THE Myt1 AND Ngn3 FEED-FORWARD EXPRESSION LOOP DRIVES PANCREATIC ISLET DIFFERENTIATION IN THE MOUSE

By

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To my parents and brother,

the most wonderful family a girl could ever have.

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CHAPTER I

INTRODUCTION

Abstract

Diabetes Mellitus is a complicated disease featured by failure of sugar metabolism regulation. This disease affects millions of people worldwide. The function of pancreatic islet β cells, which secrete insulin to maintain blood glucose homeostasis, is impaired to different extents in diabetic patients. One potential cure for diabetes is islet transplantation, yet this procedure is largely limited by the lack of transplantable islets. Many researchers therefore attempt to generate functional β cells from different sources, especially from human embryonic stem cells (hESCs). However, most of the insulin-expressing cells generated via various strategies fail to function as efficiently as endogenous mature β cells *in vivo*, suggesting that there are still major gaps in our understanding of β cell formation and function. We utilize mouse models to understand the molecular pathways that govern the production of functional islet cells. Our focus is the interactions between the basic-Helix-Loop-Helix (bHLH) transcription factor Neurogenin 3 (Ngn3) and the Myelin transcription factor (Myt) family of zinc finger proteins. In this chapter, I summarize our current understanding of the pancreas development, and focus on the role of *Ngn3* in endocrine differentiation and function.

Model Organisms for Studying Pancreatic Development

The pancreas, a heterogeneous organ functioning in both digestive and endocrine systems, is unique to vertebrates (Pieler and Chen, 2006; Slack, 1995). Given the close connection between the pancreas and various diseases, such as *Diabetes Mellitus* and pancreatic cancers, multiple model organisms have been used to study its organogenesis, including chicken, zebrafish, frogs, mice and rats. All these model organisms share a core molecular mechanism governing the pancreatogenesis (Grapin-Botton et al., 2001; Kinkel and Prince, 2009; Pearl et al., 2009). However, the pancreas in each organism differs in their structures and the developmental processes. For example, the dorsal pancreatic bud of zebrafish generates only endocrine cells, and it does not need pancreas transcription factor 1 (Ptf1a), an essential regulator of rodent pancreatic development, to do so (Field et al., 2003). Moreover, *Ngn3*, which controls the endocrine differentiation program in rodents, is not expressed in the developing pancreas of zebrafish (Wang et al., 2001). In *Xenopus*, the gut tube forms after the pancreatic buds have appeared and fused, while the mammalian gut epithelium forms a tube before pancreatic development, suggesting that different signals may be involved in pancreatic bud induction among different organisms (Pearl et al., 2009).

We utilize mice to study pancreatic development in that they share many features with humans for diabetes pathogenesis and symptoms. Mice can also be easily genetically manipulated to recapitulate human mutations. In this chapter, I focus my discussion on mouse models.

The Anatomy of the Pancreas

In mice, the pancreas is composed of multiple cell types (Figure 1.1 A & B), including acini that produce digestive enzymes, ducts that deliver digestive enzymes into the gut, and endocrine *islets of Langerhans* that secrete hormones to help maintain glucose homeostasis (Slack, 1995). The acini and ducts make up the exocrine compartment of the pancreas, contributing to approximately 95% of the pancreatic mass. The endocrine islets are globular cell clusters embedded in the exocrine pancreas (Murtaugh and Melton, 2003). They consist of 5 major cell types, each of which produces a distinct peptide hormone: **β** cells secrete insulin which down-regulates blood glucose levels, **α** cells make glucagon which up-regulate blood glucose levels, **δ** cells produce somatostatin which help to modulate insulin and glucagon secretion (Strowski and Blake, 2008), **PP** cells express pancreatic polypeptide and **ε** cells make ghrelin (Figure 1.1 B&C). The functions of the last two hormones currently remain unclear.

In adult mouse islets, β cells are the predominant cell type, making up 60-80% of islet mass, and located in the center of the islets (Edlund, 2002). Other islet cell types, which are present in different proportions (α cell: 15-20%; δ & PP & ε cells: 1-5%), surround β cells and occupy the periphery of the islets (Figure 1.1C) (Murtaugh and Melton, 2003). The spatial organization of the islets had been suggested to be important for proper islet function (Hopcroft et al., 1985). To this end, disrupted islet structure often coincides with diabetic phenotypes in mouse mutants, but the underlying mechanism is not clear (Ahlgren et al., 1998; Gannon et al., 2000a; Tweedie et al., 2006). In fact, in human islets, there are no

apparent spatial separations between different types of endocrine cell populations (Cabrera et al., 2006). Thus, islet cyto-architecture might not be substantial to islet function in humans.

The Development of the Pancreas

The organogenesis of the pancreas covers the derivation of multiple cell types from different lineages at different developmental stages, and the morphogenetic processes that organize these cells into functional organs. In mice, the onset of pancreatogenesis is at around E9.0. At this stage, two groups of cells evaginate dorsally and ventrally from the pre-patterned endodermal gut tube near the prospective stomach and liver endoderm. Later, these two buds undergo a process of branching morphogenesis while expanding, fuse at around E14 along with gut rotation and differentiate into a fully functional organ right before birth (Jensen, 2004) (Figure 1.2 A).

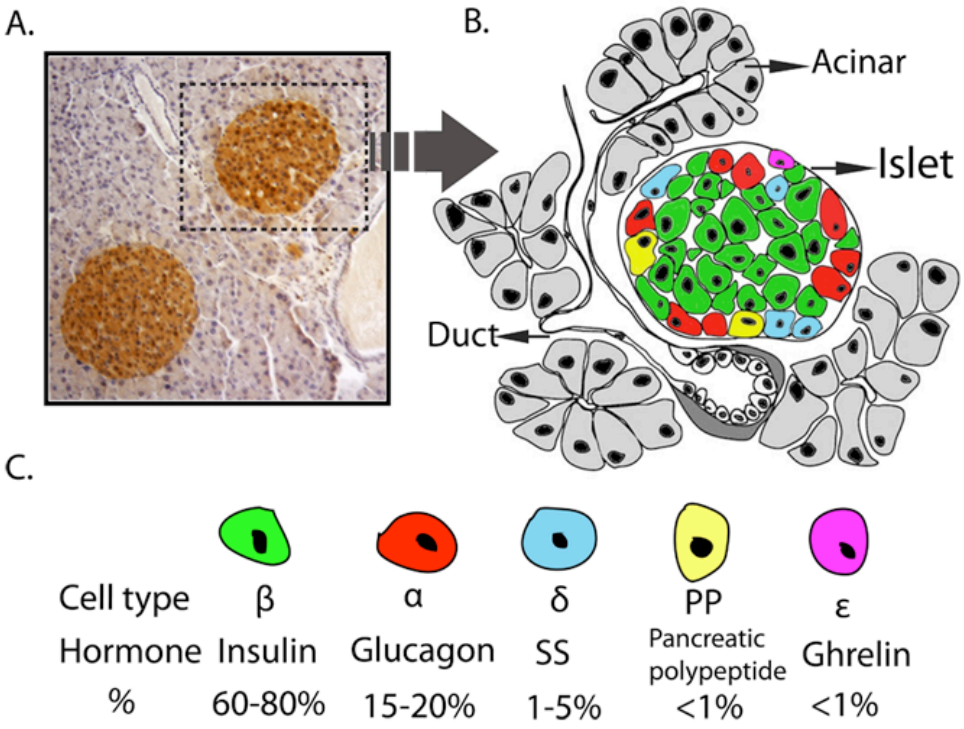


Figure 1.1 The anatomy of the mouse pancreas and islets. (A) Twelve-week-old mouse pancreas was stained with mixed antibodies against the four major endocrine hormones (insulin, glucagon, somatostatin and pancreatic polypeptide). Brown is the result of HRP staining, which visualizes the islets. Blue is the result of hematoxylin staining, which highlights nuclei. (B) The mouse pancreas is composed of islet, acinar and duct cells. Adapted from Gu et al. 2003. (C) The composition of mouse islets (Murtaugh and Melton, 2003).

Hormone-expressing endocrine cells are the earliest differentiated cells to appear in the developing pancreas. From E9.5 to E12.5, while pancreatic buds continue to grow into the surrounding mensenchyme, glucagon-expressing (Glu^+) cells and occasionally a few insulin-expressing (Ins^+) cells were found in the pancreatic epithelium. This period was generally acknowledged as the "primary transition" of the pancreas development (Jensen, 2004). During this phase, the Glu^+ cells express islet amyloid polypeptide (IAPP) and prohormone convertase1/3 (PC1/3) that are not present in adult mature α cells (Wilson et al., 2002). The Ins^+ cells fail to express some mature β cell markers like glucose transporter 2 (Glut2) (Pang et al., 1994). Furthermore, while no mature endocrine cells are generated in *Pdx1*$^{-/-}$ (pancreatic and duodenal homeobox 1) mice, the primary Glu^+ or Ins^+ cells are not affected in these mutants, suggesting that the early endocrine cells are produced via different pathways in comparison with mature islet cells (Offield et al., 1996). Based on this above observation and the fact that only a few Glu^+ or ins^+ cells are produced during the primary transition, it has been suggested that the primary endocrine cells appear to be immature cells, and may not contribute significantly to adult endocrine mass (Herrera, 2000). Between E12.5 and E16.5, a second wave of hormone-expressing endocrine cells appears, which is known as the "secondary transition". During this phase, the endocrine cell populations, which mainly arise from progenitor differentiation, increase dramatically to account for the bulk of mature islet cells. After the secondary transition, endocrine cells start to aggregate into clusters and proliferate to increase islet mass in the first two

weeks of neonatal period (Jensen, 2004; Murtaugh, 2007; Oliver-Krasinski and Stoffers, 2008).

Exocrine cells emerge in the developing pancreas during the secondary transition as well. They are strongly proliferating and form a fully functional pancreas together with endocrine cells by birth (Jensen, 2004).

Like any other organs, the production of a functional pancreas requires the correct specification and differentiation of multiple cell types in a precise temporal and spatial order. Below, I will discuss in detail on the pancreas development at the cellular and molecular level.

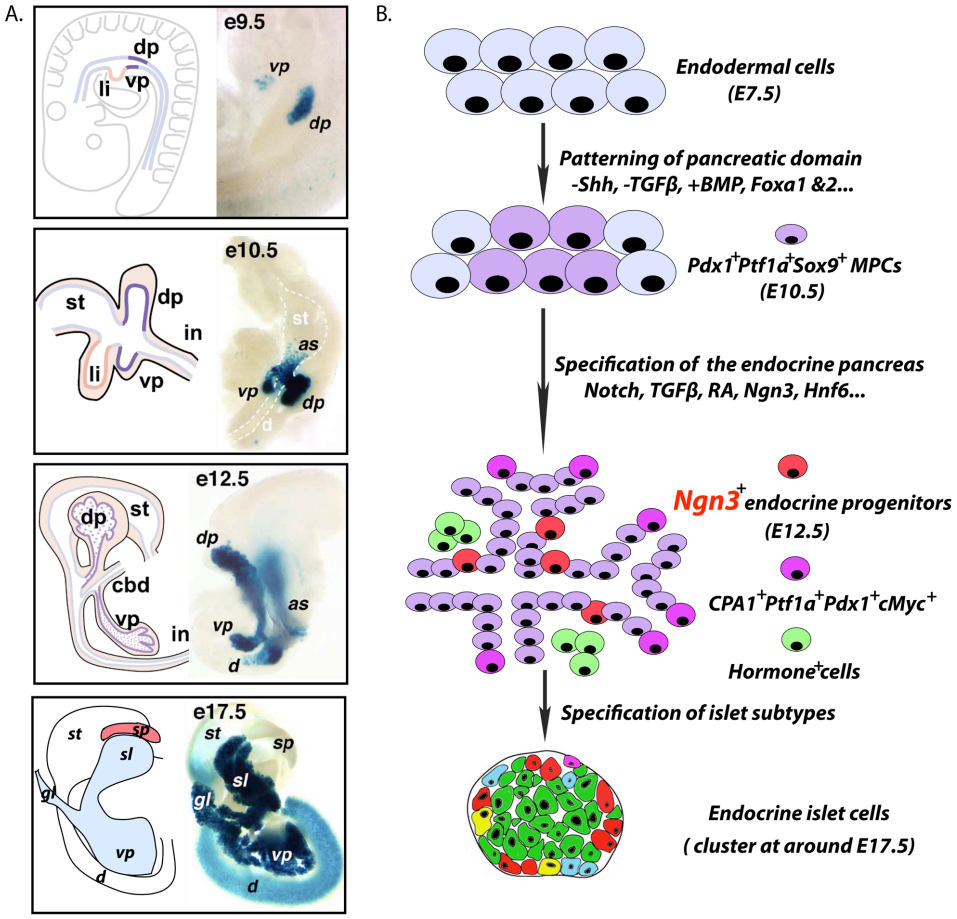


Figure 1.2 The development of the mouse pancreas. (A) At the tissue level, the pancreatic buds arise at around E8.5-E9.5 in the mouse from two strips of gut endoderm located adjacent to the forming liver. At E10.5, the pancreatic primordia evaginate into the surrounding mesenchyme. From E12.5 onward, the dorsal and ventral pancreatic buds undergo branch morphogenesis, fuse and form nearly fully differentiated pancreas before birth at around E18.5. Diagrams are adapted from Murtaugh et al., 2007. Blue staining indicates the expression of *Pdx1* (β-gal staining of $Pdx1^{tTA/+;}TetO^{LacZ}$ pancreata by Hale et al., 2005). dp: dorsal pancreas; vp: ventral pancreas; li: liver; st: stomach; sp: spleen; cbd: common bile duct; d: duodenum; gl and sl: gastric and splenic lobes of the pancreas; as: antral stomach. (B) The signaling pathways and transcription factors that are involved in the pancreatic development.

1. Patterning of the Pancreatic Domains

Before the formation of visible pancreatic buds in the gut tube, the endodermal epithelium is pre-patterned into different progenitor domains by expressing different combinations of transcription factors (Grapin-Botton and Melton, 2000; Wells and Melton, 1999). A homeodomain transcription factor Pdx1 (pancreatic and duodenal homeobox 1) and a bHLH transcription factor Ptf1a (pancreas transcription factor 1a) specifically mark the pancreatic progenitor domains at the early stages (Burlison et al., 2008; Kawaguchi et al., 2002; Offield et al., 1996). The initiation of *Pdx1* expression in the endoderm requires signals from adjacent germ layers at around E7.5 during gut tube formation (Wells and Melton, 2000). Subsequently, different mechanisms are involved in the specification of dorsal and ventral $Pdx1^+$ pancreatic progenitor domains, probably due to their distinct positions in the gut tube.

Many studies have shown that the dorsal pancreatic progenitor domain is induced by relayed interactions between gut endoderm and surrounding mesoderm. At around E8.5, the perspective dorsal pancreatic endoderm is in contact with the notochord, which sends activin-related signals to suppress Shh expression specifically in the dorsal pancreatic endoderm along the A-P axis of the gut tube. At this stage, the absence of Shh signaling is permissive for *Pdx1* expression and dorsal pancreatic fate induction (Apelqvist et al., 1997; Hebrok et al., 1998; Kim et al., 1997b). It is unclear whether there are inductive signals or what the inductive signals that actively promote pancreatic fates are at this stage. Later, at around E9.0, as the notochord moves away, endothelial cells of the

dorsal aorta interact with pancreatic endoderm, and provide additional signals to maintain *Pdx1* expression as well as induce *Ptf1a* expression in the dorsal pancreas. Even though isolated aorta endothelial cells can induce *Ptf1a* expression in endoderm explants, it is not clear what the underlying mechanism is and whether signals from the blood stream also play roles in pancreatic fate induction *in vivo* (Jacquemin et al., 2006; Lammert et al., 2001; Yoshitomi and Zaret, 2004).

The ventral pancreatic domain is specified through a different mechanism (Murtaugh, 2007). At around E9.0, endothelial cells of vitelline veins are closely located to the ventral pancreatic endoderm (Lammert et al., 2001). However, unlike in dorsal pancreatic domain induction, these endothelial cells are unnecessary for ventral *Pdx1* maintenance and *Ptf1a* induction (Yoshitomi and Zaret, 2004). It appears that the ventral endodermal cells adopt pancreatic fates in a default manner, while FGF signals from cardiac mesoderm could divert these endodermal cells to activate the liver differentiation program instead of the pancreatic program (Deutsch et al., 2001). Recently, Wandzioch et al. revealed that TGFβ signaling might function to restrain the ventral endoderm specification until endodermal cells move away from cardiac mesoderm and into a BMP signaling domain. Then, the SMAD4-dependent BMP signaling pathway leads to ventral pancreatic induction (Wandzioch and Zaret, 2009).

