) and *Myt* mutant (*6F;Cre*) β-cells at P0 (A1 and B1) and P14 (A2 and B2). Insets in A2 and B2 are images with higher magnification highlighting the size and morphology of insulin granules. Scale bar: 1 μm. (C) Quantifications of vesicle density and vesicular diameter in control and *Myt* mutant β-cells. (D) Glucose- or KCl- stimulated insulin secretion in isolated islets was measured for P7 and P28 control and *Myt* mutant mice. Note that mutant islets exhibit higher basal secretion and lower simulated secretion compared to that of control. G2.8: 2.8 mM glucose. G20: 20 mM glucose. *p<0.05, student t-test.

immature β-cells (Blum et al., 2012), suggesting that Myt factors are required for the establishment and/or the maintenance of mature insulin secretion profile.

The glucose metabolic pathway switching is a key factor contributing to β-cell maturation (Lemaire et al., 2016). Thus, it is possible that deletion of Myt factors disrupts normal glucose metabolism in mature β-cells. However, we also found that *Myt* mutant islets did not respond to the direct plasma membrane depolarizer KCl, which stimulates insulin secretion without triggering glucose metabolism (Figure 3.5D). Therefore, we reasoned that Myt factors regulate at least certain processes in GSIS other than glucose metabolism.

Persistent activation of FoxO1 in *Myt* mutant β-cells

Next, we asked whether *Myt* mutant β-cells experienced cell stress, which is often related to β-cell dysfunction and apoptosis. To address this, we examined the expression of *FoxO1*, a well-known β-cell stress marker. As a transcription factor, FoxO1 is constitutively excluded from the nucleus and is therefore inactivated in healthy β-cells. However, FoxO1 is translocated into the nucleus and becomes activated in stressful conditions, such as hyperglycemia (Guo et al., 2013), oxidative stress (Essers et al., 2004; Kitamura et al., 2005), and lipotoxicity (Wrede et al., 2002).

As expected, in β-cells of control *6F* mice, FoxO1 was only detectable in the cytoplasm throughout different postnatal stages (Figure 3.6). In contrast, FoxO1 was translocated to the nuclei of *6F;Cre* β-cells at P7, and there were more FoxO1 nucleus-enriched β-cells with age (Figure 3.6D). As *6F;Cre* mice were not hyperglycemic at P7, we reasoned that the nucleus enrichment of FoxO1 in the β-cell was not a result of glucotoxicity, but rather a direct reflection of intrinsic cell stress. We also found a dramatically reduced *Pdx1* protein level in FoxO1-activated β-cells, which was consistent with a previous report that FoxO1 and Pdx1 were mutually exclusive in the nucleus
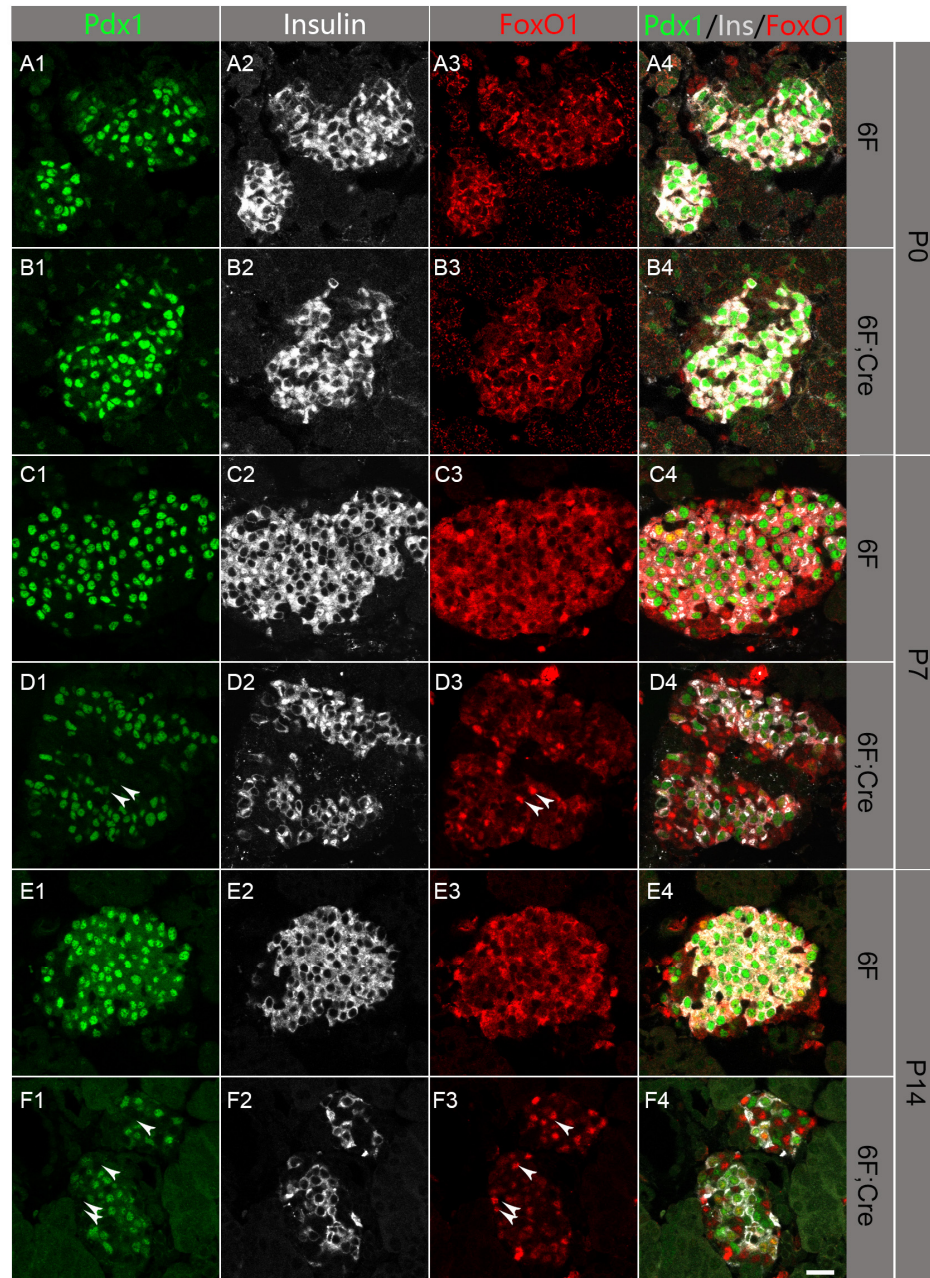
Figure 3.6 Activation of FoxO1 in *Myt* mutant islet cells. The expression and subcellular localization of FoxO1 was examined by immunofluorescence (red) at indicated stages. Pdx1 (green) and insulin (white) were co-stained to label β-cells. (A1-A4 and B1-B4) At P0, FoxO1 localizes in the cytoplasm for both control (*6F*) and *Myt* mutant (*6F;Cre*) islet cells. (C1-C4 and E1-E4) FoxO1 localizes in the cytoplasm for control islet cells at P7 and P14. (D1-D4) At P7, FoxO1 is enriched in the nucleus for some *Myt* mutant islet cells. (F1-F4) At P14, nearly all *Myt* mutant islet cells have FoxO1 enriched in the nucleus. Note that the overall Pdx1 level is reduced in *Myt* mutant β-cells. In particular, *Pdx1* expression is reduced to an undetectable level in FoxO1-activated islet cells (arrowheads). Scale bar: 20μm.

localization (Kitamura et al., 2002). At P14, almost all β-cells were found to have FoxO1-enriched nuclei, accompanied by diminished islet sizes and reduced islet cell-cell contacts (Figure 3.6F). The abnormal activation of FoxO1 suggest that *Myt* mutant β-cells have severe cell stress after birth, which may be a significant contributor to β-cell dysfunction and apoptosis. Notably, FoxO1 was not activated in *Myt* mutant β-cells at P0 (Figure 3.6B), suggesting that the cell stress was not a direct outcome of deficient β-cell differentiation but was more related to the postnatal β-cell functional failure.

***Myt* mutant mice fail to maintain fully differentiated β-cell status**

In addition to cell death, accumulating evidence have indicated that β-cell dediferentiation is another significant outcome of prolonged cell stress and functional failure (Cinti et al., 2016; Talchai et al., 2012; Wang et al., 2014). Given the deteriorating β-cell function in *6F;Cre* mice, we sought to investigate the role of Myt factors in maintaining β-cell identity. To do this, we first examined the expression of well-acknowledged β-cell marker genes, including *Pdx1*, *MafA* and *Glut2*. Their protein expression in *Myt* mutant β-cells was all normal at P0, corroborating our conclusion that deletion of Myt factors does not affect the general β-cell differentiation (Figure 3.7). However, the protein levels of these genes were reduced at P7 in *Myt* mutant β-cells and further declined in later postnatal stages (Figure 3.7 and data not shown). The temporal pattern of reduced expression of β-cell marker genes was similar with that of other β-cell defects identified in *Myt* mutant β-cells, i.e. these defects were not displayed at P0 but gradually emerged in the postnatal stage. The expression of *Nkx6.1*, another significant gene involved in postnatal β-cell function and identity maintenance (Taylor et al., 2013), was not changed (data not shown). These findings suggest that deletion of *Myt* factors selectively affects the expression of β-cell functional genes in the postnatal stage.
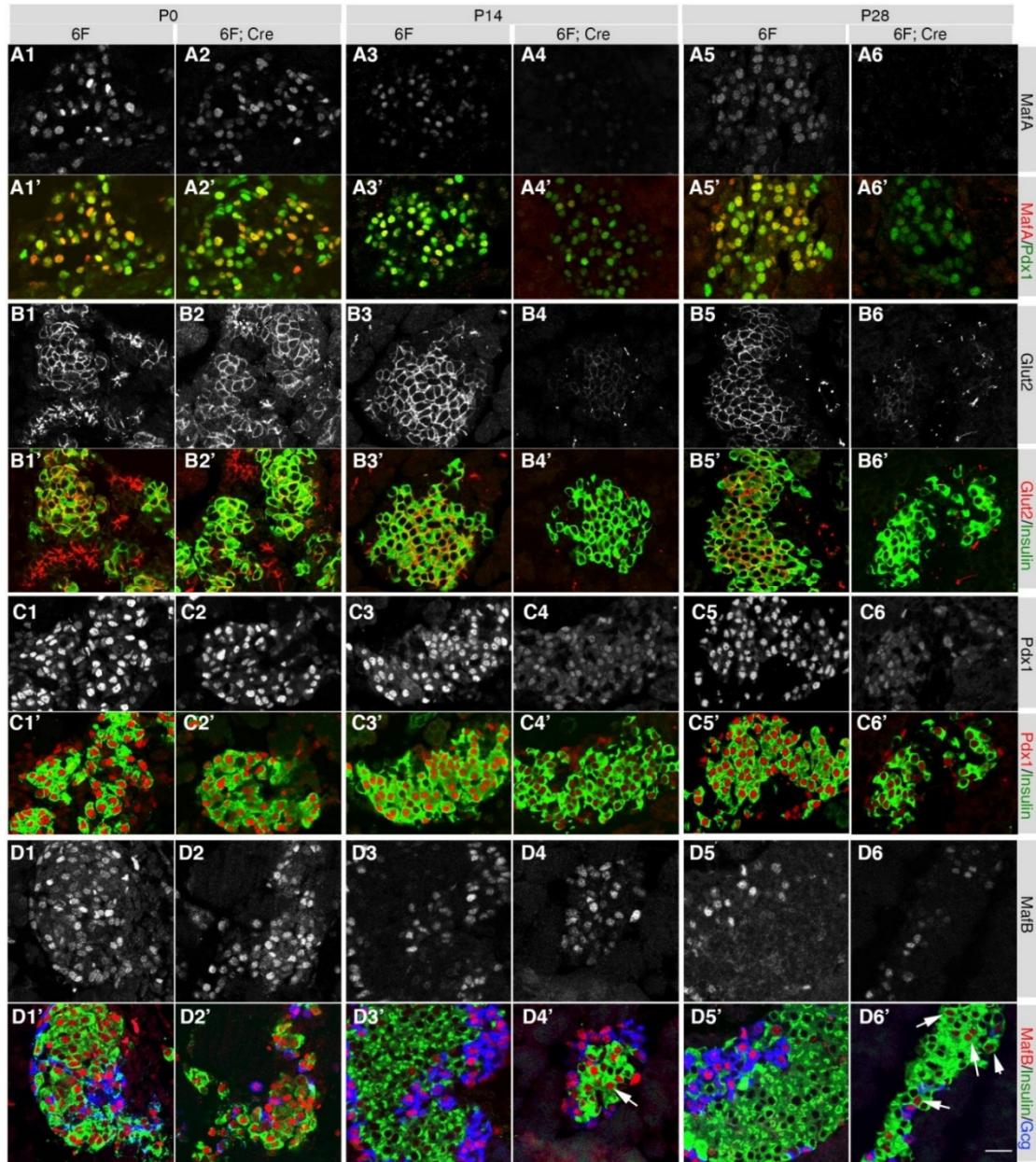
Figure 3.7 *Myt* mutant β-cells gradually lose differentiated β-cell identity.
Immunofluorescence of MafA (A), Glut2 (B), Pdx1 (C), and MafB (D) in pancreatic
sections of control and *Myt* mutant mice at indicated stages. The white channels
are used to highlight the intensity of each assayed protein. Each pair of images
detecting the same gene expression at a specific stage (e.g. A1' and A2', A3' and
A4', B1' and B2', etc.) was captured with the same parameters in order to
compare the signal intensity between the wild-type control and *Myt* mutant
samples. Arrows in D4' and D5' point to select *Myt* mutant β-cells expressing high
levels of MafB. Scale bar: 20 μm.

Pdx1 | Ngn3 | Pdx1/Ngn3/DAPI

A1 | A2 | A3 — 6F

B1 | B2 | B3 — 6F;Cre

Ins | Hes1 | Ins/Hes1/DAPI

C1 | C2 | C3 — 6F
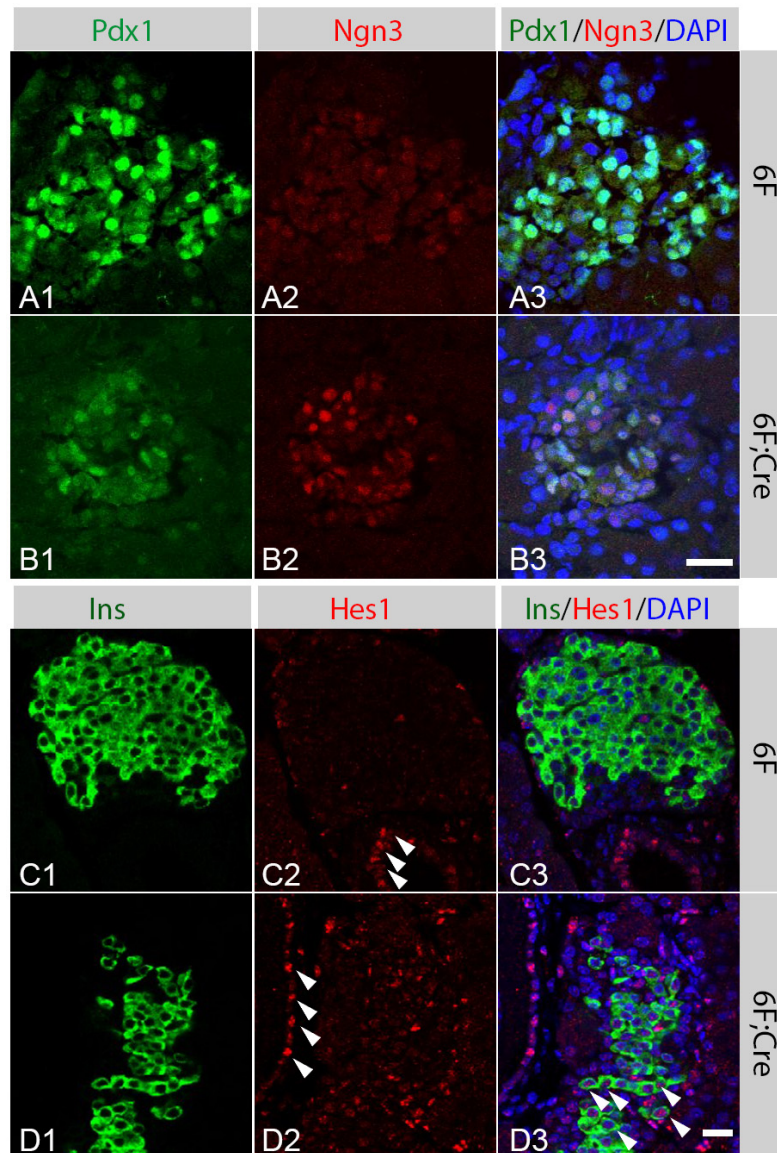
D1 | D2 | D3 — 6F;Cre

Figure 3.8 continued
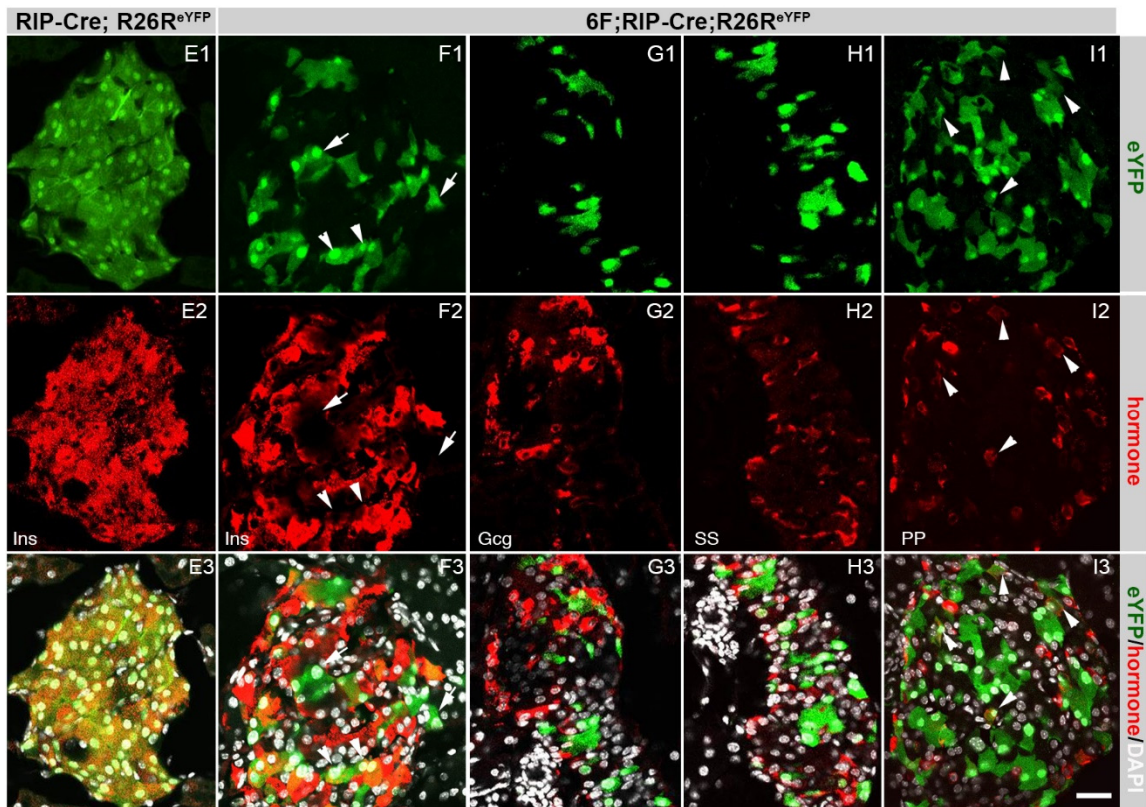
Figure 3.8 Dedifferentiation and transdifferentiation of β-cells upon deletion of Myt factors. (A-D) The expression of *Ngn3* (A and B) and *Hes1* (C and D) in pancreatic islets was detected at P14 by immunofluorescence, and the β-cells were labeled by Pdx1 or insulin. Arrowheads in C2 and D2 point to some pancreatic ductal cells with high levels of Hes1. Arrowheads in D3 point to β-cells with elevated Hes1 levels. (E-I) Insulin-expressing cells in control and *Myt* mutant mice were lineage-traced for two months. (E) The expression of *eYFP* and *insulin* in β-cells in control mice (*RIP-Cre; R26R^{eYFP}*). (F-I) The expression of *insulin* (F), *glucagon* (G), *SS* (H), and *PP* (I) in eYFP labelled cells in *Myt* mutant islets (*6F;RIP-Cre;R26R^{eYFP}*). Arrows in F1-F3 point to YFP-positive cells without detectable insulin. Arrowheads in I1-I3 point to PP-positive cells that are also eYFP positive. Scale bar: 20 μm.