It remains unclear how pancreatic inducing signals are translated into pancreatic gene expression. TGFβ/BMP/FGF signaling pathways may directly regulate *Pdx1* expression in the gut endoderm. Alternatively, they may function

together with intracellular transcription factors to initiate *Pdx1* expression. For example, Gao et al. recently revealed that Foxa1 and Foxa2, which are expressed in the gut endoderm prior to the formation of the pancreas, regulate *Pdx1* expression via co-occupation of the regulatory domains in the *Pdx1* gene. It is possible that Foxa1, Foxa2 and other unidentified factors, which can respond to the TGFβ/BMP/FGF signaling, work together to inductively initiate *Pdx1* expression and pancreatic bud formation in the gut endoderm, but this hypothesis remains to be tested (Gao et al., 2008).

2. Pancreatic Multi-potent Progenitor Cells (MPCs).

During pancreatic development, the cells that can give rise to all three pancreatic lineages (acini, ducts and islets) are defined as the pan-pancreatic multi-potent progenitor cells (MPCs) (Burlison et al., 2008; Gu et al., 2002; Kawaguchi et al., 2002; Zhou et al., 2007). Three transcription factors, Pdx1, Ptf1a and Sox9 (sex determining region Y-box 9), are highly expressed in the early (E9.0-E12.5) pancreatic MPCs. Although deletion of any of these three factors in mice results in the lack of mature endocrine and exocrine pancreas, the underlying mechanisms are different. MPCs fail to expand when *Pdx1* or *Sox9* is inactivated in mice, suggesting that these two factors are required for maintenance of the MPC pool (Offield et al., 1996; Seymour et al., 2007). When *Ptf1a* is disrupted, a portion of otherwise pancreatic MPCs adopts the duodenal fates, indicating *Ptf1a* is essential for the acquisition of pancreatic fates in the gut endoderm (Kawaguchi et al., 2002). Nevertheless, these results demonstrate that

Pdx1, *Ptf1a* and *Sox9* are required in the MPCs for their further differentiation into the mature pancreas. Moreover, Afelik et al. reported that combined ectopic expression of *Pdx1* and *Ptf1a* can expand the pancreatic progenitor domains into a more posterior region of the gut endoderm in *Xenopus*, suggesting that these factors are also capable of inducing pancreatic fates (Afelik et al., 2006; Jarikji et al., 2007). Taken together, these studies demonstrate that *Pdx1*, *Ptf1a* and *Sox9* not only mark MPCs in the early pancreatic epithelium, but also regulate pancreatic development, in directing cell proliferation and differentiation.

Interestingly, Pdx1, Ptf1a and Sox9 appear to genetically interact with each other, even thought their expression does not depend on each other. Wiebe et al. revealed that Ptf1a could bind a highly conserved region within the *Pdx1* promoter and mediate its expression in early MPCs (Wiebe et al., 2007). It would be interesting to compare the downstream gene networks controlled by these three transcription factors and elucidate how they cooperatively regulate the differentiation and maintenance of early MPCs.

During the secondary transition (E12.5-E16.5 in mice), massive numbers of differentiated exocrine and endocrine cells emerge in the developing pancreas. The expression of *Pdx1*, *Ptf1a* and *Sox9* starts to become restricted to specific differentiated cell types. While *Pdx1* and *Ptf1a* are highly maintained in differentiated β/ δ and acinar cells, respectively, *Sox9* remains exclusive to a subset of ductal cells (Burlison et al., 2008; Jensen et al., 2000a; Seymour et al., 2007). To this end, it is interesting to examine at what stage the $Pdx1^{+}Ptf1a^{+}Sox9^{+}$ triple positive MPCs disappear and whether other MPC

populations emerge to act as pancreatic MPCs. Several lines of evidence suggest that MPCs do exist at the beginning of the secondary transition (E12.5 in mice), even though the number of MPCs is significantly reduced. Gu et al. has utilized Cre^{ERT}-mediated recombination techniques to trace the $Pdx1^{+}$ cells at different developmental stages by giving the mouse embryos tamoxifen (TM) at different time points (Cre^{ERT} is active only in the presence of TM). They found that while $Pdx1^{+}$ cells can give rise to all three pancreatic lineages before and at around E12.5, they mainly contribute to acinar and islet cells after E12.5 (Gu et al., 2002). Moreover, Zhou et al. recently reported that MPCs reside in specific domains in the developing pancreas during the secondary transition, and are characterized by their co-expression of *Pdx1*, *Ptf1a*, $cMyc^{High}$ and *Cpa1* (*Carboxypeptidase A1*). They showed that $Cpa1^{+}$ cells, which locate in the tips of the pancreatic branches, can give rise to all three pancreatic lineages until E14.0 in mice (Zhou et al., 2007). These data suggest that MPCs do exist during the secondary transition, but their numbers decline quickly after E12.5.

It has been highly debated whether MPCs, particularly those that can give rise to endocrine islet cells, exist in the adult pancreas over the past decade. Dor et al. showed that new β cells arise primarily from the replication of pre-existing β cells, rather than neogenesis from MPCs or endocrine precursors, during normal life and after pancreatectomy in mice (Dor et al., 2004). This study suggests that neogenesis does not play a significant role in adult β cell replenishment under normal conditions. Nevertheless, Inada et al. recently revealed that $CAII^{+}$ (Carbonic Anhydrase II) ductal cells in the adult pancreas can serve as

progenitors for both endocrine and exocrine pancreas after Partial Ductal Ligation (PDL), suggesting that MPCs/facultative stem cells may be present in the adult pancreas (Inada et al., 2008). However, the identity of these adult MPCs/stem cells is still obscure. It is unclear whether they are de-differentiated $CAII^+$ ductal cells or putative MPCs/stem cells maintained in the duct lining, and whether they express the same markers as embryonic MPCs, such as high levels of *Pdx1*, *Ptf1a* and *Sox9*. Furthermore, progenitor cells, which can give rise to all islet cell types, have also been shown to exist in the adult mouse pancreas, and can be activated to increase the β cell mass after PDL (Xu et al., 2008). Together, these studies suggest that MPCs/precursor cells/facultative stem cells are likely present in the adult pancreas and can be activated after severe/specific injuries.

3. The Maintenance of Pancreatic MPCs

Except for intracellular transcription factors, active Notch and FGF signaling are also essential for the maintenance of the MPC pool in the early pancreatic epithelium (before E12.5 in mice),

Both Notch receptors (*Notch1*, *Notch2*) and ligands (*Delta-like-1*, *Jagged1*) are expressed in the developing pancreatic epithelium (Lammert et al., 2000). Deletion of *Delta-like-1* (*Dll1*), *Recombining binding protein suppressor of hairless* (*RBPjk*) or Notch target *Hairy and enhancer of split 1* (*Hes1*) in mice all results in depletion of the MPC pool due to premature differentiation of endocrine cells (Apelqvist et al., 1999; Fujikura et al., 2006; Jensen et al., 2000b). However,

mice without both *Notch1* and *Notch2* develop normal pancreata, suggesting that *Dll1*, *RBPjk* and *Hes1* possess Notch receptor-independent functions, or there are other unidentified Notch receptors expressed in the pancreas (Nakhai et al., 2008). Moreover, over-expression of constitutively activated Notch1 intracellular domain (NICD) significantly impairs both endocrine and exocrine development, in that most of the MPCs remain in the progenitor state and fail to differentiate (Hald et al., 2003; Murtaugh et al., 2003). Thus, the Notch signaling pathway appears to actively maintain MPCs in the progenitor state to ensure their proper expansion.

FGF receptors (*FgfR1* and *FgfR2*) are also expressed in the early pancreatic epithelium (Hart et al., 2003; Hart et al., 2000). Fgf10, produced by the mesenchymal cells surrounding the pancreatic epithelium, is required to maintain the proliferative capacity of MPCs during early pancreatic development (Bhushan et al., 2001). Ectopic expression of *Fgf10* significantly increases the proliferation of MPCs while abrogating cell differentiation by maintaining active Notch signaling in early pancreatic epithelium (Hart et al., 2003; Norgaard et al., 2003).

After E12.5, the MPC pool is significantly diminished. While active Notch signaling is still able to retain MPCs in undifferentiated states, the expression of both *FgfR2* and *Fgf10* become undetectable in the pancreatic epithelium and mesenchyme during the secondary transition (Bhushan et al., 2001; Hart et al., 2003; Murtaugh et al., 2003). In adults, the identity of MPCs is still under active investigation. It is unknown whether Notch and FGF signaling pathways also play essential roles in the maintenance/induction of putative adult MPCs.

4. Specification of the Endocrine Pancreas

It has been suggested that Notch-mediated lateral inhibition helps to select the scattered subset of pancreatic epithelial cells destined for endocrine fates, which is the same mechanism used to choose the subset of neuroepithelial cells destined for neurons in the developing nervous system (Skipper and Lewis, 2000).

The current view of lateral inhibition is largely based on the studies of neurogenesis in *Drosophila Melanogaster* and vertebrates. At the beginning, all neural progenitors are equivalent in terms of their expression levels of Notch receptors, Notch ligands (like *Dll1*) and proneural basic-Helix-Loop-Helix (bHLH) transcription factors (like *Ngn3*). The proneural bHLH transcription factors, which are repressed by Notch targets (like *Hes1*), not only stimulate the expression of Notch ligands (*Dll1*), but also drive the cells to differentiate towards specific cell fates. Due to some stochastic variations, some neural progenitor cells express higher levels of Notch ligands (*Dll1*), and more efficiently activate Notch signaling within their neighboring cells. The latter cells, which receive more Notch signals, therefore express higher levels of Notch target *Hes1*, which leads to lower expression levels of proneural factors and Notch ligands (*Dll1*) in these latter cells. As a result, the former cells are less affected by Notch signaling and further up regulate their proneural factor and Notch ligand levels. The latter cells receive even more Notch signaling and further down regulate their proneural factor and Notch ligand levels. Eventually, the former cells accumulate enough levels of proneural factors and undergo differentiation. The latter cells maintain high levels

of Notch activity and remain in the progenitor state (Beatus and Lendahl, 1998; Kageyama et al., 2008).

In the context of the developing pancreas, Notch target *Hes1* and ligand *Dll1* are highly expressed in the early pancreatic epithelium (Jensen et al., 2000b; Lammert et al., 2000). Endocrine progenitors, which are marked by the bHLH transcription factor Ngn3, emerge from the pancreatic epithelium in a scattered fashion just like the emergence of neurons from neuroepithelium (Lammert et al., 2000; Murtaugh, 2007). Thus, it is highly possible that Notch-mediated lateral inhibition controls the selection of endocrine progenitors. Several lines of evidence support this hypothesis. For instance, deletion of *Hes1*, *Dll1* and *RBPjk*, as well as over-expression of *Ngn3* in the pancreas all result in precocious differentiation of endocrine cells at the expenses of exocrine pancreatic tissues (Apelqvist et al., 1999; Schwitzgebel et al., 2000).

However, several observations need to be clarified. First, no direct evidence has been provided to show that lowering or increasing Ngn3 levels affects Notch ligand levels *in vivo* (Heremans et al., 2002; Treff et al., 2006). Second, lateral inhibition predicts that $Ngn3^+$ cells inhibit their neighbors from up-regulating Ngn3 levels. However, high-resolution immunohistochemistry results show that $Ngn3^+$ cells are sometimes located next to each other (Jensen, 2004), suggesting that mechanism(s) other than classic lateral inhibition, may operate in the pancreas.

Recently, studies in the nervous system revealed that Hes1, proneural factors (Ngn2) and Notch ligands are expressed in an oscillatory manner,

suggesting that the classic lateral inhibition model needs modification (Kageyama et al., 2008). Therefore, the role of Notch signaling in helping to select endocrine progenitors from the MPC pool remains to be investigated in the pancreas.

Besides Notch, other signaling pathways also appear to regulate endocrine specification. Follistatin, an activin antagonist, is capable of reversing the relative proportion of endocrine versus exocrine pancreas in cultured rat pancreatic buds, suggesting that activin signals can promote endocrine differentiation (Miralles et al., 1998). Supporting this notion, a reduction of Type II activin receptor levels results in islet hypoplasia (Kim et al., 2000; Yamaoka et al., 1998). Ectopic expression of Smad7, which is a potent inhibitor of all TGFβ signaling, causes decreased endocrine cell numbers, especially β cells (Smart et al., 2006). However, contrary to these data, expression of a constitutively active form of Type II activin receptor can also induce islet hypoplasia (Yamaoka et al., 1998). *Smad2*$^{+/-}$ pancreas, which should have reduced levels of activin and other TGFβ signaling, has increased numbers of endocrine cells (Smart et al., 2006). At this moment, it is still unclear how TGFβ signaling affects the specification of the endocrine lineage. Moreover, retinoic acid signaling has also been suggested to promote the generation of endocrine progenitor cells, but the underlying mechanism is currently unclear (Ostrom et al., 2008).

After/at the same time of endocrine progenitor selection, the cells that do not enter the endocrine lineage differentiate into either ductal or acinar cells. However, it does not necessarily mean that there are bi-potential exocrine

progenitors. Gu et al. showed that $Pdx1^+$ cells after E12.5 only give rise to islet and acinar cells, but not ductal cells, suggesting that ductal and acinar fates are not bundled together (Gu et al., 2002). Actually, it is not clear whether a single MPC is capable of contributing to all the three lineages of the pancreas. In other words, it is possible that the seemingly equivalent $Pdx1^+Ptf1a^+Sox9^+$ progenitor cells are indeed heterogeneous populations, with some cells only capable of differentiating into two or single cell types. Fishman et al. had tried to address this question and found that a single pancreatic progenitor cell at E11.5 could give rise to both $CPA1^+$ acinar cells and $insulin^+$ β cells by a retroviral "tagging" method (Fishman and Melton, 2002). However, they, unfortunately, didn't examine any pancreatic duct markers. Furthermore, their method hits cells in a blind manner. Differentiated cells could have also been labeled. Recently, Zhou et al. examined the clones derived from single $CPA1^+$ cells in the pancreas, and revealed that individual E11.5-E12.5 $CPA1^+$ cells are mostly (74%) tri-potent. The remaining single $CPA1^+$ cells give rise to either a mixture of two cell types or acinar cells. This result suggests that single $CPA1^+$ MPC between E11.5-E12.5 is capable of contributing to all three pancreatic lineages, yet the $CPA1^+$ MPC population still remains a certain degree of heterogeneity.

5. Islet Cell Type Specification.

In the developing pancreas, the five major endocrine cell types, which are all derived from $Ngn3^+$ endocrine progenitors, emerge asynchronously. It is not clear how these different cell types are specified from common progenitors at

different stages. Johansson et al. showed that Ngn3^{+} endocrine progenitors pass through a series of competence states, during each of which the progenitors are competent to produce a subset of endocrine cell types. Specifically, they generated transgenic mice expressing an inducible-Ngn3 protein under the *Pdx1* promoter, and crossed this transgene into *Ngn3 null* mice. Therefore, the production of endocrine cells can only be obtained when the transgenic *Ngn3* is activated. They found that activation of *Ngn3* at different developing stages resulted in the production of different subtypes of endocrine cells, a result that is confirmed by temporally controlled lineage tracing with *Ngn3*^{CreERT} knock in mice (Johansson et al., 2007). Only after E11.5, Pdx1^{+} progenitors become competent to differentiate into insulin^{+} β cells upon *Ngn3* activation. The competence to make β cells increases thereafter, whereas the competence to generate glucagon^{+} α cells begins at E8.5 and dramatically declines after E14.5. These data suggest that the Pdx1^{+} pancreatic epithelium changes its competency to make different types of endocrine cells as the development progresses prior to *Ngn3* activation. Thus, unknown factors that are intrinsic to Pdx1^{+} progenitors must control the specification of endocrine cell types. The identification of these factors might greatly facilitate our understanding of endocrine cell type specification as well as therapy-oriented β cell generation.

After the initial specification step, multiple transcription factors, most of which depend on *Ngn3* for activation, emerge to promote and maintain the differentiation of various endocrine cell types. For instance, Paired box gene 4 (Pax4) and Aristaless-related homeobox (Arx), which promote β and α cell fates

respectively, mutually repress the expression of each other to ensure the production of functional endocrine cells expressing single hormones (Collombat et al., 2005; Collombat et al., 2003; Sosa-Pineda, 2004; Sosa-Pineda et al., 1997; Wang et al., 2004). It is noteworthy that neither *Pax4* nor *Arx* appears to be the factor that provides the $Ngn3^+$ progenitors the competence to become β or α cells, since ectopic expression of either gene in early pancreatic progenitors can eliminate the production of the other cell type (Collombat et al., 2007; Collombat et al., 2009).

The Essential Role of Ngn3 in Endocrine Differentiation

The bHLH transcription factor Neurogenin 3 (Ngn3) is essential for endocrine differentiation in mice. Ngn3 was first discovered in a study searching for bHLH transcription factors that are important for mammalian neuronal determination in a neural crest stem cell derived immortalized cell line (Sommer et al., 1996). Together with Ngn1 and Ngn2, Ngn3 belongs to a family of atonal-related bHLH transcription factors. Only *Ngn3* is highly expressed in the developing pancreas (Lee et al., 2003; Yoshida et al., 2004).