We also examined the expression of pancreatic progenitor genes that are not supposed to be expressed in mature β-cells. In rodent β-cells, the expression of *MafB* is only found in endocrine progenitor cells and immature β-cells. However, we detected a substantial number of postnatal β-cells expressing *MafB*, and the percentage of MafB-positive β-cells was increased along with the loss of β-cell markers (35±4% in P14 and 68±5% in P28, n=4, Figure 3.7D). In addition, the protein expression of *Ngn3* and *Hes1* was remarkably upregulated in *6F;Cre* β-cells at P14, when these mutant β-cells were characterized by a substantial loss of β-cell markers (Figure 3.8 A-D). In pancreatic endocrine lineage, *Ngn3* and *Hes1* are both progenitor-specific genes whose expression is very low in postnatal islets, this observation suggests that Myt mutant β-cells loss the mature cell status and gain certain progenitor-like characteristics.

Since we found bihormonal β-cells in newborn *6F;Cre* islets, we were concerned whether β-cells might also be lost due to the conversion from β-cells to other islet cell types. To test this, we generated a *6F;RIP-Cre;R26R^eYFP* mouse line, in which Myt factors were deleted specifically in β-cells by an insulin promoter-driven Cre recombinase (Postic et al., 1999). In addition, the activation of the *R26R^eYFP* reporter allele upon the Cre-mediated recombination enabled us to lineage-trace the β-cell fate (Figure 3.8). We found that while eYFP-positive cells were exclusively β-cells in the *RIP-Cre;R26R^eYFP* control mice (Figure 3.8E1-E3), there were eYFP-labeled insulin-negative cells in the *6F;RIP-Cre;R26R^eYFP* mutant mice (Figure 3.8F1-F3). Additionally, we identified eYFP-positive PP-cells in the *Myt* mutant islets, suggesting a β-to-PP cell fate conversion upon deletion of Myt factors (Figure 3.8I1-I3). Together with the observation of β-cell dedifferentiation, we concluded that upon the deletion of Myt factors, β-cells failed to maintain the fully differentiated cell status.

Myt factors are essential to establish the gene expression network for functional

β-cells in postnatal stages

To uncover the mechanism underlying the β-cell defects caused by deletion of *Myt* genes, we investigated gene expression in *Myt* mutant β-cells. Notably, some *Myt* mutant β-cells underwent cell dedifferentiation and transdifferentiation, and they could not be effectively isolated by conventional β-cell isolation methods based on *insulin* expression. To this end, we isolated eYFP-positive β-cells from *6F;RIP-Cre;R26R^{eYFP}* *Myt* mutant mice and *RIP-Cre;R26R^{eYFP}* control mice at two months of age by fluorescence-activated cell sorting (FACS). Next we conducted RNA-seq to compare the genome-wide gene expression in these two cell groups.

In agreement with the defective β-cell phenotypes, we found that *Myt* mutant β-cells have dysregulated expression of genes involved in different aspects of β-cell physiology. Specifically, compared with control β-cells, the expression of genes involved in β-cell function – such as vesicle transport genes, cell-cell connection genes and cell cycle genes – was significantly reduced in *Myt* mutant β-cells (Figure 3.9A and data not shown). On the other hand, genes with upregulated expression in *Myt* mutant β-cells were mostly categorized into groups related to embryonic development and cell death (Figure 3.9A). The differential gene expression was consistent with the phenotypes we found in *6F;Cre* mice, including reduced cell proliferation, compromised GSIS, increased cell stress and apoptosis, and dedifferentiation. This observation suggests that Myt factors are required to promote and/or maintain the functional β-cell status by upregulating the expression of β-cell functional genes, as well as by repressing the expression of genes whose presence is detrimental to β-cell stability. Interestingly, we found that some genes (e,g. *Pdx1*, *MafA*, *Glut2*, *MafB* and *FoxO1*) displaying protein level change did not exhibit an obvious difference in mRNA expression levels, indicating

	CATEGORY	GENE SYMBOL
A Downregulated	**Cell-cell adhesion**	Cd27, Cd4, Cd96, S100a10, Cdh8, Cdh19, Cdh22, Cdh4, Cdh8, Clstn1, Ctnna2, Ctnnd2, Cntn1, Cntn3, Cntnap4, Cntnap5a, Ccnb2, Cyp1b1, Eif2s3xp1, Hist1h3i, Hist2h3b, Hoxd3, Hapln2, Icam4, Ifna13, Muc4, Pcdh1, Pcdha3, Pcdhgc4Slc4a1, Slc7a11, Sfn, Sdc3, Tln2, Tmem8, Troap, Tnfrsf13c, Tnfaip8l2, Umod, Zc3h12d
	vesicle-mediated transport	Stx1b, Abca7, Cadps, Rab3a, Rint1, Rapgef4, Sec13, Sh3kbp1, Scfd1, Arc, Ap1s1, Ap1s2, Ap2s1, Adora3, Amph, App, Copb2, Copg2, Copz1, Dnm3, Glrb, Lin7b, Lbp, Rims3, Nostrin, Ap2m1, Rph3a, Gnpda1, Nme1, Sorl1, Syngr, Snap91, Syt4, Sytl4, Syt10, Trappc4, Trim9, Vps45
Upregulated	**Embryonic morphogenesis**	Ldb1, Lmo4, Lhx1, Lmx1b, Tgif1, Aldh1a3, Celsr1, Chd7, Cyr61, Dlc1, Efna1, Foxc1, Fzd6, Hes1, Hhex, Lrp5, Rbp4, Sall4, Six4, Socs3, Tcf7l2, Ngn3, Mafb
	Response to ER-Stress	Eif1, Atf3, Eif1ax, Atf4, Eif2ak3, Atf5, Eif2b2, Atf7, Eif2b4, Atg3, Eif2s3y, Ddit3, Ddit4, Hsp90aa1, Hsp90ab1, Hsp90b1, Hspa12a, Hspa1a, Hspa1b, Hspa2, Hspa4
	negative regulation of cell proliferation	Cd24a, Cd9, Klf4, Tgif1, Adora1, Ang, Agt, Axin2, Cdkn1a, Cdkn2a, Dlc1, Fgfrl1, Inppl1, Nupr1, Rbp4
	Cell apoptosis	Bcap29, Faim2, Bag2, Fasl, Bag3, Tnf, Bcl2l12, Tnfsf15, Cd24a, Tnfrsf10b, Casp12, Tnfrsf11b

Figure 3.9 Differential gene expression in β-cells upon deletion of Myt factors. (A) Top functional categories containing the downregulated and upregulated genes upon deletion of Myt factors. Note that only representative genes in each functional groups are shown. β-cells from control (*RIP-Cre;R26R^eYFP*) and Myt knockout (*6F;RIP-Cre;R26R^eYFP*) mice at P60 were isolated by FACS and the genome-wide gene expression was analyzed by RNA-seq. The differentially expressed genes were functionally clustered by DAVID. (B) Differential gene expression between control (*6F*) and *Myt* mutant (*6F;Cre*) islets at P0. Islets were isolated from control and Myt knockout mice and the gene expression was detected by qRT-PCR. The expression of *GAPDH* was used as internal control across each samples. Data are presented as log2 fold change from the expression in the control islets to that of *Myt* mutant islets.

that knockout of Myt factors affects a more complicated regulatory network involving post-transcriptional regulation.

The gene expression change in *Myt* mutant β-cells could reflect a specific role of Myt factors in the adult β-cell function, or it could be due to the failure of gene regulatory network establishment in the newly differentiated β-cells. In addition, sustained high blood glucose levels might also affect β-cell gene expression (Kjorholt et al., 2005; Robertson et al., 2003). To discriminate these possibilities, we checked the gene expression in *6F* and *6F;Cre* islets at birth, when *Myt* mutant mice display normal blood glucose levels. Strikingly, quantitative RT-PCR showed gene expression changes in nearly all the gene categories that was identified in the adult stage (Figure 3.9B and data not shown). This observation suggests that the differential gene expression in the adult stage upon deletion of Myt factors is not a secondary effect of physiological changes but instead reflects the intrinsic β-cell defects. Moreover, the dysregulated β-cell gene expression at birth supports a crucial role of Myt factors in setting up a proper gene expression profile for the newly differentiated β-cells, so that these young β-cells can become functional and long-term sustainable in postnatal stages.

Myt factors expression levels are associated with β-cell function and proliferation potential

Since we found that Myt factors are closely involved in β-cell proliferation, survival and function, we asked whether the endogenous expression levels of *Myt* genes was correlated with β-cell function and proliferation under specific physiological conditions. To this end, we examined the levels of Myt factors in *db/db* mice at 7 weeks of age with wild-type littermates as controls. The *db/db* mice at this stage have enlarged β-cell mass and increased insulin secretion per β-cell in response to elevated insulin demand (Dalboge et al., 2013). We found that the protein expression of *Myt3* was
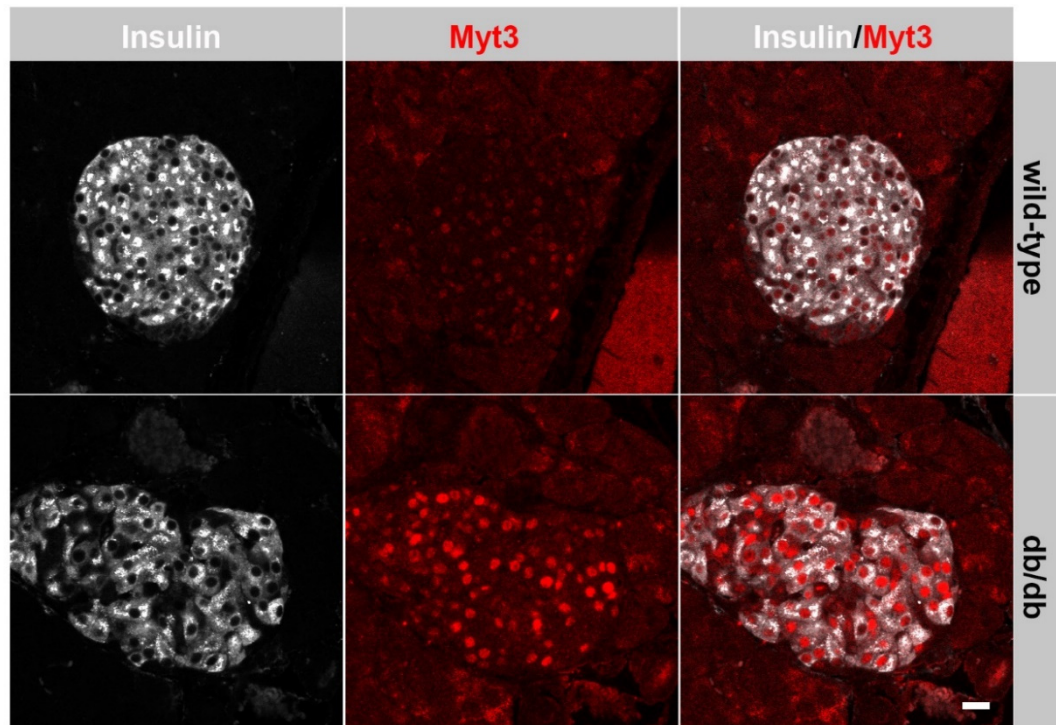
Figure 3.10 The endogenous *Myt3* protein expression level is increased in *db/db* mouse islets. Insulin and Myt3 protein levels were detected by immunofluorescence. Note that *db/db* β-cells display enlarged cell volume, which is an indicator of cell hypertrophy. Scale bar: 20 μm.

substantially upregulated, while the expression of *Myt1* or *Myt1L* remained unchanged

(Figure 3.10 and data not shown). This observation suggests that Myt3 is the major Myt

factor for compensating the insulin resistance during the pathogenesis of T2D, and Myt1

and Myt1L serve as redundant factors in this scenario.

3.4 Discussion

Pancreatic β-cells are uniquely designed to generate insulin for glucose

homeostasis. The pipeline of insulin production includes *insulin* expression, proper

protein folding in the ER, pro-insulin processing in the Golgi, and packaging into the

secretory vesicles. Moreover, GSIS also involves complex intracellular glucose

metabolism and vesicle exocytosis. Therefore, β-cells need to develop and maintain a

fine-tuned cellular machinery that adapts to each of these biological processes. Failure

to do so will result in severe cell stress, functional failure and cell apoptosis.

In this study, we provide evidence to demonstrate the crucial roles of Myt factors

in establishing proper gene expression network in postnatal functional β-cells. Triple

knockout of *Myt* genes leads to a dramatic loss of β-cell mass and functional damage.

Furthermore, *Myt* mutant β-cells are characterized by prolonged cell stress, which can

contribute to defects in β-cell function and survival. Along with the deteriorating cell

function, *Myt* mutant β-cells display loss of mature β-cell identity and gain of progenitor-

specific characteristics. Interestingly, none of these defects are observed in the newborn

Myt mutant β-cells but emerge in the postnatal stage, when β-cells start to synthesize

and secrete large amounts of insulin (Figure 3.11). Based on this observation, we

propose that Myt factors are required to generate β-cells that have the potential to

undergo functional maturation and long-term maintenance.
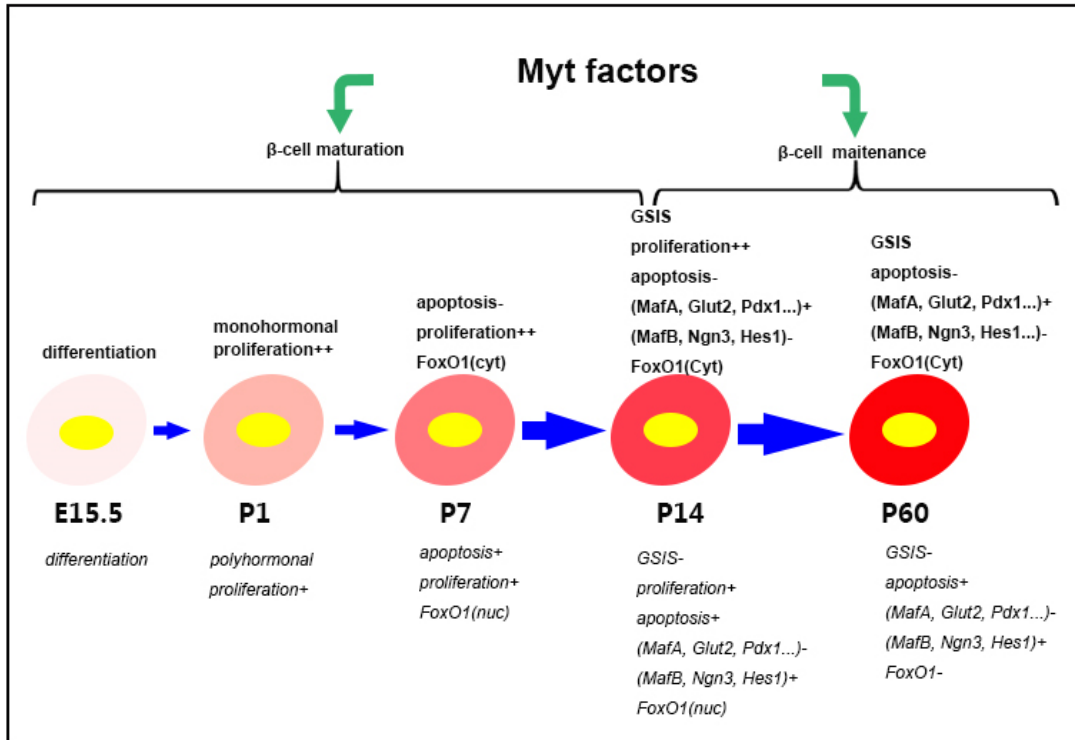
Figure 3.11 Myt factors are required to generate functional and stable β-cells. β-cell defects upon the deletion of Myt factors are listed over a time course (upper bold), with the comparison of corresponding wild-type phenotypes in each temporal stage (lower italic).

As expected from the above-described β-cell defects, our genome-wide transcription analysis revealed significant gene expression changes in *Myt* mutant β-cells, such as downregulation of genes involved in insulin secretion and upregulation of genes involved in embryonic development and cell apoptosis. Notably, we detected dysregulated gene expression in *6F;Cre* islets at newborn, suggesting that the differential gene expression is not a secondary effect of physiological changes in the postnatal stage. Instead, the altered gene expression supports a crucial role of Myt factors in setting up a gene expression network for the newly differentiated β-cells, so that these β-cells can become functional and long-term sustainable.

The next step for this study is to identify the direct transcriptional targets of Myt factors. Although we have identified several significant defects upon the deletion of Myt factors, it is possible that this group of transcription factors only directly control a small proportion of these biological processes. For instance, Myt factors may directly regulate the expression of ER chaperone genes, whose downregulation in *6F;Cre* β-cells will cause insulin misfolding and severe ER stress. This abnormality alone can trigger the cascade of β-cell functional failure and cell apoptosis. However, based on previous investigations of Myt factors in other systems, it is more likely that their direct transcriptional targets are categorized into multiple functional groups. For instance, Myt1 was first demonstrated as a transcriptional activator to increase the expression of *hplp* in oligodendrocytes (Kim and Hudson, 1992). Also, there were reports that Myt1 and Myt3 can both act as transcription repressors (Romm et al., 2005; Yang et al., 2008). These combined findings suggest that Myt factors may directly activate β-cell functional genes while simultaneously repressing progenitor-specific genes and apoptotic genes. A more comprehensive genome-wide identification of Myt factors' direct target genes will help to elucidate their molecular working model.

As described above, based on loss-of-function analysis and gene expression profiling, several transcription factors have been shown to be essential for postnatal β-cell function (Pan and Wright, 2011). Among them, MafA, Pdx1 and Nkx6.1 are three best characterized factors, whose ablation results in β-cell dysfunction, β-cell mass decline and/or β-cell identity loss (Ahlgren et al., 1998; Gao et al., 2014; Hang et al., 2014; Nishimura et al., 2006a; Sachdeva et al., 2009; Taylor et al., 2013). All these defects are found in *Myt* mutant mice, suggesting that these transcriptional factors cooperate with Myt factors to establish and/or maintain the gene expression network for postnatal β-cell stability and functional maintenance. Notably, the expression levels of MafA, Pdx1 and Nkx6.1 are all normal in the newborn *Myt* mutant β-cells. This observation suggests that, during the establishment of β-cell function, Myt factors do not act upstream of these factors. It is possible that Myt factors govern a β-cell functional pathway that is independent of these factors, or Myt factors interact synergistically with them to regulate target genes. These possibilities need to be addressed by identifying the direct transcriptional targets of all these transcription factor in the newly differentiated β-cells and by analyzing the genetic networks governed by these factors.

Another interesting perspective raised by our study is the role of Myt1 in connecting embryonic β-cell differentiation and postnatal β-cell function. The expression of *Myt1* in the pancreas is first activated by Ngn3 during the primary transition of pancreas development (E9.5) (Gu et al., 2004; Wang et al., 2007). *Myt1* expression is then restricted to the endocrine lineage, i.e. endocrine progenitor/precursor cells and differentiated endocrine cells. In embryonic stages, *Myt1* is unlikely to regulate endocrine cell-fate determination, as total endocrine cell number and the ratio of different endocrine cell types are both normal in *Myt* mutant mice. Therefore, it is possible that by activating *Myt1*, one role of the Ngn3-mediated embryonic differentiation program is to set up the gene regulatory network for the postnatal β-cell function.

Conventional *in vitro* β-cell directed differentiation deploys the step-wise strategy, in which specific inducing factors are utilized to achieve a set of predesigned intermediate steps (e.g. endodermal progenitor-like cells, pancreatic progenitor cells, endocrine progenitor cells, etc) (Pagliuca and Melton, 2013). Our studies showed that Myt1 *expression* is activated much earlier (i.e. in the endocrine progenitor cells) than the stage when the major function of Myt1 is exerted (i.e. after the appearance of β-cells). Therefore, our results provide a novel idea to activate the expression of pro-maturation genes in the early step during the direct differentiation.

Finally, while this study provided evidence that Myt factors are essential to generate functional β-cells with a long-term sustainable activity, whether this group of transcription factors are also needed after the establishment β-cell function has not been addressed. Based on our analysis of possible direct targets of Myt factors, it is more likely that their existence is required throughout the β-cell cycle. Indeed, our preliminary study has found β-cell dysfunction and loss of β-cell mass in a *6F;Pdx1-CreER* mouse model, in which Myt factors are deleted specifically in mature β-cells (Huang and Gu, unpublished observations).

In summary, the conditional *Myt* triple knockout mouse model provided us with an unprecedented platform to preclude any intra-family functional redundancy. Our study in this mouse model showed that Myt factors are essential in generating functional β-cells, which are not only glucose responsive but also have the long-term functional stability. Our results therefore suggest that Myt factors can be useful targets in strategic optimization for therapeutic β-cell regeneration to treat diabetes.

3.5 Material and Methods

Mouse strains and care

Mouse husbandry and genotyping were performed following protocols approved by the Vanderbilt Medical Center IACUC for Dr. Gu. For initial knockout mice production, C57BL/6 strain was used (Charles River Laboratories, Inc. Wilmington, MA). Subsequent strain maintenance and crosses utilized CD1 mice. Mouse strains used in this study included *6F* (derived from crossing between *Myt1^{F/F}*, *Myt1L^{F/F}* and *Myt1L^{F/F}* mice, see below for the targeting strategy), *Flpe* (used to delete the selection marker in the targeted allele) (Rodriguez et al., 2000), *Pdx1-Cre* (Gu et al., 2002), *RIP-Cre* (Postic et al., 1999) and *R26R^{eYFP}* (Srinivas et al., 2001). Genotyping was performed by standard PCR protocols. Genotyping primers used in this study were listed in Table 1.