1. Ngn3 is both Necessary and Sufficient for Endocrine Differentiation

In the mouse pancreas, the cells expressing detectable levels of Ngn3 by immunohistochemistry (IHC) first appear at around E9.0. The number increases gradually thereafter, reaches a peak at around E15.5, and then declines (Murtaugh, 2007). After birth, $Ngn3^+$ cells are rarely detected by regular IHC

under normal conditions. However, Real time-PCR shows that isolated adult islets express significant levels of *Ngn3* transcripts (Dror et al., 2007; Gu et al., 2002). Recently, we show that low levels of Ngn3 are maintained in adult islet cells, and can be detected with a Guinea Pig anti Ngn3 antibody following a specific protocol (Wang et al., 2009). While high levels of Ngn3 during pancreatogenesis promote endocrine differentiation, low levels of Ngn3 are important for islet cells to maintain their proper functions in adults. More interestingly, in a single pancreatic cell, Ngn3 is only transiently expressed. Within about 6-12 hours, $Ngn3^{+}$ cells differentiate into $hormone^{+}$ cells, and down-regulate Ngn3 to low levels (Miyatsuka et al., 2009).

The essential role of *Ngn3* in pancreatic endocrine differentiation has been demonstrated in many studies. Gradwohl et al. showed that *Ngn3 null* mutant mice, which have a relatively normal exocrine pancreas, fail to develop any endocrine cells, suggesting that *Ngn3* is required for endocrine differentiation (Gradwohl et al., 2000). Besides, *Ngn3* is sufficient to initiate endocrine differentiation. Several groups have shown that over-expression of *Ngn3* under the *Pdx1* promoter in transgenic mice can induce the appearance of premature endocrine cells in the pancreas or ectopically in the antral stomach (Apelqvist et al., 1999; Schwitzgebel et al., 2000). These studies not only establish Ngn3 as a core regulator of endocrine differentiation, but also highlight the importance of understanding the regulation of the spatiotemporal expression of *Ngn3* and the transcription network that is controlled by *Ngn3*.

2. Ngn3 Controls the Endocrine Differentiation Program

Ngn3 controls a network of factors, especially transcription factors that are important for endocrine differentiation/function (Gasa et al., 2004; Gu et al., 2004; Juhl et al., 2008; Miyatsuka et al., 2009; Petri et al., 2006; White et al., 2008). Based on their function and position in the network, the factors downstream of *Ngn3* can be divided into two major groups (Figure 1.3).

The first group of factors responds to *Ngn3* activation rapidly. These genes, including *Insulinoma-associated 1* (*IA1*), *NK2 homeobox 2* (*Nkx2.2*), *Pax4* and *Neurogenic differentiation gene* (*NeuroD*), are direct downstream targets of *Ngn3* (Breslin et al., 2007; Huang et al., 2000; Mellitzer et al., 2006; Smith et al., 2003; Watada et al., 2003). They not only share common features, but also possess some distinct characteristics.

In terms of the expression pattern, *IA-1/Nkx2.2/Pax4/NeuroD* start to appear in the pancreatic epithelium at around E9.5, the same time $Ngn3^+$ endocrine progenitors emerge. Later, they are all maintained in differentiated endocrine cells until adulthood (Collombat et al., 2009; Gierl et al., 2006; Naya et al., 1997; Sussel et al., 1998; Theis et al., 2004; Wang et al., 2004). It had been shown that IA-1 and Nkx2.2 are present in a portion of $Ngn3^+$ cells during development, suggesting that they may play essential roles in endocrine progenitors (Gierl et al., 2006; Schwitzgebel et al., 2000). Although it remains to be determined as to whether *Pax4* and *NeuroD* are expressed in $Ngn3^+$ endocrine progenitors, lineage-tracing experiment revealed that $Pax4^+$ cells could contribute to all endocrine cell lineages (Greenwood et al., 2007; Wang et al.,

2008a). Therefore, *IA-1/Nkx2.2/Pax4/NeuroD* may all play important roles in endocrine progenitors.

In fact, targeted gene disruption experiments revealed that these four factors affect different aspects of endocrine differentiation despite their similar expression patterns.

- *IA-1 null* mice accumulate undifferentiated endocrine precursors that do not express any hormones and have reduced endocrine mass, indicating that *IA-1* drives the endocrine progenitors towards a more committed differentiation state downstream of *Ngn3* (Gierl et al., 2006).
- *Nkx2.2 null* mice expand their ghrelin-expressing ε cell population at the expense of β, α and PP cells (Prado et al., 2004; Sussel et al., 1998).
- *Pax4 null* mice have dramatically reduced β and δ cell numbers and coordinately increased α and ε cell numbers (Prado et al., 2004; Sosa-Pineda et al., 1997). It is possible that these ε cells represent a type of undifferentiated endocrine precursors, but the hypothesis remains to be determined.
- *NeuroD null* mice show no obvious developmental defects by E14.5 in the pancreas. After E14.5, the numbers of each islet cell types are significantly reduced due to increased apoptosis. Thus*, NeuroD* seems to be essential for the survival of endocrine cells (Naya et al., 1997).

The capabilities of *IA-1/Nkx2.2/Pax4/NeuroD* to induce endocrine differentiation are also unequal. Ectopic over-expression of *NeuroD* in pancreatic progenitors can induce endocrine differentiation, a feature shared by *Ngn3*

(Schwitzgebel et al., 2000). *IA-1* alone cannot induce *Ngn3* target genes in adult human duct cells, but can enhance Ngn3's ability to induce its own targets (Mellitzer et al., 2006). *Pax4* ectopic expression in early pancreatic progenitors or Glucagon$^+$ α cells can re-specify these cells towards a β cell fate (Collombat et al., 2009). Currently, it is not clear whether *Nkx2.2* are also able to enhance endocrine differentiation on its own or in cooperation with *Ngn3*.

Overall, transcription factors in the first group respond to *Ngn3* activation rapidly, and probably function in Ngn3$^+$ cells to promote the endocrine progenitors towards a more committed state. Beside the four factors discussed above, several other transcription factors may also belong to this group, such as transcription factor V-maf musculoaponeurotic fibrosarcoma oncogene homolog B (MafB) and Paired box protein 6 (Pax6), which are critical for endocrine differentiation (Artner et al., 2007; Artner et al., 2006; Ashery-Padan et al., 2004; Sander et al., 1997; St-Onge et al., 1997). It is not clear whether *Ngn3* directly regulates the expression of these two genes, but *MafB* and *Pax6* are expressed in a subset of Ngn3$^+$ progenitors. Their expression levels appear to increase rapidly after *Ngn3* activation (Artner et al., 2006; Heremans et al., 2002; Jensen et al., 2000a).

The second group of factors downstream of *Ngn3* responds to *Ngn3* activation slowly. They define the functional identities of mature endocrine cells, and are controlled by the factors in the first group. Transcription factors such as V-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA), Pdx1 and NK6 homeobox protein 1 (Nkx6.1), hormone secretion related factors like

Glucose transporter 2 (Glut2), and peptide hormones like insulin are included in this group. These factors help to define the mature β cell identity, and often emerge late after Ngn3 activation (Heremans et al., 2002). For instance, it has been shown that as insulin expressing β cells mature, they become $MafA^{+}Glut2^{+}Insulin^{+}$, and express high levels of Pdx1 and Nkx6.1 (Ahlgren et al., 1998; Brissova et al., 2002; Dutta et al., 1998; Jensen et al., 2000a; Jensen et al., 1996; Nishimura et al., 2006; Pang et al., 1994; Sander et al., 2000).

Several studies have suggested that the factors of the first group play key roles in promoting the expression of the factors in the second group. In *IA-1*$^{-/-}$, *Pax4*$^{-/-}$, *Nkx2.2*$^{-/-}$ and *NeuroD*$^{-/-}$ mutant pancreata, the level of Pdx1 is significantly down regulated starting from E16.5 until birth, even though initial *Pdx1* expression at E10.5 is not affected (Gierl et al., 2006; Naya et al., 1997; Sussel et al., 1998; Wang et al., 2004). More directly, NeuroD could bind to the *Pdx1* promoter and promote its expression (Sharma et al., 1997). The level of *Nkx6.1* is also reduced in *IA-1*$^{-/-}$, *Pax4*$^{-/-}$, *Nkx2.2*$^{-/-}$ pancreata around birth (Gierl et al., 2006; Sussel et al., 1998; Wang et al., 2004). Sander et al. had suggested that *Nkx6.1* lies downstream of *Nkx2.2* during the process of β cell differentiation, in that *Nkx6.1*$^{-/-}$*Nkx2.2*$^{-/-}$ double mutant pancreata are indistinguishable from that of *Nkx2.2*$^{-/-}$ mice (Sander et al., 2000). How *MafA* and *Glut2* expression is regulated is still not clear. It has been shown that MafB is capable of occupying the promoter regions of *MafA* and *Glut2 in vivo* and positively regulate their expression (Artner et al., 2007). For the *insulin* gene, NeuroD can specifically bind to the E-box of the *insulin* gene promoter with high affinity (Naya et al.,

1995). Together, these studies demonstrate that the factors of the second group, which terminally determine functional mature endocrine cells, are extensively regulated by the factors of the first group.

Besides, within the second group, different factors cross talk and interact with each other to promote endocrine differentiation. *MafA* and *Pdx1* are transcriptional targets of each other (Raum et al., 2006; Samaras et al., 2003). They may up-regulate their expression levels through a feed-forward mechanism. Moreover, MafA, Pdx1 and NeuroD together could synergistically enhance *insulin* gene transcription (Aramata et al., 2005; Zhao et al., 2005).

In summary, *Ngn3* controls a network of factors, which not only relay differentiation signals to their downstream targets, but also actively cooperate with each other to accomplish the endocrine differentiation program.

3. Ngn3 is Tightly Regulated in the Pancreas

$Ngn3^+$ cells emerge from a seemingly equivalent epithelial sheet in a scattered manner. Each single pancreatic cell expresses high levels of *Ngn3* in a short time period. This unique expression pattern implies that at least three important issues need to be addressed:

- How are $Ngn3^+$ cells selected from the pancreatic epithelium?
- What are the signals or factors that up-regulate Ngn3 levels when cells are released from Notch-inhibition?
- How are Ngn3 levels down-regulated in differentiated hormone expressing cells?

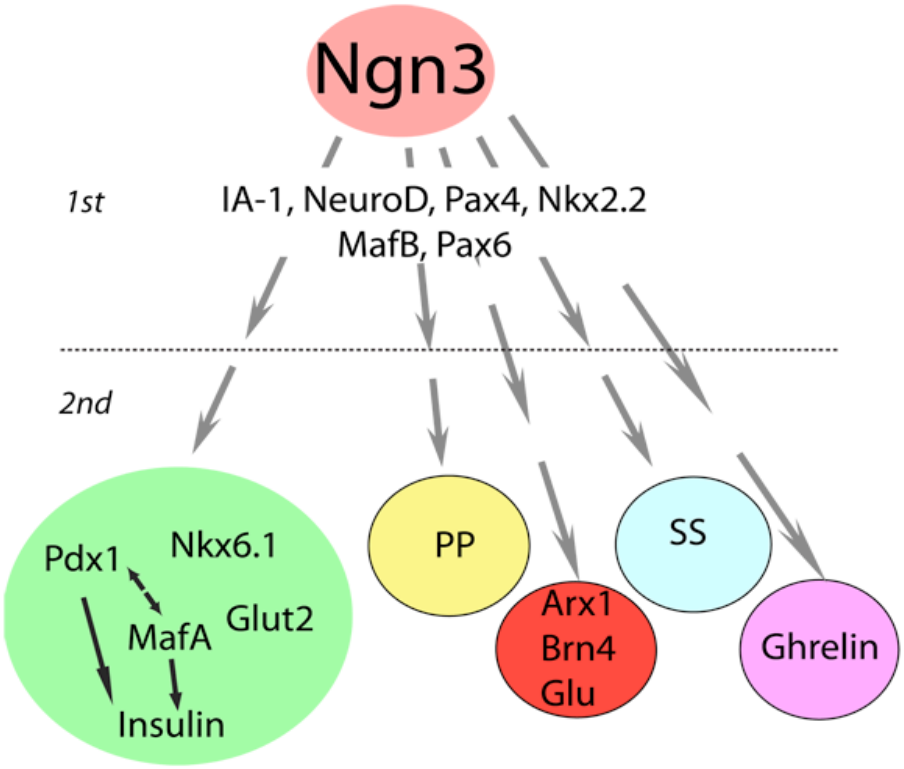


Figure 1.3. *Ngn3* controls the endocrine differentiation program. 1st: genes in this group are directly downstream of *Ngn3*, and can respond to *Ngn3* activation rapidly. They mostly function in $Ngn3^+$ cells to help the endocrine progenitors differentiate towards a more committed state. 2nd: genes in this group respond to *Ngn3* activation slowly. The expression of these genes, which defines the functional identities of mature endocrine cells, is regulated by factors in the 1st group. Cross talk within the 2nd group also contributes to the differentiation of endocrine cells.

For the first question, it is clear that (see above) Notch-mediated lateral inhibition together with other signaling pathways play essential roles in selecting $Ngn3^+$ cells, even though the working details of the mechanism remain to be clarified. In this part, the latter two issues will be discussed (Figure 1.4).

3-1. Up-Regulation of Ngn3 Levels in $Ngn3^+$ Endocrine Progenitors

As previously mentioned, the Notch target Hes1 can directly bind to the *Ngn3* promoter region and inhibit *Ngn3* transcription. However, simple release from Hes1 repression is not sufficient to up-regulate Ngn3 levels in endocrine progenitors, since a longer *Ngn3* promoter sequence than the region containing just the Hes1 binding sites is necessary to direct strong and selective *Ngn3* expression in the pancreas (Lee et al., 2001). Thus, additional signals/factors are required to enhance *Ngn3* expression.

Intercellular signals, such as TGFβ, GDF11, retinoic acid and activin signals from the surrounding pancreatic mesenchyme, can promote the generation of $Ngn3^+$ cells and endocrine differentiation (see “Specification of endocrine pancreas”). However, it is not clear whether these signaling pathways directly regulate *Ngn3* expression or if it is through some indirect regulatory mechanisms.

Intracellularly, several transcription factors have been shown to positively regulate *Ngn3* expression. Lee et al. reported that Forkhead box protein A2 (Foxa2, Hepatocyte nuclear factor 3β, Hnf3β) and Hepatocyte nuclear factor 1α (Hnf1α) could bind to the *Ngn3* promoter and probably enhance *Ngn3* expression

(Lee et al., 2001). However, when *Foxa2* was specifically deleted from the mouse endoderm (by *Foxa3-Cre*), endocrine specification was not affected (Lee et al., 2005). *Hnf1α*$^{-/-}$ pancreata showed relatively normal endocrine differentiation but with defects in β cell growth and function (Pontoglio et al., 1998). Thus, these two hepatic nuclear transcription factors are not required for *Ngn3* level up-regulation. Another hepatic nuclear factor Hnf6 has also been revealed to play essential roles in regulating *Ngn3* expression. It directly binds to the *Ngn3* promoter and can stimulate the promoter activity *in vitro*. More importantly, *Ngn3* expression is nearly abolished in *Hnf6*$^{-/-}$ pancreata during development, suggesting that Hnf6 is necessary for the up-regulation of Ngn3 levels (Jacquemin et al., 2000). Currently, it remains to be determined whether Foxa2, Hnf1α and Hnf6 individually are sufficient to promote Ngn3 levels to a certain threshold that is enough to initiate endocrine differentiation in the absence of active Notch signals.

Besides these hepatic nuclear factors, other transcription factors also function to regulate *Ngn3* expression levels. Sox9 has been shown to bind the human *Ngn3* promoter and activate its expression *in vitro* (Lynn et al., 2007). However, Seymour et al. found that the number of Hes1^{+} cells is significantly decreased, whereas the percentage of glucagon^{+} cells (Glu^{+}) increases in *Sox9*$^{-/-}$ mouse pancreas at around E10.5 (Seymour et al., 2007). Since *Ngn3* is a direct target of Hes1 and able to actively induce Glu^{+} cell differentiation, this study suggests that Sox9 suppresses *Ngn3* expression via up-regulation of *Hes1*. To unravel the discrepancy between these two studies, more detailed examinations

need to be performed *in vivo* to investigate the role of *Sox9* on regulating *Ngn3* expression. Moreover, Oliver-Krasinski et al. recently showed that mice homozygous for a *Pdx1* hypomorphic allele displayed a marked reduction of *Ngn3* mRNA levels and $Ngn3^+$ cell numbers from E13.5 onwards, suggesting that *Pdx1* is also involved in promoting Ngn3 levels. This result is further confirmed by the observation that Pdx1 can bind to the *Ngn3* promoter region and directly regulate *Ngn3* expression (Oliver-Krasinski et al., 2009).