Myt1L and Myt3 targeting constructs and animal derivation

Conditional *Myt1L* and *Myt3* alleles were designed similarly to that of *Myt1* (Wang et al., 2007). Briefly, exons 12 and 13 in *Myt1L*, as well as exons 9 and 10 in *Myt3* were flanked with LoxP sites after gene targeting. In both cases, the floxed exons to be deleted contained (3n + 1 or 2) nucleotides so that the recombination not only deleted part of the gene product, but also caused a reading frame shift to further disable the allele (Figure 3.12).

For homologous recombination, we used BAC recombineering to obtain 5' arms and 3' arms from BAC clones (RP22-191C21 for Myt1L and RP22-214C11 for Myt3, respectively). These arms were subcloned into the targeting vector pGKNeo-DTA, which contains the positive selection marker Neo and negative selection marker DTA (Zhang et al., 2001).
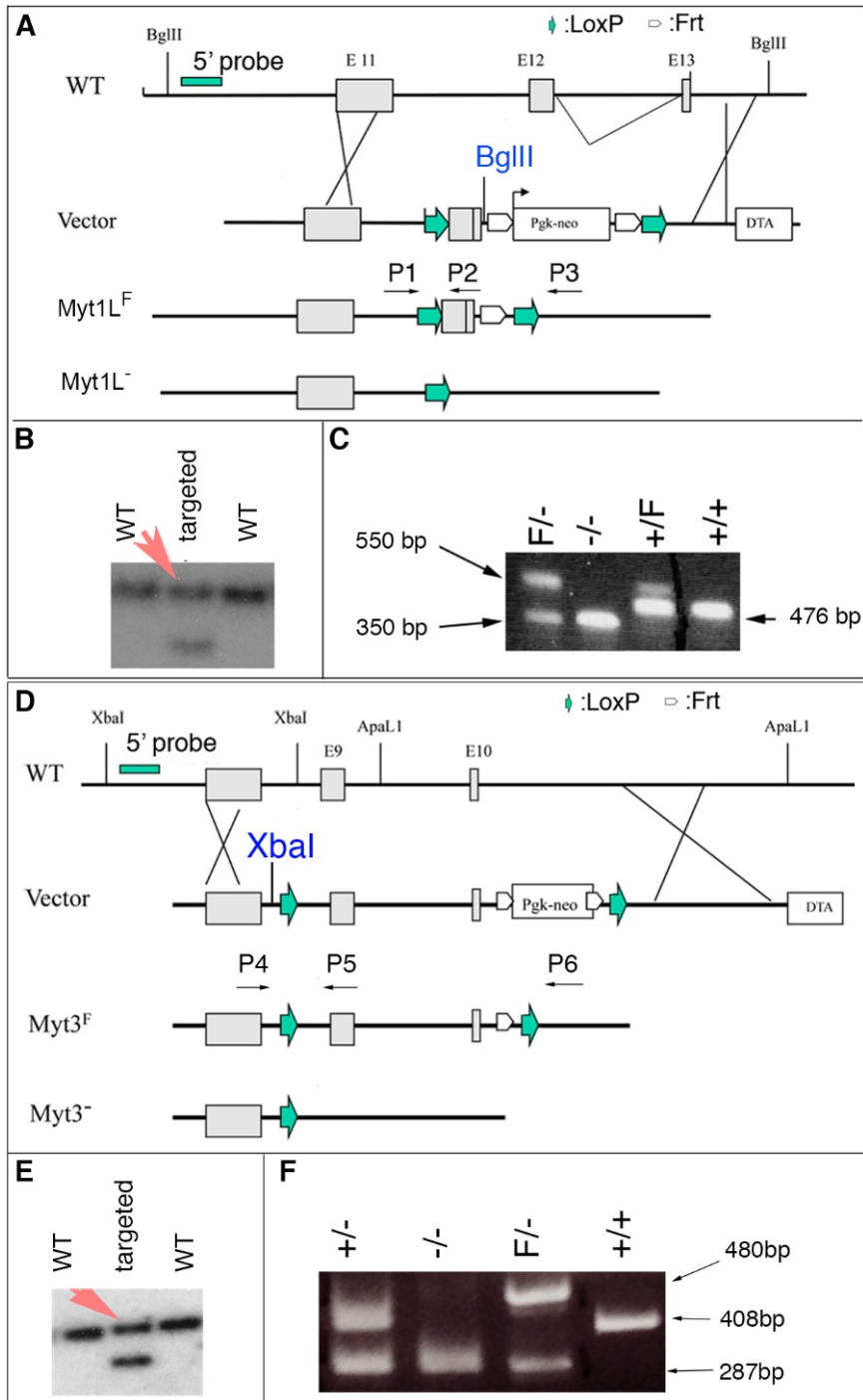
Figure 3.12 Gene targeting strategies to derive _Myt1L_ and _Myt3_ conditional alleles. (A) Schematic diagram showing the targeting constructs for _Myt1L_, _Myt1L^F_ and _Myt1L⁻_ alleles. Exons 12 and 13 were flanked by LoxP sites after targeting. Note that homologues recombination induced an extra BglII restriction site. 5' probe: DNA southern blot probe. P1, P2, and P3: primers for PCR-based genotyping. (B) Southern blots of _Myt1L_-targeted ES cell clones (wildtype band: 14kb and targeted band: 12kb). Genomic DNA was digested by BglII. (C) PCR-based genotyping using primers P1, P2 and P3. (D) Schematic diagram showing the targeting constructs for _Myt3_, _Myt3^F_ and _Myt3⁻_ alleles. Exons 9 and 10 were flanked by LoxP sites after targeting. Note that homologues recombination induced an extra XbaI restriction site. 5' probe: DNA southern blot probe. P4, P5, and P6: primers for PCR-based genotyping. (B) Southern blots of _Myt3_-targeted ES cell clones (wildtype band: 11.2kb and targeted band: 9.6kb). Genomic DNA was digested by XbaI. (C) PCR-based genotyping using primers P4, P5 and P6.

ES cell manipulation, including clone selection, expansion, blastocyst injection, and germ line transmission were performed by Vanderbilt Transgenic Mouse Core following established protocols. Genotyping primers were listed in table 1.

Generation of Myt1L and Myt3 antibodies

The cDNA sequences corresponding to amino acid residues 208-424 of Myt1L and 59-298 of Myt3 were fused with that of the maltose binding protein. Recombinant proteins were expressed in *E.coli* and affinity-purified as antigens to generate antibodies from guinea pig and rat, respectively. The specificity of the antibodies was tested by immunofluorescence in pancreas sections (Figure 3.1) and in HEK293T cells transfected with each protein-expressing plasmid.

RNA preparation, RNA-seq and quantitative RT-PCR

RNA was prepared from flow-sorted β-cells or isolated islets as described in Chapter II. Quantitative RT-PCR was performed following the standard protocol (Wang et al., 2009). The primers for RT-PCR were listed in table 2.

RNA-seq was performed by the Vanderbilt Technologies for Advanced Genomics. RNA-seq raw data were mapped to the mouse genome by TopHat2 (Kim et al., 2013). The mapped reads were then counted by HTSeq2 (Anders et al., 2013). Differential gene expression was identified by DEseq2 and was then functionally analyzed by DAVID (V6.7) (Huang da et al., 2009).

Glucose tolerance test and *in vivo* insulin secretion assay

Intraperitoneal glucose tolerance test (IPGTT) and *in vivo* insulin secretion assay were performed as described in Chapter II.

Islet isolation *and in vitro* insulin secretion

Islet isolation was performed as described in Chapter II.

For *in vitro* insulin secretion assays, isolated islets were first recovered by culturing for 2 hours in RPMI medium (containing 5.6 mM glucose and 10% FBS) and balanced in the Krebs-Ringer buffer with basal glucose (2.8 mM) and KCl (5 mM) for 30 minutes. Islets were then incubated at the Krebs-Ringer buffer with the basal glucose and KCl levels for 45 minutes before switching to the stimulating condition (20 mM glucose or 20 mM KCl). Supernatants containing secreted insulin were collected from different culture conditions, and islets were lysed by the ethanol/HCl lysis buffer to obtain total insulin contents. Insulin levels were assayed using an ELISA assay kit (Linco) following the manufacturer protocol. The percentage of insulin secretion was calculated by dividing secreted insulin contents by the total insulin contents.

Immunofluorescence (IF) and immunohistochemistry (IHC)

The IF and IHC of all target proteins were based on 6 μm paraffin sections, except in the cases of Ngn3, which were based on 10 μm frozen sections and were performed as previously described (Wang et al., 2009). The sample processing and immunostaining of paraffin sections were performed as described in Chapter II. For IF, fluorophore-conjugated secondary antibodies were used. For IHC, HRP-conjugated secondary antibodies were used, and IHC signals were developed by a DAB substrate kit (Pierce). The information of primary antibodies used in this study, including antibody sources, usages and antigen retrieval methods was listed in table 3.

Quantification of β-cell mass and β-cell proliferation

The paraffin sectioning for β-cell mass and β-cell proliferation qualification was performed as described in Chapter II. One third or one sixth of all sections were

collected to analyze β-cell mass for mice before or after weaning, respectively. β-cells

were detected by an insulin IHC, and the total slide was counterstained by eosin to

visualize the pancreatic tissue area. The percentage of β-cell areas was calculated by

dividing the insulin-positive areas by the total eosin area. β-cell mass was further

calculated by multiplying the percentage of β-cell areas by the pancreas weight. The

quantification of β-cell proliferation was performed similarly as described in Chapter II,

except that some tissue samples (i.e. the pancreas samples from BrdU injected mice)

were labeled by BrdU.

Microscopy and statistical analysis

All confocal images were collected on an Olympus FV-1000 confocal

microscope. When expression levels of a protein between samples were compared,

slides or cells were processed side-by-side, and images were captured utilizing identical

optical and electronic settings.

Statistical analyses utilized standard student t-test. A p-value of 0.05 or lower

was considered significant. All quantification data were presented as (mean ± SEM).