In fact, *Foxa2*, *Hnf6, Sox9* and *Pdx1* not only regulate *Ngn3* expression individually, but also function in concert with each other to form a cross-regulatory network. Pdx1 not only physically interacts and cooperates with Hnf6 to directly regulate *Ngn3* promoter activity, but also occupies the conserved regulatory region of the *Foxa2* gene within the E13.5 pancreas *in vivo* (Oliver-Krasinski et al., 2009). Sox9 also binds to conserved regions upstream of *Foxa2* and *Hnf6* genes. Reduction of Sox9 levels in the pancreatic ductal cell line, mPAC, results in decreased expression of *Foxa2* and *Hnf6*. Reduced *Foxa2* expression in these cells also lead to a dramatic decrease of *Sox9* levels (Lynn et al., 2007). These results suggest that a complicated cross-regulatory transcription factor network function to regulate *Ngn3* expression.

In addition to the above listed transcription factors that can recognize/bind specific DNA sequence, several none-specific histone deacetylases (HDACs), which modify histones post-translationally, also regulate the number of $Ngn3^+$ endocrine progenitors (Haumaitre et al., 2008). However, it is still unclear how this regulation is achieved.

In order to search for factors that can potentially regulate or cooperate with *Ngn3*, the global gene expression profile of $Ngn3^{+}$ endocrine progenitors had been investigated by microarray-based analysis (Gu et al., 2004). A zinc-finger transcription factor Myt1 (Myelin transcription factor 1) was identified in this study as a factor specifically enriched in the endocrine pancreas. In this thesis work, we will provide evidence to show that Myt1 forms a feed-forward expression loop with Ngn3 to promote endocrine differentiation.

Taken together, after *Ngn3* gene expression is released from Notch inhibition, its expression is tightly up-regulated by both intercellular and intracellular signaling pathways.

3-2. Down-Regulation of Ngn3 Levels in Differentiated Endocrine Cells

In endocrine progenitor cells, *Ngn3* levels gradually decline as the cells progress towards a more differentiated state. As a result, hormone-expressing endocrine cells maintain very low levels of *Ngn3* expression (Wang et al., 2009). It remains unclear how Ngn3 levels are down regulated in these differentiated endocrine cells and whether this down-regulation is required for the normal function of endocrine cells.

Smith et al. showed that while Ngn3 activates the endocrine differentiation program, it represses its own expression (Smith et al., 2004). This mechanism may contribute to the down-regulation of Ngn3 levels in differentiated cells.

However, it seems that Ngn3 cannot directly act as a transcriptional repressor (Smith et al., 2004). Whether Ngn3 represses its own promoter by competing with other transcription activators or by inducing the expression of other repressors, remains to be determined (Smith et al., 2004). Nevertheless, the fact that Ngn3 represses its own expression offers a possible explanation for the "pulse-like" expression of *Ngn3* in the endocrine progenitors. Besides, several microRNAs, which can potentially bind to *Ngn3* transcripts, are highly expressed in the adult and regenerating mouse pancreas, and may silence *Ngn3* expression post-transcriptionally in differentiated endocrine cells (Joglekar et al., 2007).

It is currently not clear whether down-regulation of *Ngn3* levels is required for the proper function of differentiated endocrine cells. To test this idea, we over-expressed *Ngn3* in adult mature β cells (under the *Pdx1* promoter) in mice. The percentage of adult β cells expressing high levels of *Ngn3* is relatively low, and varies between individual animals, suggesting that unknown mechanisms regulate Ngn3 levels post-translationally in at least a portion of mature adult β cells (Wang S. & Gu G., unpublished data). The consequences of maintaining high Ngn3 levels in mature β cells remain to be examined. As discussed previously, *Ngn3* controls a network of factors essential for endocrine differentiation. It is possible that up-regulation of *Ngn3* expression in differentiated cells may drive these cells towards an unstable-progenitor-like state, and therefore interfere with their proper function, such as insulin secretion. However, thinking from a diabetes therapist's point of view, these β cells re-

expressing high levels of *Ngn3*, may be a good source for repopulating the reduced or dysfunctional β cells in human patients with diabetes.

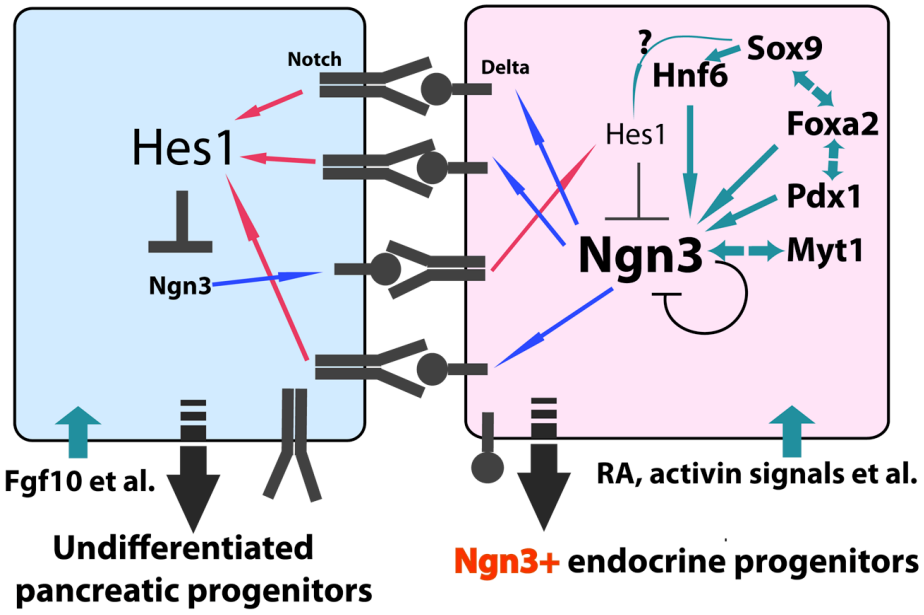


Figure 1.4 *Ngn3* expression is tightly regulated. Multiple signaling pathways and transcription factors are involved in regulating *Ngn3* levels in the pancreas.

4. Ngn3 Functions Differently at Different Stages.

The human genome contains less than 30,000 genes, which is only increased by about 30% compared to the genome of *C. elegans*. However, human genes obviously conduct much more sophisticated and complex biological/physiological processes than that of nematodes. It appears that many genes in humans/mammals are used repeatedly during development and in adulthood. For instance, in the mouse pancreas, *Pdx1* plays very different roles throughout the development. At the beginning of pancreatic development (E9.0 in mice), *Pdx1* is expressed in all the pancreatic progenitors (MPCs), and required for the expansion of the progenitor pool (Offield et al., 1996). During the secondary transition (E12.5-E16.5 in mice), *Pdx1* is expressed in low levels in exocrine cells and endocrine progenitors, and probably is required for the differentiation and maintenance of these cells (Hale et al., 2005; Oliver-Krasinski et al., 2009). During the late gestation stage and adulthood, *Pdx1* expression is restricted to β and δ cells, and is necessary for the proliferation/function of β cells in mice (Ahlgren et al., 1998; Brissova et al., 2002; Gannon et al., 2008). Based on these studies, it is clear that *Pdx1* carries out distinct functions in different cell types at different developmental stages, probably through regulation of different sets of downstream targets. Beside *Pdx1*, *Nkx2.2*, *Nkx6.1*, *MafB* and *NeuroD* et al. function differently in different contexts as well, but *Ngn3* was only considered as an endocrine progenitor marker previously (Aramata et al., 2005; Artner et al., 2007; Artner et al., 2006; Henseleit et al., 2005; Naya et al., 1997; Raum et al., 2006; Sander et al., 2000; Sussel et al., 1998).

Recent studies from our lab suggest that *Ngn3* also conducts different roles within different developmental contexts. During embryogenesis, *Ngn3* is highly expressed in endocrine progenitors, and is essential for endocrine differentiation. After differentiation, Ngn3 levels are down regulated in the adult pancreas. While several groups had claimed that *Ngn3* expression was not maintained in the adult pancreas, others showed that significant amount of *Ngn3* mRNA could be detected in isolated adult islets (Dror et al., 2007; Gu et al., 2002; Jensen et al., 2000a; Schwitzgebel et al., 2000). By optimizing the immunohistochemistry detecting protocol and utilizing sensitized *Ngn3* reporting strategies, we recently revealed that *Ngn3* mRNA and protein are maintained in low levels in most of the adult pancreatic endocrine cells. More importantly, specific deletion of *Ngn3* in β cells impairs proper endocrine function in adults (Wang et al., 2009). Therefore, Ngn3 is required not only for initiating the endocrine differentiation program, but also for islet cell maturation and proper function.

It is largely unknown what controls the stage/context-dependent functions of transcription factors, such as Ngn3 and Pdx1. Different factors might be involved in regulating *Ngn3* expression in different developmental contexts. Moreover, Ngn3 differentially regulates its downstream target genes at different stages (Wang et al., 2009). Thus, some differentially expressed factors may restrict Ngn3's capability to activate specific downstream targets. It is also possible that chromatin modification states and promoter accessibility vary in different contexts, which causes Ngn3 to possess different activities.

The Application of Ngn3 in Diabetes-Related Therapies

Diabetes Mellitus, a metabolic disorder characterized by the failure to maintain glucose homeostasis in the body, has rapidly become a worldwide disease in the past decade. It is estimated that approximately 246 million people had diabetes worldwide in 2007, and the figure is expected to reach 380 million by 2025 (International Diabetes Federation, http://www.idf.org). This alarming increase of the diabetes prevalence has urged biological and medical researchers to understand the pathogenesis of this disease and to forage for possible cures.

Based on the underlying causes, diabetes can be categorized into different types. Type I and type II diabetes are the two major forms, contributing to approximately 10% and 90% of all diabetic cases respectively. In type I diabetes, β cells, which are the major source of insulin production in the body, are attacked and eliminated by the autoimmune system. The loss of insulin then causes a lasting increase of blood glucose levels that can lead to long-term complications and eventually harm all of the organ and systems (Sordi et al., 2008). In type II diabetes, β cells fail to compensate for obesity-associated insulin resistance. As a result, patients gradually progress from normoglycemia to hyperglycemia (Prentki and Nolan, 2006). Even though administration of exogenous insulin can relieve the symptoms of both type I and type II diabetes, there is no cure for this disease currently.

Considering that it is the β cell that is impaired/dysfunctional in diabetes, replenishing patients with renewable, functional β cells would possibly offer a real

cure. There are two major ways to achieve such a goal. One is through generating mature β cells *in vitro* or *ex vivo* that could correct insulin insufficiency when transplanted into patients. The other way would be stimulating β cell regeneration *in vivo* (Bonner-Weir and Weir, 2005). *Ngn3* has been implied to play essential roles in both of these strategies.

To generate mature β cells *in vitro*, many efforts have been put on directed-differentiation of various cell types, including hESCs/mESCs (human/mouse embryonic stem cells), pancreatic duct cells, hepatic progenitor cells, neural progenitor cells, bone marrow-derived stem cells, intrahepatic biliary epithelial and gall bladder epithelial cells (Ai et al., 2007; Baetge, 2008; Heremans et al., 2002; Hori et al., 2005; Jiang et al., 2007; Jin et al., 2008; Phillips et al., 2007; Treff et al., 2006; Yechoor et al., 2009). The idea is to mimic endogenous embryonic endocrine differentiation *in vitro* and therefore generate mature β cells. As discussed previously, *Ngn3* controls the endocrine differentiation program during embryogenesis. Thus, several groups have tested its role in directing endocrine differentiation *in vitro*. Heremans et al. showed that adult human pancreatic duct cells could be converted into insulin-expressing cells after adeno-virus mediated *Ngn3* over-expression (Heremans et al., 2002). Inducible expression of *Ngn3* in mESCs promotes pancreatic endocrine differentiation and insulin expression (Treff et al., 2006). These results suggest that *Ngn3* can promote endocrine differentiation *in vitro*. However, the insulin-expressing cells induced by *Ngn3* over-expression do not seem to be mature β cells in terms of their poor response to glucose stimulation. This is predictable to

some extent, in that *Ngn3* initiates the endocrine differentiation program, but is not responsible for the specification of different islet cell types even during embryogenesis *in vivo* (Johansson et al., 2007). Other factors that can specifically promote β cell genesis remain to be determined for successful generation of mature functional β cells *in vitro*.

Another interesting way to reverse diabetes would be stimulation of adult β cell regeneration *in vivo*. β cell mass is maintained mainly through self-replication of pre-existing β cells in a fairly slow rate (0.5%/6 hour in rodents) (Bonner-Weir, 2000a; Bonner-Weir, 2000b; Dor et al., 2004). Under physiological and pathological stresses such as pregnancy and obesity, the β cell replication rate significantly increases to compensate for the rising demand (Lee and Nielsen, 2009). Several factors have been identified playing important roles in β cell replication, including cyclin/CDK, menin, transcription factors (Pax4//Nkx6.1), hormones and growth factors (Lee and Nielsen, 2009). Although treatments targeting these replication-related factors may markedly help type II diabetic patients, type I diabetic patients are hardly able to benefit from these treatments since they have lost most of their β cells and endogenously regenerated β cells are still under the attack of their immune system.

Stimulating β cell neogenesis in the adult pancreas is another promising way to enhance β cell regeneration. The existence of β cell neogenesis in the adult pancreas remains highly controversial. On one hand, Dor. et al. showed that new β cell arise solely from replication of existing β cells; on the other hand, several studies have shown that ductal cells in the pancreas might contribute to β

cell neogenesis (Bonner-Weir et al., 2004; Dor et al., 2004). Until recently, Xu et al. revealed that β cell progenitors could be activated in injured adult mouse pancreas after Partial Ductal Ligation (PDL) (Ackermann Misfeldt et al., 2008; Xu et al., 2008). More importantly, these adult β cell progenitors are located in the ductal lining, and depend on *Ngn3* to further differentiate into all islet cell types, including glucose responsive β cells (Xu et al., 2008). Based on this study, it would be intriguing to examine whether induced *Ngn3* expression in adult pancreatic duct cells can enhance β cell neogenesis *in vivo*. Furthermore, it had been shown that β cells could be produced via the reprogramming of other cell types. Zhou, Q *et al.* reported a strategy that could successfully reprogram adult pancreatic exocrine cells into insulin producing cells *in vivo*, through virus-mediated forced expression of *Pdx1*, *Ngn3* and *MafA* (Zhou et al., 2008). This strategy successfully restores euglycemia in diabetic animals, but more details remain to be explored prior to clinical purposes, such as the life span and mitotic features of the induced insulin-expressing cells. Moreover, generating β-like cells at the expense of the exocrine pancreas may cause some side effects in the long run. Nevertheless, this study suggests that *Ngn3* together with other key transcription factors can reprogram terminally differentiated exocrine cells into insulin-expressing cells *in vivo*. More intriguingly, *Ngn3* was revealed to be sufficient for trans-determination of hepatic progenitor cells into islet-like cells *in vivo* (Yechoor et al., 2009). Overall, these studies not only indicate that transcription factors like Ngn3 can actively reprogram either differentiated or

determined cells into islet cells, but have also brought new directions into the diabetic therapy field.

In summary, important progress has been made regarding how to generate mature β cells *in vitro* and *in vivo* during the past several decades. However, even though very promising, none of the above-discussed strategies are able to produce β cells as efficient as endogenously generated β cells, which highlights the fact that there is still many unknowns about pancreatic development and islet biology. A comprehensive understanding of endocrine islet differentiation and function would significantly benefit therapy-oriented researches.

The Significance of the Thesis Research

The connection between the pancreas and various diseases such as diabetes, has urged biologists and medical researchers to understand the molecular programs that control the formation and homeostasis of the pancreas. During the past decade, a network of transcription factors has been identified and determined to be essential for pancreatic development and maintenance. More importantly, based on this information, various strategies have been developed and tested to repopulate β cell populations *in vitro* or *in vivo*, with the hope of benefiting diabetic patients. However, some important issues remain mysterious.

The existence of stem-cell-like progenitors in the adult pancreas has been a big debate in the field. Only until recently, has solid lineage tracing data been acquired to demonstrate that pancreatic or β cell progenitors are present in the

adult pancreas. Inada et al. revealed that carbonic anhydrase II$^+$ (CAII$^+$) ductal cells act as pancreatic progenitors in adults after severe injury (Inada et al., 2008). However, it is still unknown whether these adult progenitors exist as a special population that resides in adjacent to ductal cells or actually comes from de-differentiated ductal cells. If the former situation were true, it would be important to examine whether these adult progenitors sit in special niches like adult intestine stem cells and what contributes to the pancreatic niches. It would be even more intriguing if these adult progenitor cells could be isolated, expanded *in vitro and* differentiated into desired cell types. In another study, Xu et al. showed that Ngn3$^+$ β cell progenitors could be activated in the adult mouse pancreas after PDL (Ackermann Misfeldt et al., 2008; Xu et al., 2008). Although these β cell progenitors are located within the ductal lining, their identity is not quite clear. It is also unknown whether they differentiate from adult pancreatic progenitors as proposed by Inada et al., or represent a distinct population of adult endocrine progenitors with their own niches.

Another issue of pancreatic development that remains mysterious concerns the specification of different islet cell types. *Ngn3* is the key regulator of endocrine differentiation, but it obviously does not control the islet cell type specification, nor do the signals from tissues adjacent to the pancreatic epithelium (Johansson et al., 2007). It appears that the pancreatic progenitors are pre-patterned for which islet subtypes they are fated to be. However, little is known about the signals/factors that control islet subtype specification, which is integral for generating mature β cells *in vitro* or *in vivo*.

Moreover, considering Ngn3's essential role in endocrine differentiation, more needs to be explored regarding its upstream regulators and major downstream targets. Several signaling pathways and transcription factors have been implicated in regulating Ngn3 levels in endocrine progenitors. But individually, none of them is sufficient to induce high Ngn3 activity *in vivo*. Additional factors appear to be required to up regulate Ngn3 levels. Moreover, it is substantial to know whether Ngn3 levels in individual endocrine progenitor cells are important for endocrine differentiation. These questions will be discussed and addressed in the following chapters. Furthermore, the mechanisms that down regulate Ngn3 levels in differentiated endocrine cells have not been thoroughly examined. It is also unknown how *Ngn3* is maintained in a low level in differentiated endocrine cells in adults, and whether the same signals/factors regulating *Ngn3* expression during embryogenesis function in the adult pancreas. Careful examination of *Ngn3* promoter occupancy at different development stages might provide useful information and clues needed to address these questions.

Downstream of *Ngn3*, there is a complicated network of transcription factors that are essential for endocrine differentiation. It remains unclear how these different factors cross talk with each other, and what the major targets of *Ngn3* are. In fact, several groups have tried to isolate $Ngn3^+$ cells via various strategies to examine their expression profiles (Gu et al., 2004; Juhl et al., 2008; Miyatsuka et al., 2009; White et al., 2008). However, due to the nature of Ngn3's transient expression and long duration of reporter proteins like Enhanced Green

Fluorescent Proteins (EGFP), the isolated cells remain a mixed population of $Ngn3^{+}$ cells and differentiated endocrine cells, making it hard to identify direct *Ngn3* targets or Ngn3 co-factors. Moreover, the generation of mutant mouse models (knock out/in, transgenic mice), which are predominantly used within the field to examine gene function, is very complicated and time-consuming. This greatly slows down the identification of significant factors from the massive number of candidate genes that stand out from various screens. Considering the convenience of gene manipulation in the chick system, such as siRNA/cDNA electroporation, establishment of a platform in chicks to quickly test candidate gene function may significantly accelerate the experimental process.

This thesis research work focuses on understanding how pancreatic endocrine differentiation/function is regulated, especially the role of *Ngn3* in the pancreas. The results from this work will not only help us to understand the basic principles of organogenesis, but will also provide valuable information to favor the generation of unlimited mature β cells, which is the first essential step towards curing the worldwide disease *Diabetes Mellitus*.

CHAPTER II

LOSS OF *Myt1* FUNCTION PARTIALLY COMPROMISES ENDOCRINE ISLET CELL DIFFERENTIANTION AND PANCREATIC PHYSIOLOGICAL FUNCTION IN THE MOUSE

This chapter is published under the same title in *Mechanisms of Development*, Nov-Dec, 2007 (Wang et al., 2007).

Abstract

Myelin transcription factor 1 (Myt1) was identified as a factor that is specifically enriched in the endocrine cell lineage. Ectopic gene expression and dominant-negative-based loss-of-function studies suggest that *Myt1* may regulate endocrine differentiation and function in the pancreas. Here, we analyzed the expression pattern of *Myt1*, as well as the effects of *Myt1* gene disruption to further understand how *Myt1* functions in the mouse pancreas. *Myt1* is expressed in both $Ngn3^{+}$ endocrine progenitors during embryogenesis and differentiated endocrine islet cells in adulthood. $Myt1^{-/-}$ animals die postnatally due to non-pancreatic related defects. The endocrine compartment of the $Myt1^{-/-}$ pancreas contains abnormal endocrine cells that co-express multiple hormones, while maintains relatively normal numbers of endocrine cells. Furthermore, pancreatic-specific *Myt1* mutant mice, which are viable and fertile, displayed similar pancreatic phenotypes as $Myt1^{-/-}$ mice during development. Male mice

that have lost *Myt1* in their pancreatic cells display poor glucose tolerance, attenuated insulin secretion and reduced Glut2 expression. Taken together, these findings demonstrate that *Myt1* is important for proper endocrine differentiation and islet function in the mouse pancreas.

Introduction

In the pancreas, endocrine islets, whose malfunction is associated with *Diabetes Mellitus*, are generated from $Ngn3^+$ endocrine progenitors in a multi-step process. Genetic and functional studies have identified several signaling pathways and transcriptional factors that are essential for pancreatic endocrine differentiation, but a global analysis of regulatory signals/factors in the endocrine pancreas is still missing (Murtaugh, 2007; Oliver-Krasinski and Stoffers, 2008). Previously, Gu et al. compared the gene expression profiles of $Ngn3^+$ endocrine progenitor cell pool with $Ngn3^-$ cell pool of the developing mouse pancreas by microarray-based analyses. *Myt1* (*Myelin transcription factor 1*) was discovered as a candidate gene that is highly expressed in endocrine progenitors (Gu et al., 2004).

Myt1 encodes a C2HC type zinc finger transcription factor that is conserved in vertebrates (Bellefroid et al., 1996; Jiang et al., 1996; Matsushita et al., 2002). It has two paralogs, *Myt1L* and *Myt3*. Proteins encoded by these three genes share high similarity in terms of their protein sequences and structures (Figure 2.1) (Jiang et al., 1996; Kim and Hudson, 1992; Matsushita et al., 2002; Yee and Yu, 1998).

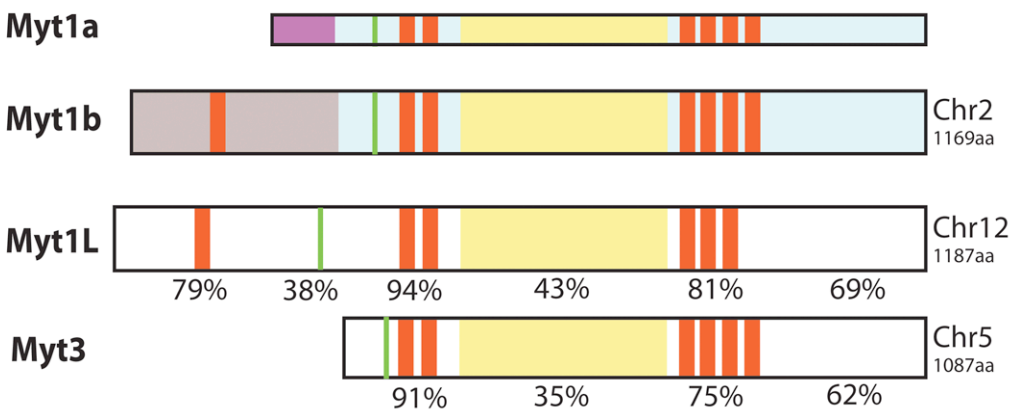


Figure 2.1 *Myt* family members share high similarities. The protein structures of *Myt* family members were shown. Genome location and protein length were indicated on the right. Percentages of amino acid similarities of various regions of Myt1L and Myt3 to Myt1 were shown. The *Myt1* locus produces two proteins (Myt1a and Myt1b) that differ in their N-termini. The first 103 aa residues (light cherry rectangle) of Myt1a are not shared with Myt1b (light brown rectangle, 152 residues). Orange boxes: zinc fingers; Yellow box: putative transactivation domain; Green bars: the exons that were deleted to generate null alleles in the following chapters; Chr: chromosome.

Myt1 gives rise to two transcripts (*Myt1a* and *Myt1b*), differing only in their 5' regions by utilizing alternative transcription start sites (Kim and Hudson, 1992; Matsushita et al., 2002). *Myt1b* is the predominantly expressed form in almost all of the tissues examined (Matsushita et al., 2002). In *Xenopus*, *xMyt1* (the homolog of *Myt1* in *Xenopus*) is expressed in the neural plate during neurogenesis, and possibly cooperates with *xNgnr1* (the homolog of *Ngn3* in *Xenopus*) to promote neuronal differentiation (Bellefroid et al., 1996; Quan et al., 2004). In rodents, *Myt1* is highly expressed in the nervous system (brain, spinal cord, neural crest, sensory organs and peripheral ganglia) and the endocrine pancreas (Kim et al., 1997a; Matsushita et al., 2002). It remains unclear whether/how *Myt1* functions in any *in vivo* systems.

In tissue cultures, *Myt1* modulates the proliferation and differentiation of myelin-forming oligodendrocytes (Armstrong et al., 1995; Nielsen et al., 2004). Since Myt1 can interact with SIN3 homolog B (Sin3B), a chromatin modifier that serves as scaffolds tethering histone deacetylases (HDACs) activity (Romm et al., 2005), it was proposed that *Myt1* might regulate its target genes by modulating the chromatin structure (Romm et al., 2005). However, no direct study has been performed to address this possibility.

In the mouse/chicken pancreas, both *Myt1a* and *Myt1b* are expressed in the pancreatic epithelium during embryogenesis, with *Myt1b* being the predominantly expressed form (Matsushita et al., 2002). A dominant negative Myt1 without the putative transactivation domain can impair endocrine differentiation in the mouse pancreas (Gu et al., 2004). Mis-expression of *Myt1b*

in the chicken hindgut endoderm results in the ectopic expression of glucagon and stomatostatin (Gu et al., 2004). *Myt1* is also able to partially antagonize Notch activity to facilitate endocrine differentiation in the chicken pancreas (Ahnfelt-Ronne et al., 2007). These data suggest that *Myt1* may play important roles during pancreatic endocrine differentiation.

In this chapter, the protein expression pattern of *Myt1* was carefully examined in the mouse pancreas. Furthermore, *Myt1* global and pancreatic specific knock out mutant mice were generated and characterized. *Myt1*$^{-/-}$ pancreata contain abnormal islet cells that co-express multiple hormones. Pancreatic specific *Myt1* knock out male mice display impaired glucose clearing ability, reduced Glut2 expression and attenuated glucose induced insulin secretion (GSIS). These findings demonstrate that *Myt1* is important for proper pancreatic endocrine differentiation and islet function in mice. Interestingly, the expression of *Myt1L* and *Myt3*, which are not expressed in the wild type pancreas, is induced upon *Myt1* deletion, suggesting that *Myt1L* and *Myt3* may compensate for the loss of *Myt1* function in mice.

Materials and Methods

1. Mouse strains and care

Mouse husbandry and genotyping were performed following standard protocols (M/03/363 and M/03/354) approved by the Vanderbilt Medical Center Institutional Animal Care and Use Committee (IACUC). For embryonic

staging, vaginal plug appearance was counted as embryonic day 0.5 (E0.5). For routine mouse embryo production, the ICR mouse strain was utilized (Charles River Laboratories, Inc. Wilmington, MA). For initial knockout mice production, C57BL/6 strain was used (Charles River Laboratories, Inc. Wilmington, MA). Subsequent strain maintenance and crosses utilized CD1 mice. *Flpe* (used to delete the selection marker in the targeted allele), *Pdx1-Cre* and *Sox2-Cre* mice were previously reported (Dymecki, 1996; Gu et al., 2002; Hayashi et al., 2002). Genotyping followed published methods with minor modification. Briefly, mouse tissue from ear puncture were collected and boiled in 40mM NaOH for 30 minutes. Equal volume of 0.1mM Tris (pH: 4.0) was then added for neutralization. 1μl of the lysate was used for PCR-based genotyping.

2. Generation of Myt antibodies

To produce Myt1 antibodies, an open reading frame encoding Myt1 amino acid residues 109–298 (as numbered in mKIAA0835) was fused with that encoding a maltose binding protein (New England Biolabs, Beverly, MA). The fusion protein was purified and used as an antigen. The antibody production in rabbits was performed by Strategic Bio-solution (Newark, DE). The specificity of the antibodies was verified by the lack of positive signals in *Myt1*$^{-/-}$ mouse tissues, which produces *Myt1L* and *Myt3.* Production of Myt1L and Myt3 antibodies followed the similar design, except that amino acid residues 215-424 (as numbered in AAC53457) for Myt1L and 63-302 (as numbered in mKIAA0535) for Myt3 were used.

3. In situ hybridization and immunohistochemistry/immunofluorescence (IHC/IF)

In situ hybridization and IHC/IF followed established protocols. Briefly, mouse tissues were fixed in 4% paraformaldehyde overnight at 4°C or 4h at room temperature. For *in situ* hybridization, tissues were prepared as 6μm paraffin sections. All cRNA probes were made by using cDNA clones as templates. The *Myt1* cDNA (#LDN 147) was a gift from L. Hudson (Kim et al., 1997a). This cDNA clone was digested with *XhoI* and transcribed with T7 RNA polymerase. The *Myt1L* (#6844118) and *Myt3* (#5366688) cDNA clones were purchased from Open Biosystem (Huntsville, AL). These cDNA clones were digested with *Sal-I* or *AvrII* and transcribed with T3 or T7 RNA polymerase for cRNA probe production, respectively. For IHC/IF, either frozen or paraffin sections were utilized. Primary antibodies used: guinea pig anti-insulin, goat anti-C peptide (recognizing the same cells as anti-insulin antibodies), and guinea pig anti-glucagon are from Dako, Carpinteria, CA; Rabbit anti-glucagon, rabbit anti-SS, goat anti-SS, guinea pig anti-PP are from Invitrogen, Carlsbad, CA; Rabbit anti-Glut2 is from Chemicon, Temetula, CA; Rabbit anti-MafA and MafB are gifts from R. Stein (Matsuoka et al., 2004); Goat anti-Pdx1 is a gift from C.V. Wright; Mouse anti-Ngn3 is from Hybridoma Bank, Iowa (F25A1B3); Guinea pig anti-Ngn3 is a gift from M. Sander (Seymour et al., 2007). Secondary antibodies used: FITC-conjugated donkey anti-rabbit IgG, Cy3-conjugated donkey anti-rabbit IgG, Cy3-conjugated donkey anti-mouse IgG, Cy3-conjugated donkey anti-guinea pig IgG and Cy3-conjugated donkey anti-goat IgG are from Jackson Immunoresearch, West Grove, PA; Cy5-conjugated donkey anti-rabbit IgG and

Cy5-conjugated donkey anti-guinea pig IgG are from Chemicon, Temetula, CA. For all antibodies, a 1:500–1:2000 dilution was used, depending on the amount of tissue on each slide.

4. Generation of Myt1$^{-/-}$ animals

The targeting vector *pGKNeo-DTA* was a gift from A. P. McMahon (Zhang et al., 2001). This vector contains a *pBluescript KSII* backbone, a diphtheria toxin A subunit (DTA) sequence as a negative selection marker, and a *FRT*-flanked *pGKneo* gene as a positive selection marker. A genomic fragment flanking the third exon of *Myt1a* was PCR amplified, sequenced, and cloned into the FseI site of the vector. The primers used were: forward: 5'-aggccggccaaagattaaagtttagag-3' and reverse: 5'-aggccggccctaccatgccccagcttaat-3'. The template was BAC #519N6 in library RPCI22, purchased from Children's Hospital of Oakland, Oakland, CA (Warming et al., 2005). Another 3.5kb genomic DNA fragment 3' of the deleted region was PCR-amplified, sequenced and cloned between the *SalI–PmeI* sites of the vector. The primers used were: forward: 5'-agtttaaacgaaattagggctgggcctgt-3' and reverse: 5'-agtcgacgcagttttaaaatcaccctcag-3'. Finally, a 8.2kb genomic fragment 5' of the deleted region, derived through BAC-based recombineering, was ligated into the *NotI–AscI* sites to complete the targeting vector construction. The oligos used for BAC recombineering were: 5'-ggcgcgccctcgagtgctgagactacaggtatgcaccaccatgctctgctaaggatagaatccctccagagag tgagtagcttcatgtagggttatcccgcggccgc-3' and its complimentary strand. Oligos used

to amplify the 5' probe were: forward: 5'-atattaggaatattaaaacttgt-3' and reverse: 5' ggtcacaatgatgggcactaac-3'. Oligos used to amplify the 3' probe: forward: 5'-ctacataaaccttcaaggtc-3' and reverse: 5'-gaattcaattcccagcaaccaca-3'.