Table 1. Genotyping primers

Allele	Primer sequence (5'→3')
Myt1^F	TCCACCTGTCCAGTTCAGTG
	AGATCCTTCCAGGGTGGAGA
	TGTCTGTCCAGAACCTATTCCA
Myt1L^F	GTATGGGGAAACTGCTGAATGAA
	GCATCCAGACAGACTGCGGTGA
	GAGGAGGCAACATAACTGAAGA
Myt3^F	TGAGACTGAGACTACTTGTTAGC
	GGAGCATGTTCACCTTCTGG
	GCTTTCTGGGTTTCATTTCTG
Pdx1-Cre	TTG AAACAAGTGCAGGTGTTCG
	TTCCGGTTATTCAACTTGCACC
RIP-Cre	GTGCTTTGGACTATAAAGCTAGTGG
	TTCCGGTTATTCAACTTGCACC
R26R^{EYFP}	GGAGCGGGAGAAATGGATATG
	AAAGTCGCTCTGAGTTGTTAT
	GCGAAGAGTTTGTCCTCAACC
Pdx1-CreER	CTTGACAGCTGACCAGATGGTCAGT
	ATATGGGAAAGAATGAGAAGGAGC
RIP-rtTA	GTGAAGTGGGTCCGCGTACAG
	GTACTCGTCAATTCCAAGGGCATCG
TetO-Syt4	AAGAAGCACAGAGTGAAGACCAG
	GGATCTGAAAGTCCAGACACATC
Syt4$^{-/-}$	CACTTCCCTCACGTCAGAGGAG
	GCAAGGAGAGCT CTTGGATGTG
	AACCACACTGCTCGACATTGGG

Table 2. qRT-PCR primers

mRNA	Primer sequence (5'→3')
kcnj12	TGTGCCTGGATTTGTTTTGA
	CAGGCTTGTGTGGCCTAGAG
GZMC	AGGGAGCTTCCTTTGAGGAG
	ATCCATCAGTTTGCCCGTAG
Cdkn1	TTGCACTCTGGTGTCTGAGC
	TTGCACTCTGGTGTCTGAGC
Cdkn1b	ACGCCAGACGTAAACAGCTC
	TTCAATGGAGTCAGCGATATG
cdk15	TCCCAGTTGTACCAGCTTCC
	GAAGCCGAAAGGTCACACAT
kcnb2	ACTTTGGGACTTGCTGGAGA
	GCAATGGTGGAAAGAACGAT
Syt12	CCAATGAGAAGAGCACAGCA
	ATGGCTGGCACTGAGAAGAT
Syt15	CTTCCTCCAGTCCAAGACCA
	GGGTGACACTTTTGCTGGAT
Syt3	TGGCAAAGGATTGTCAGAGA
	GGACATTATGGCTTCGAGGA
GZMA	GTGGAAAGGACTCCTGCAAT
	ATGCCTCGCAAAATACCATC
GZMB	GACCCAGCAAGTCATCCCTA
	AGGGGCCTCACAGCTCTAGT
IRS-1	GCCAGAGGATCGTCAATAGC
	AAGACGTGAGGTCCTGGTTG
Foxm1	GAGCACTTGGAATCACAGCA
	AGCAGAGGCTTCATCTTTCG
Kcnj12	CCCAAGCCTCAGCTAAACAA
	CCCGTCCTCCTCTGATGATA
Kcnf1	GAGCAGACCTTTGCCAAGAC
	GGGGACACAAAAGCAGATGT
Kcnj5	CTCGTGCTCAACTGGAACAG
	GACCCCAGAGCACCTCTGTA
Kcng3	ACCCTCCGGGATAATTGAAG
	ATAGGGCGTGATTGCCAGTA
Kcnc1	TGTTCGAGGACCCCTACTCA
	GAAGAAGAGGGAGGCAAAGG
Kcnb1	TGCTGTGCTGAGAAGAGGAA
	GCAATGGTGGAGAGGACAAT
	GCTGAAGCCTGGAAGATCAC

Sfn	GCAGGAGCGTCTTGGATTAC
Ywhaq	CCATCGCAGAGCTTGATACA
	TCCTGCACTGTCTGATGTCC
Rims3	CCTCCCAGCCACCTATATCA
	CATGTTTTCTTGGCCACCTT
Rab9b	CTCCCCATTGTGTTGGAATC
	GCACCAAACAGGGAGAAGTC
GAPDH	AACTTTGGCATTGTGGAAGG
	GGATGCAGGGATGATGTTCT
FAS1	CAGACATGCTGTGGATCTGG
	CATGGTTGACAGCAAAATGG
Atf3	TGCCAAGTGTCGAAACAAGA
	CCTTCAGCTCAGCATTCACA
Atf4	GAAACCTCATGGGTTCTCCA
	TCTCCAACATCCAATCTGTCC
Kcnj10	AGAGCAGCCACTTCACCTTC
	CGGCTCTCTGTCTGAGTCGT
Stx1a	AAGAAGGCCGTCAAGTACCA
	ATGATGCCCAGAATCACACA
Stx1b	GTATCAGAGCAAGGCCAGGA
	GAAGGGTAGGGGCCTACAAG
Syt10	TCCACTGTGCTACCACAGAAAG
	CCAGCAGTTGGCAAATAGCAA
Eef1a2	CCCAGTTCACCTCTCAGGTTA
	TCTCCTTTAGCTCGGCAAAC
Cdh8	ATTTTGCTGCTCGTCATTGTG
	TTTCTCGAACGTCTTCGTCA
Zcchc12F	CTGGTTGTAGAGCTGGTCTTGA
	GATCAGCTGGGAAACTGGAG
Hist2h3c	TACCTGAGGAGGCCAAAAGA
	TCAATTCCCAGAACCCACAT
Gng2	CATCGACAGGATAAAGGTGTCC
	CTTAAAGGATGGCGCAGAAG
FAS	CAGACATGCTGTGGATCTGG
	CATGGTTGACAGCAAAATGG
Atf3	TGCCAAGTGTCGAAACAAGA
	CCTTCAGCTCAGCATTCACA
Syt16	AGCAAAGAAGTGGCCTTCAA
	TGATCACCTCCTCGTCACTG
Syt13	ACGGGACATCTGTGCCTCTA
	CGGGAGGTAGCTGATGGAT

Table 3. Antibodies

Target	Host	Source	Usage	Dilution	Antigen Retrieval for IF
Myt1	Rbt	Gu lab	IF, WB, IP	1:1000 (IF) 1:3000 (WB) 1:500 (IP)	pH 6.0
Myt1L	GP	Gu lab	IF	1:1000	pH 6.0
Myt3	Rat	Gu lab	IF	1:500	pH 6.0
Pdx1	Gt	Wright lab	IF	1:1000	pH 6.0
Ngn3	GP	Gu lab	IF	1:2000	pH 6.0
Nkx6.1	Rbt	Sander lab	IF	1:2000	pH 6.0
Insulin	GP	Dako	IF, IHC	1:1000 (IF) 1:1000 (IHC)	N/A
Insulin	Gt	Santa Cruz	IF	1:100	N/A
Glucagon	GP	Dako	IF	1:1000	N/A
Somatostatin	Rbt	Dako	IF	1:500	N/A
Pancreatic peptide	Rbt		IF	1:500	N/A
Ki67	Rbt	Abcam	IF	1:1000	pH 6.0
BrdU	Ms	Abcam	IF	1:500	pH 6.0
Syt4	Rbt	SySy	IF	1:500	pH 6.0
GM130	Ms	Cell signaling	IF	1:2000	N/A
FoxO1*	Rbt	Cell signaling	IF	1:2000	pH 9.0
Syt7	Rbt	SySy	IF	1:2000	pH 6.0

PDI	Rbt	Cell signaling	IF	1:2000	pH 6.0
MafA	Rbt	Novus	IF	1:500	pH 6.0
MafB*	Rbt	Novus	IF	1:2000	pH 9.0
Glut2	Rbt	Alpha diagnostic	IF	1:200	pH 9.0
HA	Ms	Vanderbilt Antibody and Protein Resource	IF, WB, IP	1:500 (IF) 1:2000 (HA) 1:500 (IP)	pH 6.0
Hes1*	Rbt	Stanger lab	IF	1:1000	pH 6.0

Abbreviations:

IF: immunofluorescence**; IHC:** immunohistochemistry**; WB:** Western blot**; IP:**
immunoprecipitation**;**

pH 6.0: pH 6.0 10 mM sodium citrate buffer; **pH 9.0:** 10 mM TEG buffer;

Antibodies marked with (*) required tyramide signal amplification (TSA)

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Introduction

As β-cell loss is the major factor contributing to the pathogenesis of both type I and type II diabetes, replenishing β-cell mass has long been considered as a promising therapeutic strategy (Vetere et al., 2014). There are generally three ways in achieving this goal. First, β-cells can be derived from pluripotent stem cells, such as embryonic stem cells (ESCs) or induced pluripotency stem cells (iPSCs). Second, β-cell mass can be increased by activating the proliferation potential of remnant β-cells. Last, other differentiated cell types, such as α-cells and acinar cells, can be transdifferentiated into β-cells via genetic manipulations or physiological impacts. Thanks to decades of basic and transitional research, insulin-expressing β-like cells are now obtainable from the above-mentioned regeneration approaches (Pagliuca and Melton, 2013).

However, compared to their in *vivo* developed mature β-cells, β-like cells generated in these "unconventional" ways usually lack full glucose-stimulated insulin secretion (GSIS) competency. Moreover, whether they are durable for long-term functional maintenance under potential cell stress has yet to be examined. For instance, human ESC-derived β-like cells share similar phenotypes and gene expression profiles with immature β-cells, which are characterized by a high cell turnover rate (Hrvatin et al., 2014; Teta et al., 2005).

Notably, transplantation of human fetal immature islets or human ESC-derived pancreatic progenitor cells into immunodeficienct rodents gives rise to glucose-responsive β-cells (Hayek and Beattie, 1997; Rezania et al., 2012; Xie et al., 2013).

These findings suggest that β-cell functional maturation can be promoted by certain unknown factors from the *in vivo* microenvironment. Therefore, further efforts should be directed toward identifying these pro-maturation factors, which can be exploited to improve β-cell regeneration for clinical usages.

In this thesis research, I have identified two important gene/gene families that contribute to β-cell functional maturity and longevity. I took advantage of the mouse model, which shares similar gene usage, pancreatic islet constitution and β-cell physiology with humans and therefore provides an efficient and reliable system to investigate biological events involved in β-cell function.

In the first study, I systematically examined the Ca^{2+}-coupled vesicle exocytosis during β-cell maturation and found that insulin vesicles in immature β-cells are more sensitive to Ca^{2+} influx than those in mature β-cells. This result is consistent with the previous finding that the glucose threshold for insulin secretion is lower in immature β-cells than that in adult β-cells (Blum et al., 2012). Next, I sought to identify factors that are responsible for tuning this Ca^{2+} sensitivity during β-cell maturation. By temporal gene expression analysis, I found that the expression of *Syt4* is increased along with β-cell maturation. My further studies based on genetic manipulation of mouse models found that *Syt4* expression not only correlates with, but also promotes β-cell glucose response. Moreover, our mechanistic studies suggest that Syt4 attenuates the Ca^{2+} sensitivity by interfering with Syt7-dependent Ca^{2+}-secretion coupling. Intriguingly, lentivirus-based *Syt4* overexpression in human β-like cells successfully lowered basal insulin secretion, suggesting that its function is well conserved between mouse and human. Our results not only revealed that tuning Ca^{2+} sensing is a key step for β-cell functional maturation, but also identified Syt4 as a potential factor that can be exploited to promote β-cell function.