Targeted ES cells were utilized to derive chimera mice following standard procedure. After mouse line establishment, *Flpe* mice were used to delete the *FRT*-flanked *pGK-Neo* selection cassette, deriving the $Myt1^{fl}$ allele (Dymecki, 1996). Oligos for PCR genotyping: Pr1: 5'-agatccttccagggtggagaagc-3'; Pr2: 5'-gtctgtccagaacctattccaga-3'; Pr3: 5'-cagacttccattcccacagtt-3'. Expected DNA fragment lengths for the wild type, the floxed $Myt1^{fl}$, and the deleted $Myt1^{-}$ alleles are 490, 380 and 340bp, respectively. Oligos for assessing the stability of mutant *Myt1* messages: 5'-gagctatctagtcctaaacctga-3' and 5'-ttgggaggatctcctgtctgcaa-3'. Expected length of wild-type fragment: 608bp, mutant fragment: 517bp. After RT-PCR, the intensities of the bands were quantified by using BIOQUANT true-color windows system (R &M Biometrics, Nashville, TN). Gene targeting was performed by using TL1 ES cells. Three correctly targeted clones were identified from 219 colonies screened. Blastocyst microinjections were performed by the Vanderbilt Transgenic/ES Cell Shared Resource, following standard procedures.

5. Intraperitoneal glucose tolerance test (IPGTT) and insulin secretion assay

IPGTT followed published procedure (Lammert et al., 2003). Briefly, after overnight fasting (~16h), mice were injected intraperitoneally with glucose at 2 gram per kg body weight. Blood glucose levels were monitored by a compact glucose analyzer (BD/Medtronic MiniMed Blood Glucose Monitor) at different

time points after glucose administration.

For insulin secretion assay, blood samples were taken before or 30 min after glucose injection from the mouse saphenous vein. Serum insulin levels were assayed using Elisa Assay Kit from Linco following manufacturer-recommended protocol (St. Charles, MO).

6. Islet isolation and glucose stimulated insulin secretion assay (GSIS)

Pancreatic islet isolation was achieved by collagenase perfusion (Gu et al., 2004). Batches of 10–15 similar sized hand-picked islets were incubated in RPMI 1640-10% fetal calf serum at 37°C for overnight. These islets were washed and incubated in 1 ml Krebs-Ringer Hepes-buffered saline in 2.8 mM glucose at 37°C for 30 min and then transferred to 1 ml Krebs-Ringer Hepes-buffered saline in 20 mM glucose for another 30 min. Supernatants were collected for GSIS. Islets were extracted for total insulin assay. Insulin secretion was calculated as percent of insulin released within the 30 min glucose stimulation. All assays utilized triplicate samples.

7. Confocal microscopy and statistical analysis

All fluorescent images in the figures were taken by confocal microscopy. Typically, optical sections with 0.4–0.6μm intervals were taken. A single or a projection of two adjacent optical sections was used for a high quality image. All confocal parameters were kept consistent throughout our experiments. For quantification, we utilized BIOQUANT true-color windows system (R & M

Biometrics, Nashville, TN). This system measures both cell number and fluorescence intensities (Zhang et al., 1997). The hormone amount quantified using this approach matched well with our Elisa-based total insulin or glucagon assay data from E18.5 embryonic pancreata. For Myt1 staining, 10–15μm frozen sections were used. For Glut2 expression or co-hormone expression analyses, 4–8μm paraffin sections were used. For the quantification of co-hormone expressing cells at E15.5 and E18.5, adjacent 10 μm frozen sections were separated onto groups of three slides, with one staining for glucagon/insulin, one for insulin/PP and one for PP/SS. Co-stained cells were counted on optical sections at 6μm apart. Two optical sections from each tissue section were counted. All tissue sections from each E15.5 pancreas were analyzed. A third of the tissue sections from each E18.5 pancreas were counted. For quantification in adults, each pancreas was cut into 25–30 small pieces and embedded into a single paraffin block. One of every four 4μm sections was collected, with sets of three being mounted onto groups of three slides for glucagon/insulin, insulin/PP, and SS/PP staining respectively. Slides were then counterstained with DAPI and co-stained cells were scored using a Carl Zeiss Axioplan 2 fluorescence microscope. At least 25 sections were scored for each hormone combination in each pancreas. Statistical analyses utilized standard Student's t-test. A p-value of 0.05 or less was considered significant. All quantification data were presented as (mean$\pm$standard deviation).

Results

Myt1 is expressed in endocrine progenitors and differentiated islet cells

Previous mRNA-based studies showed that *Myt1* is specifically expressed in the endocrine pancreas during pancreatic development in mice (Gu et al., 2004). To determine which endocrine cell types express *Myt1*, specific antibodies for Myt1 was developed (see "Materials and methods").

During embryogenesis, robust *Myt1* expression could be detected in the developing mouse pancreas starting from E9.0, corresponding with the initiation of pancreatic development (Figure 2.2 A1). At E9.0, most $Myt1^+$ cells expressed Pdx1 (Figure 2.2 A1&B1), suggesting their pancreatic origins. Later, Myt1 was detected in all of the four major endocrine cell types (α, β, δ and PP cells), but not in the exocrine pancreas (Figure 2.2 A3-A4, B3-B4, C1-C1 & D1-D2). Specifically, Myt1 was expressed in all $insulin^+$ (ins^+) cells at E10.5 (Figure 2.2 A2). Then, the percent of ins^+ cells expressing Myt1 decreased with age (E13.5: $91.4\pm7.6\%$ of ins^+ cells are $Myt1^+$; E18.5: $94.3\pm2.9\%$ of ins^+ cells are $Myt1^+$; 12-weeks: $58.6\pm6.7\%$ of ins^+ cells are $Myt1^+$, n=3). The percentage of glucagon (glu), somatostatin (ss) and pancreatic polypeptide (PP)-expressing cells that co-express Myt1 remained relatively constant from E10.5 to 12 weeks of age (95% of $glu^+/ss^+/PP^+$ cells are $Myt1^+$). These data suggest that mature β cells are a heterogeneous population, whereas the other types of endocrine cells are relatively uniform.

Since *Myt1* is specifically enriched in the $Ngn3^+$ cell pool, the expression

of Myt1 in $Ngn3^+$ endocrine progenitors was also examined (Gradwohl et al., 2000; Gu et al., 2004; Jensen et al., 2000a; Murtaugh, 2007; Schwitzgebel et al., 2000). Two antibodies for Ngn3, a monoclonal mouse anti-Ngn3 antibody and a guinea pig anti-Ngn3 antibody (Seymour et al., 2007), were utilized to examine whether Myt1 is expressed in $Ngn3^+$ cells. Both antibodies revealed that Myt1 expression partially overlapped with that of Ngn3 (Figure 2.3), suggesting that a portion of $Myt1^+$ cells is endocrine progenitors at different stages (E10.5, E13.5, and E15.5). It seems that Myt1 expression is activated in $Ngn3^+$ endocrine progenitors, and maintained in these same cells after differentiation when Ngn3 expression is down regulated. This result was further supported by the findings that Myt1 expression overlapped with the expression of Isl1, MafB, Pax6 and MafA, which label endocrine progenitors under different commitment states or mature islet cell types (Figure 2.4) (see Chapter 1).

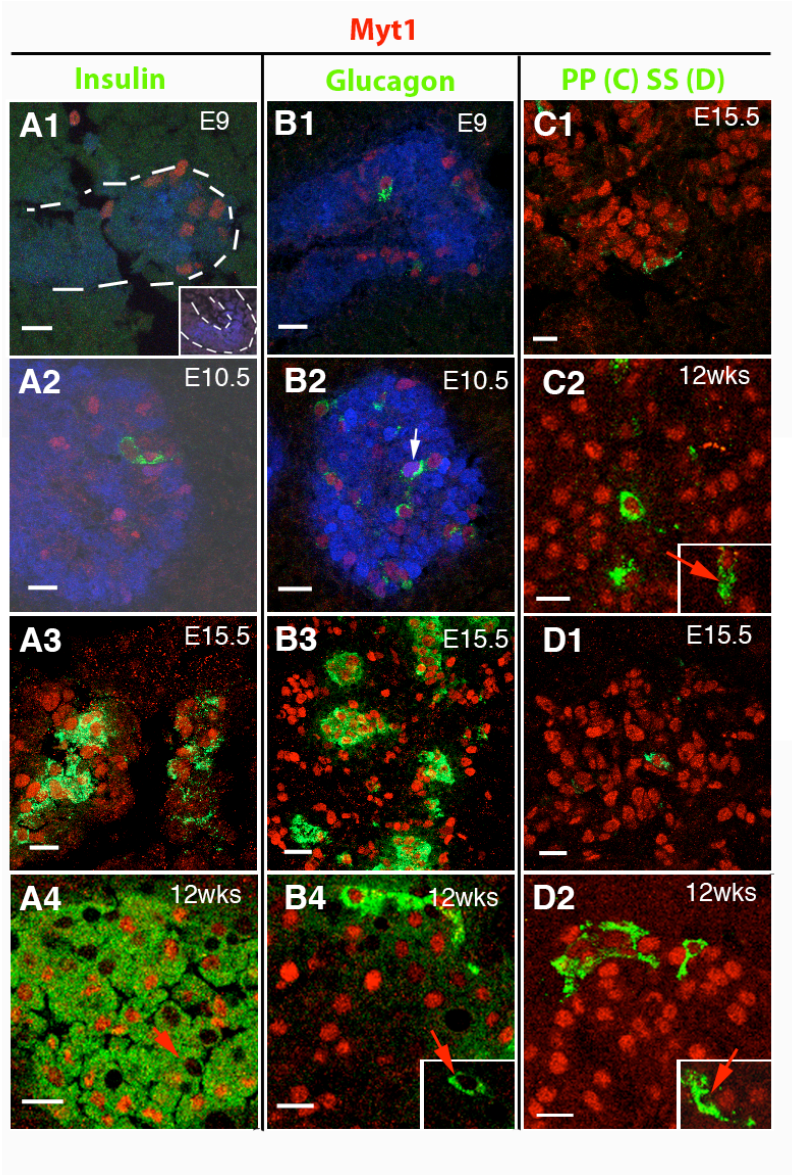


Figure 2.2 Myt1 is expressed in hormone-expressing endocrine cells in the pancreas. (A1-A4) Most ins^{+} cells expressed Myt1 from E10.5 to 12 wks (12 weeks of age). Blue: Pdx1; Green: insulin; Red: Myt1. In A1, the pancreatic region was circled by white broken lines. Inset represented an E8.5 pancreatic epithelium stained for Myt1, insulin and Pdx1. (B1-B4) Glu^{+} cells maintained Myt1 expression from E9 to 12wks. Blue Pdx1; Green: glucagon; Red: Myt1. The white arrow in B2 and green arrow in B4 pointed to examples of Glu^{+}Myt1^{-} cells. (C1-2 &D1-2) Most ss^{+} and PP^{+} cells were Myt1^{+}. Red: Myt1; Green: PP or ss. Green arrows in C2 and D2 pointed to PP^{+}Myt1^{-} and ss^{+}Myt1^{-} cells, respectively. Scale bars: 20µm.

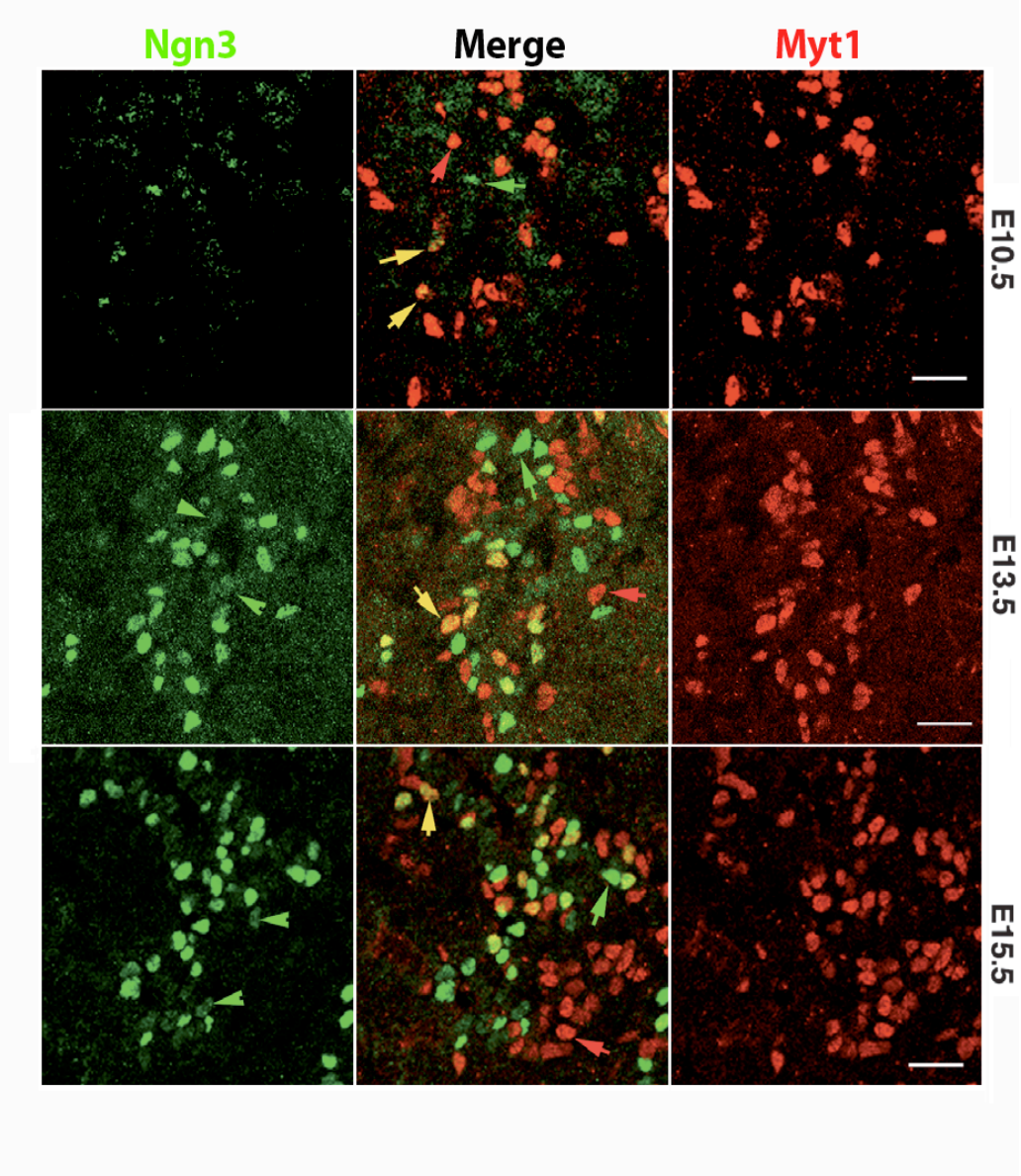


Figure 2.3 Myt1 expression overlaps with that of Ngn3. E10.5, E13.5 and E15.5 pancreatic tissues were co-labeled with Myt1 and Ngn3 antibodies. Images in the middle column were merges of the left and right images. Yellow arrow: $Myt1^{+}Ngn3^{+}$ double positive cells; Green arrow: $Ngn3^{+}Myt1^{-}$ cells; Green arrowhead: cells with low levels of Ngn3. Scale bars: 20μm.

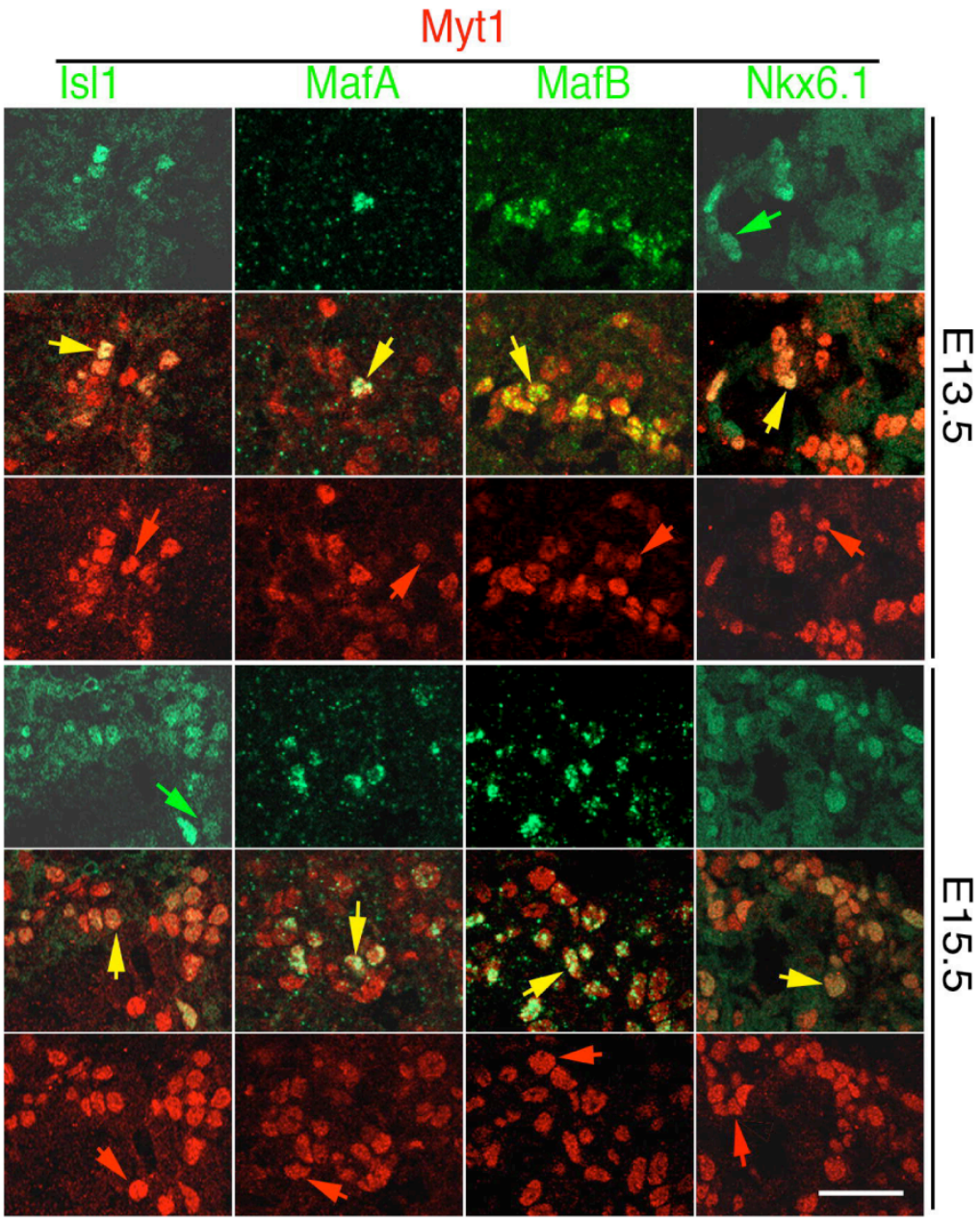


Figure 2.4 Myt1 is co-expressed with several endocrine markers. Results from two stages, E13.5 and E15.5, were shown. Red: Myt1; Green: Isl1, MafA, MafB, and Nkx6.1. For each co-staining result, three panels were shown. The top and bottom panels were single staining and the middle panels were merged images. Yellow arrows: co-stained cells. Red or green arrows: cells stained only for Myt1 or other specified transcription factors respectively. Sacle bars: 20μm.

Myt1 is the only paralog expressed in the embryonic mouse pancreas

The presence of *Myt1* paralogs *Myt1L* and *Myt3* in the mouse pancreas was examined. During embryonic development, *Myt1L* and *Myt3* were detected in neural or glial cells within the mesenchymal tissues that surround the gut epithelium (Figure 2.5). However, they were not detected in the developing mouse pancreas by *in situ* hybridization at E13.5, E14.5 and E15.5 (Figure 2.5) (Kim et al., 1997a). Moreover, specific antibodies for Myt1L and Myt3 were generated to examine their expression pattern. Neither Myt1L nor Myt3 was detected in the developing mouse pancreas (Figure 2.11 I-L). These results suggest that *Myt1* is the only *Myt* family member expressed in the developing pancreas, and likely involved in pancreatic development.

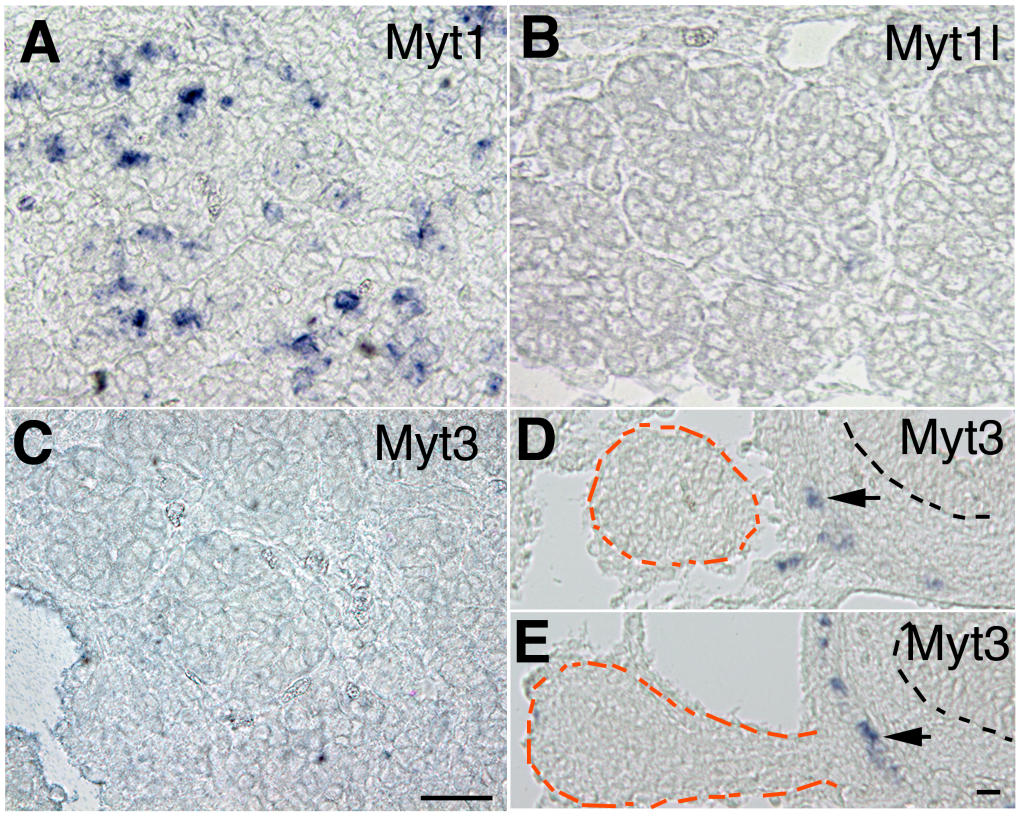


Figure 2.5. *Myt1* is the only *Myt* family member expressed in the developing pancreas. (A) *Myt1* was detected in the E14.5 pancreas by *in situ* hybridization. (B & C) *Myt1L* and *Myt3* transcripts were not found in the E14.5 pancreas. (D & E) *Myt3* was expressed in cells surrounding the duodenal epithelium at E13.5. Red broken lines circled the pancreatic regions. Black broken lines mark the duodenal epithelium. Scale bars: 20μm.

Myt1 inactivation compromises pancreatic endocrine differentiation

To directly examine the role of *Myt1* in the mouse pancreas, a conditional *Myt1* knockout allele (*Myt1*^{fl}) was derived. The 91-base-pair exon common to both *Myt1* transcripts (#3 exon of *Myt1a* or #9 exon in *Myt1b*) was flanked with tandem LoxP sites to simultaneously inactivate *Myt1a* and *Myt1b* by Cre-mediated recombination (Figure 2.1 & Fig. 2.6). The frame-shift introduced by the deletion in the mutant allele produced truncated Myt1a or Myt1b peptides, which have no similarity to any known proteins, or contain a single zinc finger, respectively.

Sox2-Cre animals were used to delete the floxed exon and generate the loss-of-function *Myt1* allele (*Myt1*$^{-}$) in the germ line (Hayashi et al., 2002). *Myt1*$^{-/-}$ animals displayed no visible abnormality yet died immediately after birth. The diaphragms of *Myt1*$^{-/-}$ mice were not properly innervated, which may result in the death after birth (Figure 2.7). Because these diaphragm-related defects could be readily scored, we used them to determine whether the *Myt1*$^{-}$ allele has dominant negative effects. If the mutant Myt1 products had dominant negative effects, we would expect to observe abnormal diaphragm innervations or reduced viability in heterozygous animals. In fact, *Myt1*$^{+/-}$ diaphragms were innervated normally. To date, 156 wild type and 329 heterozygous but no homozygous adult animals were obtained. This is consistent with the Mendelian ratio, suggesting that the *Myt1*$^{-}$ allele does not behave in a dominant negative manner. Moreover, *Myt1* mRNA levels in wild type, *Myt1*$^{+/-}$ and *Myt1*$^{-/-}$ pancreata were analyzed by *in situ* hybridization and semi-quantitative RT-PCR. The cRNA probe, derived from the

full-length *Myt1* cDNA (see Materials and Methods), recognized both wild type and mutant *Myt1* mRNA. The mRNA level of the *Myt1*$^{-}$ allele was reduced by more than 4 folds comparing with that of the wild type *Myt1* allele (Figure 2.6 D, E, and F), making the *Myt1*$^{-}$ allele unlikely to be a dominant negative allele.

The expression of endocrine hormones (insulin, glucagon, ss and pp) in the *Myt1*$^{-/-}$ pancreas was examined at E10.5, E13.5, E15.5, and E18.5. There was no significant difference in the expression levels of these hormones between wild type and *Myt1*$^{-/-}$ pancreata, as assayed by the number of hormone^{+} cells (Figure 2.8 A1-A2), and the relative fluorescence intensity within each hormone-expressing cell (Figure 2.8 A3-A4). The number of Ngn3^{+} endocrine progenitors, as well as the *Ngn3* expression level within individual cells, did not vary as well in wild type, *Myt1*$^{+/-}$and *Myt1*$^{-/-}$ pancreata at E10.5, E13.5, and E15.5 (data not shown).

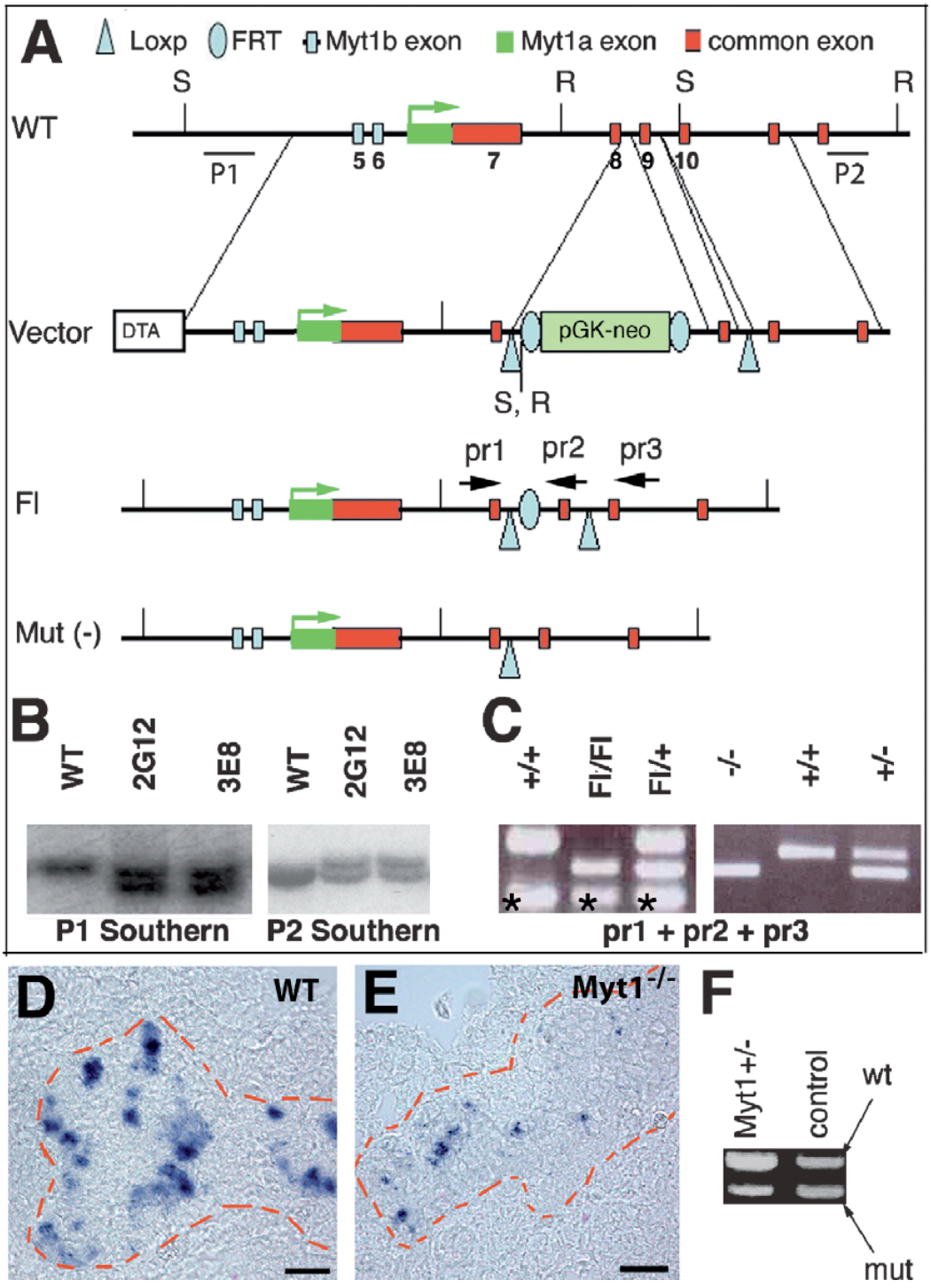
However, *Myt1*$^{-/-}$ pancreata contained a substantial number of endocrine cells co-expressing insulin and PP, or SS and PP, at all stages (Figure 2.8 B–E). Other hormone co-expression combinations were not found at significant numbers (Figure 2.8 F&G). Specifically, while less than 1.5% of PP^{+} cells expressed detectable insulin in wild type and *Myt1*$^{+/-}$ pancreata, 11.6$\pm$2.4% (mean $\pm$ standard deviation) of PP^{+} cells co-expressed insulin in *Myt1*$^{-/-}$ pancreata at E15.5. Moreover, 19.9 $\pm$ 3.1% of PP^{+} cells in *Myt1*$^{-/-}$ pancreata co-expressed SS, while less than 2% of PP^{+} cells expressed SS in wild type and *Myt1*$^{+/-}$ pancreata at E15.5 (Figure 2.8 B1-3, C1-3). Similar phenotypes were also found at E18.5 (Figure 2.8 D1-3, E1-3). These findings suggest that *Myt1* is

required for proper endocrine differentiation, or for differentiated cells to maintain specific hormone production in the mouse pancreas.

During pancreatic development, in consistent with previous findings, *Myt1$^{fl/-}$;Pdx1-Cre* pancreata contained significant numbers of ins$^+$PP$^+$ and PP$^+$ss$^+$ cells, in comparison with wild type pancreata at E15.5 and E18.5 (Figure 2.8 F&G). In adults (12-week of age), the overall islet morphology remained normal in *Myt1$^{fl/-}$;Pdx1-Cre* pancreata. Yet there was a significant portion of PP$^+$ cells expressing either insulin or ss in *Myt1$^{fl/-}$;Pdx1-Cre* islets. Specifically, 2.4 $\pm$ 0.6% PP$^+$ cells co-expressed ss in wild type islets, while 10.2 $\pm$ 5.4% of PP$^+$ cells co-expressed ss in *Myt1$^{fl/-}$;Pdx1-Cre* islets ($n = 3$, $p < 0.01$). Similarly, 0.2 $\pm$ 0.1% of PP$^+$ cells co-expressed insulin in wild type islets, whereas 4.1 $\pm$ 0.6% of PP$^+$ cells co-expressed insulin in *Myt1$^{fl/-}$;Pdx1-Cre* islets ($n = 3$, $p< 0.01$). In addition, a significant number of glu$^+$ins$^+$ cells was observed in *Myt1$^{fl/-}$;Pdx1-Cre* islets, accounting for 2.7 $\pm$ 0.8% of total glu$^+$ cells and distributing in 8% of islet sections. However, only 0.3 $\pm$ 0.1% of glu$^+$ cells expressed detectable levels of insulin in wild type islets, which is consistent with the previous finding that glucagon and insulin double positive cells (glu$^+$ins$^+$) can be rarely found in wild type pancreata (Guz et al., 1995; Herrera, 2000).