The second project was based on our previous studies regarding the regulatory role of *Myt1* in pancreatic endocrine cells. (Wang et al., 2008; Wang et al., 2007; Xu et al., 2006). Mice with a pancreatic deletion of *Myt1* were partially deficient in glucose clearance and had a small portion of bihormonal endocrine cells. However, as the expression of the other two Myt family members, *Myt1L* and *My3*, was activated upon *Myt1* ablation, the defects we identified might not reflect the comprehensive regulatory function of Myt factors. In addition, whether Myt factors are mostly involved in embryonic β-cell differentiation or postnatal β-cell function was determined.

Our lab addressed these issues by generating a conditional triple knockout mouse model (*6F;Cre*), in which all three *Myt* paralogs are simultaneously deleted. In this thesis study, I performed a time course characterization of the phenotype displayed by these *Myt* mutant mice. I found that although *Myt* mutant mice had severe postnatal damages in β-cell function and survival, their embryonic β-cell fate specification appeared to be mostly unaffected, as judged by *insulin* expression and β-cell number at birth. However, these newborn *Myt* mutant β-cells, despite being morphologically normal, had aberrant gene expression: the expression of many functional genes was reduced, and the expression of many progenitor-specific and apoptotic genes was activated. This findings suggest that Myt factors are crucial to establish a gene expression network in young β-cells, which is required for further β-cell maturation and functional maintenance.

4.2 Future directions

In this thesis research, I provided evidence that the proper Ca^{2+} sensitivity of insulin vesicles is essential for β-cells to develop robust GSIS during maturation. I further identified Syt4 as a key factor in downregulating this Ca^{2+} sensitivity and repressing

basal insulin secretion. In addition, I found that Myt factors regulate a gene expression network for β-cell maturation and functional maintenance. These findings are anticipated to provide novel insights into generating functional and sustainable β-cells for diabetes treatment. Following this thesis research, there are several important future directions worth pursuing.

Comprehensive understanding of Syt4 molecular mechanism

In my study, Syt4 was found to physically interact with the Ca^{2+} sensor Syt7. Also, *Syt4* overexpression phenocopied the loss of *Syt7* in GSIS and insulin vesicle docking. These combined findings provide a possible Syt4 functional mechanism that this atypical synaptotagmin regulates insulin secretion by interfering with Syt7-dependent Ca^{2+}-secretion coupling.

However, where Syt4-Syt7 interaction occurs and how Syt4-Syt7 heterodimer affects insulin vesicles docking are both unknown. It is likely that on the insulin vesicle, the Syt7 protein structure changes upon binding with Syt4, which in turn modifies the affinity of Syt7 to Ca^{2+}. Interestingly, we identified that only a small portion of Syt4 proteins are located in the Golgi apparatus or on insulin vesicles, the two cellular compartments directly involved in insulin secretion. Instead, the majority of Syt4 proteins are located within the ER compartment. This distribution pattern raises a possibility that Syt4 may trap Syt7 within the ER by direct interaction, which restricts the access of Syt7 to the Golgi and insulin vesicles. Subsequently, less density of Syt7 proteins on insulin vesicles results in reduced Ca^{2+} affinity of vesicles (Figure 4.1).

To determine whether there is a Syt4-Syt7 complex in the ER, several strategies aiming to examine protein-protein interaction with ultra-high resolution can be conceived. For instance, immunogold labeling combined with transmission electron microscopy (TEM) can be used to detect the co-localization between Syt7 and Syt4 and further
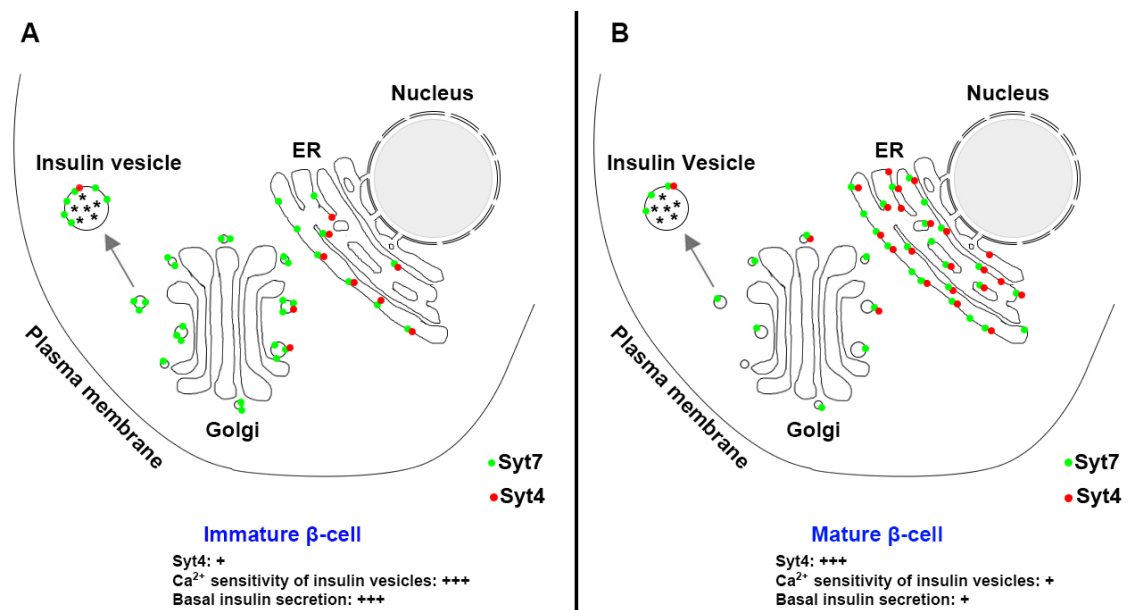
Figure 4.1 A proposed Syt4 working model. In immature β-cells, the expression level of *Syt4* is low, and Syt7 proteins bind freely with insulin vesicles to increase the Ca^{2+} sensitivity of vesicles, resulting in high basal insulin secretion. Conversely, in mature β-cell cells, ER-located Syt4 proteins trap Syt7 into this cellular compartment by a direct Syt4-Syt7 interaction. In this way, the proportion of Syt7 proteins that can bind with insulin vesicles are substantially reduced. Therefore, insulin vesicles are less sensitive to Ca^{2+} simulation due to less Ca^{2+} sensors distributed on the vesicle surface, and subsequently basal insulin secretion is reduced.

determine their location based on the subcellular morphology revealed by TEM. We have tried this approach in our previous study. However we did not detect obvious Syt4 or Syt7 signals, most likely due to poor antibody qualities and/or inefficient permeabilization of gold particles into intact islets (Figure 2.8). These technique issues need to be addressed in the future by developing antibodies with better quality and optimizing the detergents for the islet permeabilization.

Besides TEM, another approach for the same purpose is the fluorescence resonance energy transfer (FRET) assay. In FRET, an excited "donor" fluorophore transfers energy to an "acceptor" fluorophore when the physical distance between the donor and the acceptor is closed enough ($\leq$ 10 nm) (Piston and Kremers, 2007). In this case, Syt4 and Syt7 can be tagged with fluorescence proteins (e.g. CPF and YFP) that can provide donor and acceptor fluorophores, respectively. A Syt4-Syt7 interaction will be indicated by FRET signals, such as sensitized emission from the acceptor fluorophore or increased donor fluorescence intensity after photobleaching of the acceptor. Furthermore, by labeling the ER with a third fluorophore, it is possible to discriminate whether the interaction is located with this organelle. One caveat of performing the FRET assay is that tagging a fluorescent protein to Syt4 or Syt7 may affect the original Syt protein structures. This possibility can be tested by analyzing the function of fluorescent protein-tagged Syt4 and Syt7 in β-cell lines by a routine gain-of-function assay.

How many synaptotagmins are directly involved in β-cell maturation?

The synaptotagmin (Syt) family has least 17 members that share an N-terminal transmembrane domain and two C-terminal Ca^{2+} binding domains (C2A and C2B) (Sudhof, 2002). Previous studies in the neuronal system and β-cells suggest that typical Ca^{2+}-binding Syts (such as Syt1, 3, 5 and 7) act as Ca^{2+} sensors during vesicle

exocytosis by promoting vesicle fusion upon Ca^{2+} influx (Fernandez-Chacon et al., 2001; Gauthier and Wollheim, 2008a; Gustavsson et al., 2009). However, some Syts (such as Syt4, 13 and 16) have little to no Ca^{2+} affinity due to certain amino acid substitutions in the C2A and/or C2B Ca^{2+} binding domains (Sudhof, 2002). Much less is known about these atypical Syts. Based on the finding that different Syts can form heterodimers, it was hypothesized that atypical Syts may play inhibitory roles in the vesicle exocytosis by interfering with the function of Ca^{2+}-sensing Syts (Chapman, 2002). In our study, we provided evidence that the Syt4-Syt7 interaction partially accounts for the mechanism by which Syt4 represses basal insulin secretion.

Interestingly, overexpression of two other atypical *Syts* (*Syt13* and *Syt16*) can also repress basal insulin secretion in human β-cells. This finding raises a possibility that these Syts with abolished Ca^{2+}-binding capability may share a common function in attenuating the Ca^{2+} sensitivity of insulin vesicles and increasing the glucose threshold for insulin secretion. Further studies are needed to discriminate subtle differences among these atypical Syts. For instance, they may use different molecular mechanisms to fulfill the role in regulating exocytosis, e.g. by interacting with certain Ca^{2+} sensors, or by interacting with other components of the exocytotic machinery (such as SNARE proteins). In particular, it will be intriguing to identify the kinetics of each atypical Syt in repressing basal insulin secretion, for the sake of choosing the most efficient Syt paralog for therapeutic β-cell regeneration *in vitro*.

How is the expression of Syt4 regulated during β-cell maturation?

Our results suggest that the endogenous *Syt4* expression is increased to repress basal insulin secretion during β-cell maturation. However, it remains unknown what transcription factors are responsible for activating *Syt4* expression. Based on our knowledge of genes involved in postnatal β-cell function, Pdx1, Myt1, MafA, NeuroD1

and Nkx6.1 are likely to be such candidates. To this end, loss-of-function assays for these genes can be conducted to test whether their existence is required for the expression of *Syt4*. Moreover, ChIP assays can be performed to determine whether they directly bind *Syt4* promoter/enhancer regions.

Another equally, if not more, important question to ask is what growth factors and signal pathways can increase *Syt4* expression. Addressing this question would provide more useful information for β-cell regeneration, as administration of growth factors is more achievable than forced gene expression. There are several growth factors and related signal pathways that have been identified to potentiate GSIS, such the insulin secretagogues GIP and GLP-1(Vetere et al., 2014). However, most of their pro-GSIS effects are only found on mature β-cells; whether they can promote β-cell maturation has not been determined.

The signal pathways activated by islet innervation are interesting candidates for promoting *Syt4* expression. Previous studies have demonstrated that islet innervation occurs specifically at the neonatal stage, during which endogenous *Syt4* expression is increased (Borden et al., 2013). In addition, loss of sympathetic or parasympathetic innervation compromises β-cell functional maturation (Rossi et al., 2005), a phenotype shared by loss of *Syt4* function. It is therefore worth testing if downstream genes related to islet innervation have any effects on *Syt4* expression. Furthermore, a Syt4 reporter cell system (such as cells engineered with a *Syt4* promoter/enhancer-driven luciferase) can be established to identify growth factors and small molecules that can activate *Syt4* expression based on large scale screening.

What are the direct transcriptional targets of Myt factors?

Although our RNA-seq identified hundreds of genes whose expression was altered upon deletion of Myt factors, which genes are direct transcriptional targets of Myt

factors remains unknown. It is possible that only a small portion of them are directly regulated by Myt factors, while others are affected secondarily by the change of physiological conditions. The discrimination of these two gene groups is necessary to determine what physiological processes are directly regulated by Myt factors. To this end, a genome-wide ChIP-seq is required to identify transcriptional targets of Myt factors and reveal the whole gene expression network controlled by this zinc finger transcription factor family. My previous efforts in performing this experiment included affinity-purification of Myt1 antibody and optimization of the experimental conditions for the ChIP on isolated mouse islets (Figure 4.2). Previous studies in the Roland Stein lab have shown that Myt1 forms a complex with MafA (Scoville et al., 2015), and a preliminary ChIP by using this purified Myt1 antibody was able to pull down an insulin promoter region bound by MafA, suggesting that this antibody is well-adapted for this experiment.

What are the molecular mechanisms involved in Myt-mediated transcription?

Myt factors exert their function by activating or repressing the expression of their transcriptional targets. Transcription factors do no act alone; instead, they interact with transcriptional coactivator or corepressors, which are usually histone modifiers or chromatin remodeling complexes, to modulate the chromatin status of their target genome regions. In the case of β-cell-specific transcription factors, for instance, MafA recruits MLL3 and MLL4 H3K4 methyltransferase to increase the expression of *insulin* (Scoville et al., 2015) and Pdx1 interacts with Swi/Snf chromatin remodeling complex to facilitate the transactivation of target genes (McKenna et al., 2015). In agreement with these findings, a previous study in neuronal cells showed that Myt1 recruits histone deacetylase (HDAC) via the transcription corepressor Sin3B to silence certain neuronal transcription (Romm et al., 2005). Recent investigations from our lab found that loss of *Sin3A* and *Sin3B* in the pancreas led to similar phenotypes observed in the *Myt* triple

131
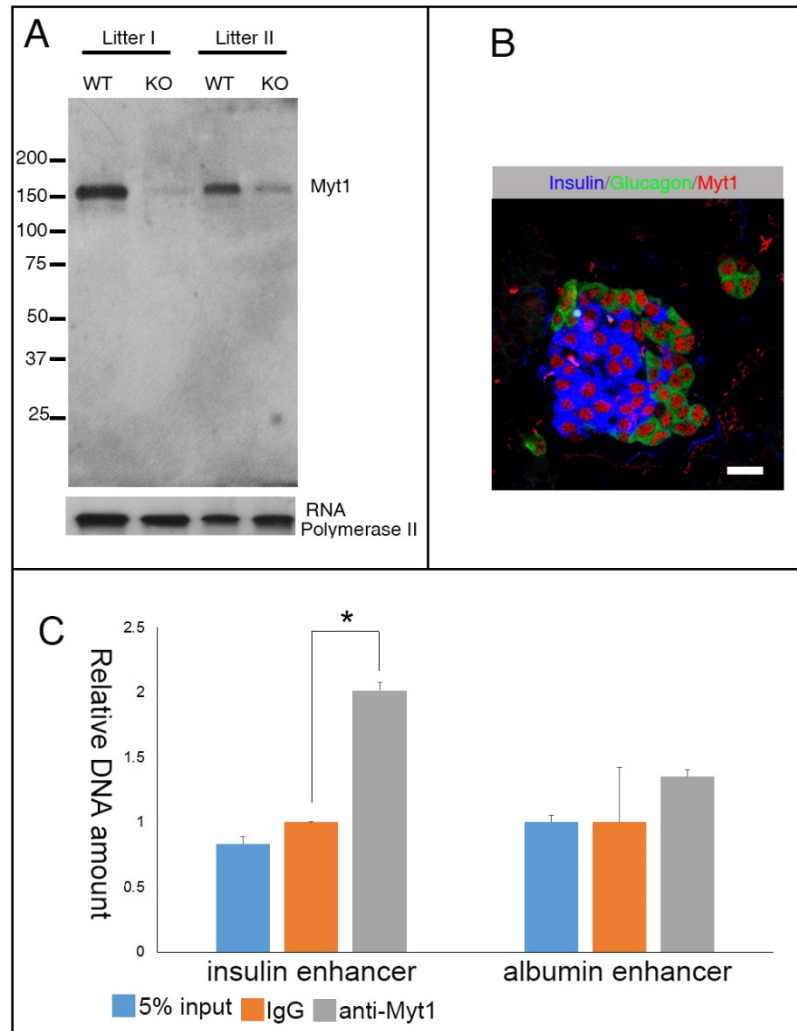
Figure 4.2 Assessment of the purified Myt1 antibody. (A) The antibody was tested by western blot. Isolated islets from newborn *6F;Cre* and *6F* littermates were lysed for western blot, and Myt1 protein was detected by this purified antibody. Note that the weak band in the *6F;Cre* islet extracts is due to incomplete deletion mediated the Pdx1-driven Cre recombinase (also see Figure 3.1). The protein levels of RNA polymerase II were used as the internal control. (B) The antibody was tested by immunofluorescence. The expected *Myt1* expression pattern was observed in the adult mouse islet section, i.e. in all types of islet cells. Scale bar: 20 μm (C) A preliminary ChIP by this antibody. ~500 adult mouse islets were used for the ChIP, by using either the purified Myt1 antibody or host-matched IgG. A pair of qPCR primers aligning to an insulin enhancer was used to detect target DNA enrichment. Moreover, another pair of qPCR primers aligning to an albumin enhancer was used to analyze the specificity of the ChIP, i.e. Myt1 antibody did not pull down non-specific DNA. * $p < 0.05$, student t-test.

knockout mice, including compromised GSIS and β-cell demise (Yang and Gu, unpublished results). Therefore, similar to that in the neuronal system, there may also be functional interactions between Myt factors and Sin3A/B complex in pancreatic endocrine cells.

However, Sin3A/B and the HDAC complex are not likely the only functional partners of Myt factors. Based on our gene expression analysis, Myt factors can also act as transcription activators (see chapter III). Also, in oligodendrocytes, Myt1 directly binds to the promoter of *proteolipid protein 1 (plp1)* to activate its expression (Bellefroid et al., 1996; Kim et al., 1997). Therefore, there are certain pro-transcription histone modifiers/chromatin remodeling complexes remaining to be identified as Myt1 coregulators. To this end, a simple yet effective strategy is to perform a co-immunoprecipiation assay followed by mass spectrometry to identify all proteins bound by Myt1. Subsequently, gene manipulation assays (such as RNAi-mediated knockdown) can be performed in stable β-cell lines to check whether these binding partners are indeed required for the function of Myt factors, i.e. whether their absence will compromise the expression of Myt target genes.

Do Myt factors perform similar regulatory roles in human β-cell function?

Due to the difficulty in genetic manipulation of human tissues, the function of Myt factors has not been investigated in human β-cells. To determine if this group of transcription factors play similar roles in generating functional β-cells in human pancreas, the first step would be to examine their expression profile.

Recent studies based on immunofluorescence have identified the temporal and spatial expression of a set of regulatory genes, particularly transcription factors, during human pancreas development and β-cell differentiation (Jennings et al., 2013; Jeon et al., 2009; Lyttle et al., 2008). Most of these transcription factors share a similar

expression pattern with their mouse counterparts, a strong indication that their roles in setting up gene expression networks during β-cell differentiation can be inferred from the knowledge obtained from mouse models (Jennings et al., 2015). However, there are some subtle, but significant expressional differences between mouse and human for certain genes. For instance, in the adult mouse pancreas, the expression of *MafA* and *MafB* is restricted to β-cells and α-cells, respectively; however, both of Maf family members are expressed in human adult β-cells (Hang and Stein, 2011). This finding suggests that distinct gene paralogs may be employed to fulfill a specific biological regulation in different species. Therefore, the expression of each Myt factors needs to be examined individually in human β-cells, even though they are all expressed in mouse β-cells.

Following gene expression analysis, some exploratory functional characterization for Myt factors can be performed in the newly-derived human β-cell lines, such as endoC-βH1 and endoC-βH2 cells (Ravassard et al., 2011; Scharfmann et al., 2014). Conventional genetic manipulations, such as RNAi-based knockdown or electroporation-based overexpression, can be conducted on these cell lines to study the roles of human Myt factors in different biological processes involved in GSIS, including glucose metabolism, insulin synthesis, ER stress, vesicle exocytosis, etc. Similar experiments can be also performed in primary human islets. Additionally, RNA-seq and ChIP-seq can be performed in human β-cell lines and primary islets to identify direct target genes of Myt factors for a mechanistic interpretation of their function.

4.3 Concluding Remarks

This thesis research aims to understand what factors can promote β-cell maturation and postnatal functional maintenance. We have demonstrated that Syt4 is

necessary for β-cells to repress basal insulin secretion and establish robust GSIS in the neonatal stage, and Myt factors are crucial to set up a gene regulatory network for long-term postnatal β-cell function. These findings provide two levels of insights into therapeutic β-cell regeneration. Firstly, the endogenous expression levels of *Syt4* and Myt factors can be used to assess the maturity and functionality of β-like cells derived from select regeneration methods. Secondly, growth factors or small molecules that can increase the levels of Syt4 and/or Myt factors are likely to improve glucose response and longevity of these β-like cells. Consequently, identification of these factors will be helpful to generate glucose-responsive β-like cells to treat type I diabetes and reverse β-cell failure in type II diabetes.

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