We also examined the maturity of these double-hormone expressing cells in the adult *Myt1$^{fl/-}$;Pdx1-Cre* pancreas. As insulin-expressing β cells mature, they activate *MafA* expression while down regulating *MafB* expression. As a result, adult mature β cells are MafA$^+$MafB$^-$ (Nishimura et al., 2006). MafA was detected in most β cells in wild type islets, but absent in any of these ins$^+$PP$^+$ and glu$^+$ins$^+$

Figure 2.6 Generation of *Myt1*^{fl} and *Myt1*$^{-}$ alleles. (A) Targeting strategy. Targeted *fl* allele had two LoxP sites flanking exon 3 of *Myt1a* (exon 9 of *Myt1b*). P1 and P2: DNA Southern blot probes. Pr1, Pr2, and Pr3: oligos for genotyping. (B) Southern blots of two targeted ES cell clones, 2G12 and 3E8. *SpeI* (S) digestion and blotting with P1 probe produced a wild type band of 14.5kb and a targeted band of 12kb. *EcoRI* (R) digestion and blotting with P2 probe resulted in a wild type band of 6kb and a targeted band of 7kb. (C) PCR-based genotyping using DNA oligos pr1, pr2, and pr3. Bands marked with ‘‘*’’ were primer dimmers. (D and E) *Myt1* mRNA was detected by *in situ* hybridization in wild type and *Myt1*$^{-/-}$ pancreata at E13.5. cRNA was generated from a full-length *Myt1* cDNA, which also recognized the *Myt1* mutant mRNA. Red broken lines circled the pancreatic epithelial region. (F) *Myt1* mRNA levels in control and *Myt1*$^{+/-}$ pancreata, determined by semi-quantitative RT-PCR. Left lane: RT (reverse transcription) products of RNA samples from *Myt1*$^{+/-}$ pancreata were used as templates. The higher and lower bands were the wild type and mutant bands, respectively. A comparison of the intensity of these two bands (1:3.1 ratio as determined by BIOQUNT) revealed the relative abundance of the mutant and wild type mRNA. Right lane: a mix of wt and mutant DNA products at 1:1 molecular ratio were used as control templates, to show the amplification efficiency of these two fragments. Scale bars: 20μm.


A
Loxp
FRT
Myt1b exon
Myt1a exon
common exon
S
R
S
R
WT
P1
5 6
7
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9
10
P2
Vector
DTA
pGK-neo
S, R
pr1
pr2
pr3
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Mut (-)
B
WT
2G12
3E8
WT
2G12
3E8
P1 Southern
P2 Southern
C
+/+
Fl/Fl
Fl/+
-/-
+/+
+/-
pr1 + pr2 + pr3
D
WT
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F
Myt1+/-
control
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mut

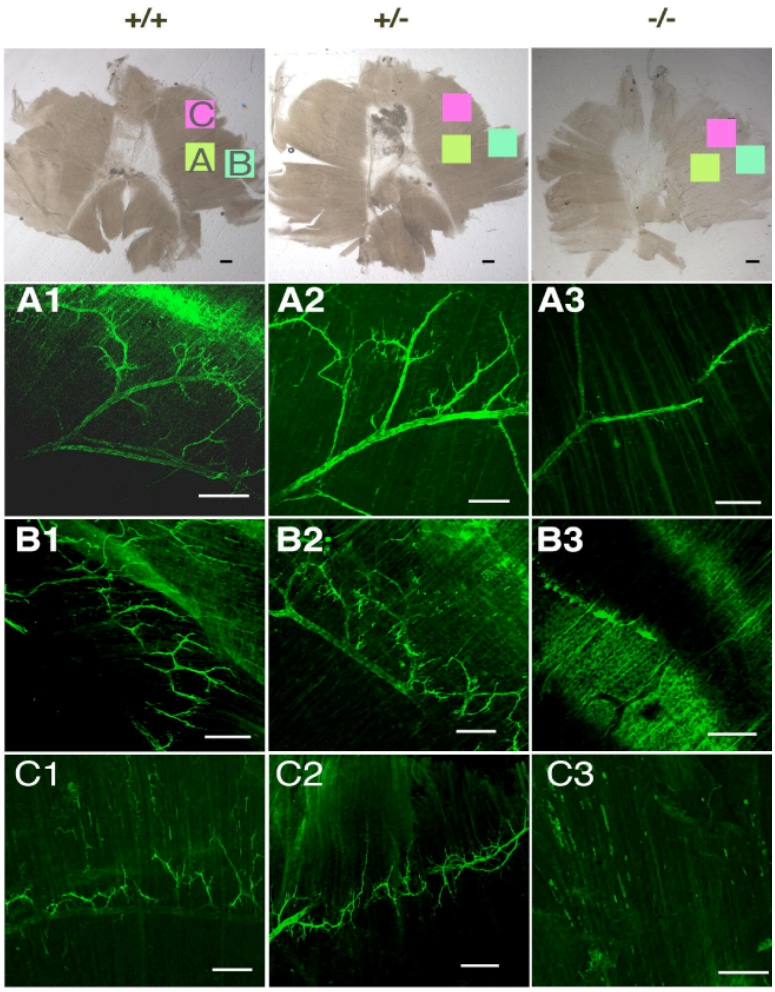
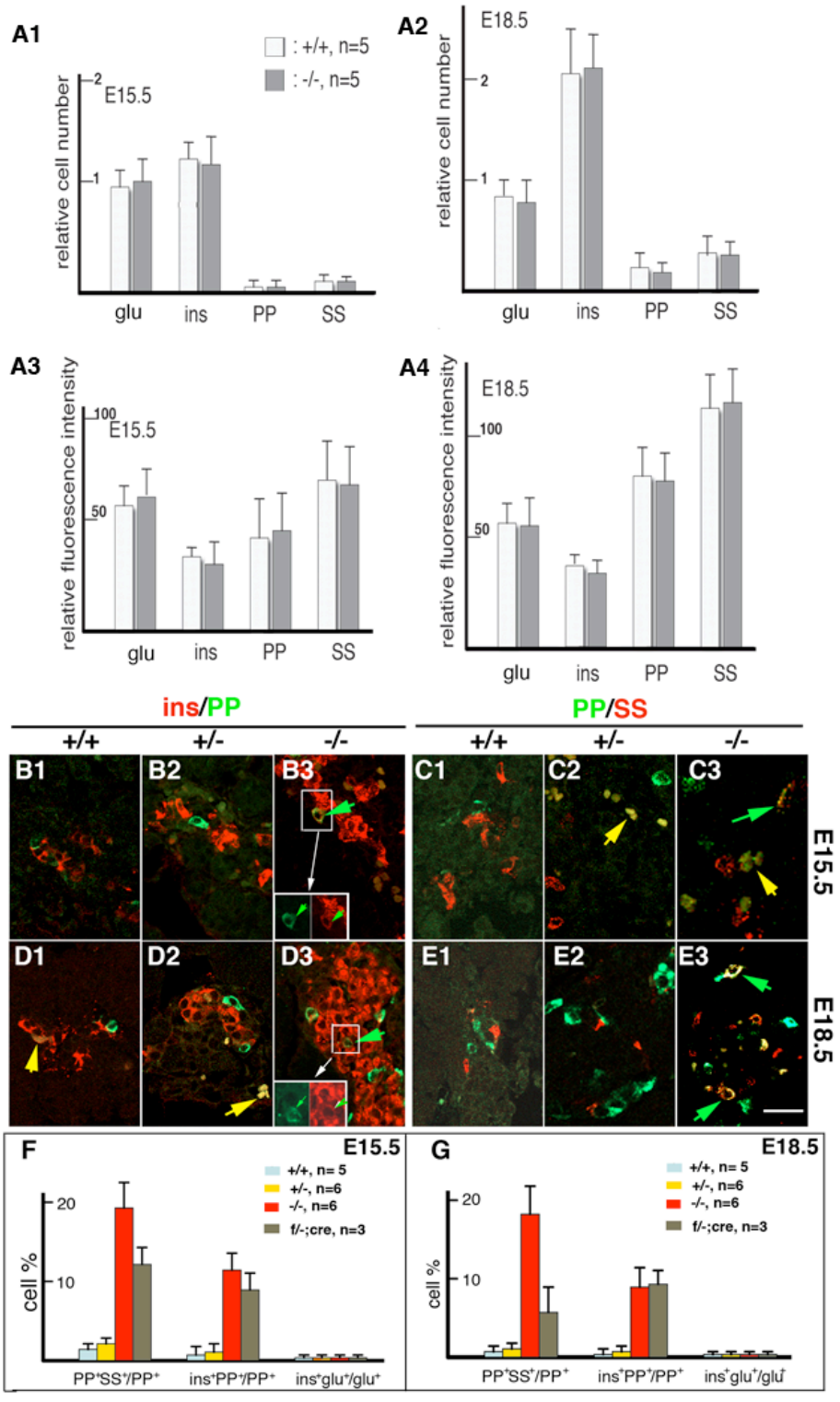


Figure 2.7 Diaphragms in *Myt1*$^{-/-}$ embryos are not properly innervated. Whole mount staining by mouse anti-β tubulin antibody. Images were light microscopy and confocal images (Z-stack). One animal of each genotype, wild type (+/+), heterozygous (+/−), and homozygous (−/−) was shown. (A), (B), and (C) represented the boxed region of the diaphragm shown in their corresponding color. Scale bars: 200μm.

Figure 2.8 *Myt1*$^{-/-}$ pancreata contain abnormal endocrine cells co-expressing multiple hormones. (A1-4) Individual endocrine hormone productions were normal in *Myt1*$^{-/-}$ pancreata (E15.5 and E18.5). For each stage, the relative number of endocrine cells (number of cells/area of the pancreas) and the relative hormone expression level (artificial level) within each cell were shown. (B1-3 & D1-3) PP and insulin co-staining were performed on E15.5 and E18.5 wile type, *Myt1*$^{+/-}$ and *Myt1*$^{-/-}$ pancreata. (C1-3 & E1-3) SS and PP co-staining were performed on E15.5 and E18.5 wild type, *Myt1*$^{+/-}$ and *Myt1*$^{-/-}$ pancreata. Yellow arrows: cells co-expressing two hormones. Yellow arrowhead: blood cells, which also show yellow color. Yet they can be distinguished from the double hormone expressing cells by their specific appearance. Insets in B3 and D3 showed boxed regions within each panel, split as green and red channels (green arrows). (F & G) The abundance of hormone co-expressing cells. The percentage of PP^+SS^+ or PP^+ins^+ cells over total PP^+ cells, and the percentage of ins^+glu^+ cells over total glu^+ cells were indicated in these charts. *f/-;Cre* refers to *Myt1*$^{fl/-}$*;Pdx1-Cre*. Ins: insulin. Glu: glucagon, ss: somatostatin, PP: pancreatic polypeptide. Scale bars: 20μm.


A1
: +/+, n=5
: -/-, n=5
E15.5
relative cell number
glu
ins
PP
SS
A2
E18.5
relative cell number
A3
E15.5
relative fluorescence intensity
A4
E18.5
relative fluorescence intensity
ins/PP
PP/SS
+/+
+/-
-/-
B1
B2
B3
C1
C2
C3
D1
D2
D3
E1
E2
E3
E15.5
E18.5
F
E15.5
+/+, n= 5
+/-, n=6
-/-, n=6
f/-;cre, n=3
cell %
PP+SS+/PP+
ins+PP+/PP+
ins+glu+/glu+
G
E18.5

cells in the adult *Myt1$^{fl/-}$;Pdx1-Cre* pancreas (Figure 2.9 A&B). Instead, most of the abnormal ins^{+} cells (19/21 ins^{+}PP^{+} and 26/26 glu^{+}ins^{+} cells) maintained MafB expression (Figure 2.9 B&D). These findings suggest that double-hormone positive cells, even though they express insulin, are not mature β cells.

Adult Myt1$^{fl/-}$;Pdx1-Cre male mice have impaired glucose tolerance and insulin secretion abilities

To investigate whether loss of *Myt1* compromises islet function, we examined the ability of adult *Myt1$^{fl/-}$;Pdx1-Cre* mice to maintain glucose homeostasis. *Myt1$^{fl/-}$;Pdx1-Cre* and wild type mice showed no difference in their fasting glucose levels. Yet intraperitoneal glucose tolerance test (IPGTT), a more sensitive assay for general islet function, revealed that *Myt1$^{fl/-}$;Pdx1-Cre* male mice developed glucose intolerance starting form 6 weeks of age (Figure 2.10 A). Consistent with this finding, 10-week old *Myt1$^{fl/-}$;Pdx1-Cre* males displayed attenuated glucose-induced insulin secretion. Thirty minutes after glucose challenge, serum insulin levels increased 100% in wild type male mice, but only increased 30% in *Myt1$^{fl/-}$;Pdx1-Cre* male mice (Figure 2.10 B), while serum glucagon levels altered similarly in these animals. Moreover, Glut2 protein levels were significantly reduced in *Myt1$^{fl/-}$;Pdx1-Cre* islets compared with that of wild type islets (Figure 2.10 C). Therefore, the attenuated insulin secretion and reduced Glut2 expression are likely responsible for the glucose intolerance in *Myt1$^{fl/-}$;Pdx1-Cre* male mice.

To further confirm the above finding, glucose-stimulated insulin secretion assay (GSIS) was performed on isolated 7-week and 12-week old *Myt1*$^{fl/-}$*;Pdx1-Cre* and wild type male islets (Figure 2.10 D). Seven-week-old *Myt1*$^{fl/-}$*;Pdx1-Cre* islets displayed no statistically significant reduction in GSIS compare with wild type islets, in that *Myt1*$^{fl/-}$*;Pdx1-Cre* males just started to become glucose intolerant at this stage. At 12 weeks of age, *Myt1*$^{fl/-}$*;Pdx1-Cre* islets showed statistically significant reduction in their ability to secrete insulin under glucose stimulation (Figure 2.10 D). Taken together, these data suggest that *Myt1* is required for the proper function of adult islets.

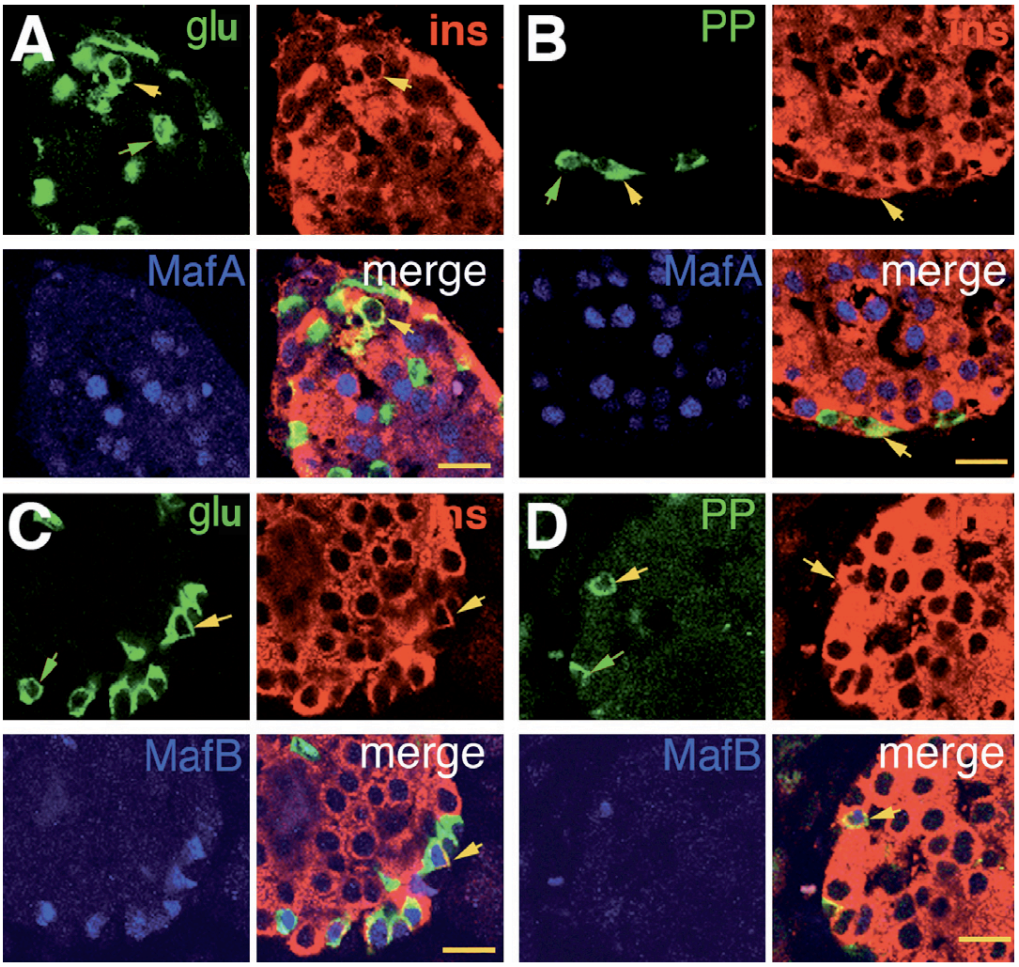


Figure 2.9 Insulin^{+} cells co-expressing PP or glucagon in adult *Myt1$^{fl/-}$;Pdx1-Cre* pancreata are not mature β cells. Adult *Myt1$^{fl/-}$; Pdx1-Cre* islets were co-stained with anti glucagon (glu), insulin (ins), PP, MafA and MafB antibodies. (A & C) Insulin and glucagon co-expressing cells were MafA^{-}MafB^{+} (yellow arrows). (B & D) Insulin and PP co-expressing cells were MafA^{-}MafB^{+} (yellow arrows). Green arrows in all panels: single hormone^{+} cells (internal controls). Scale bars: 10μm.

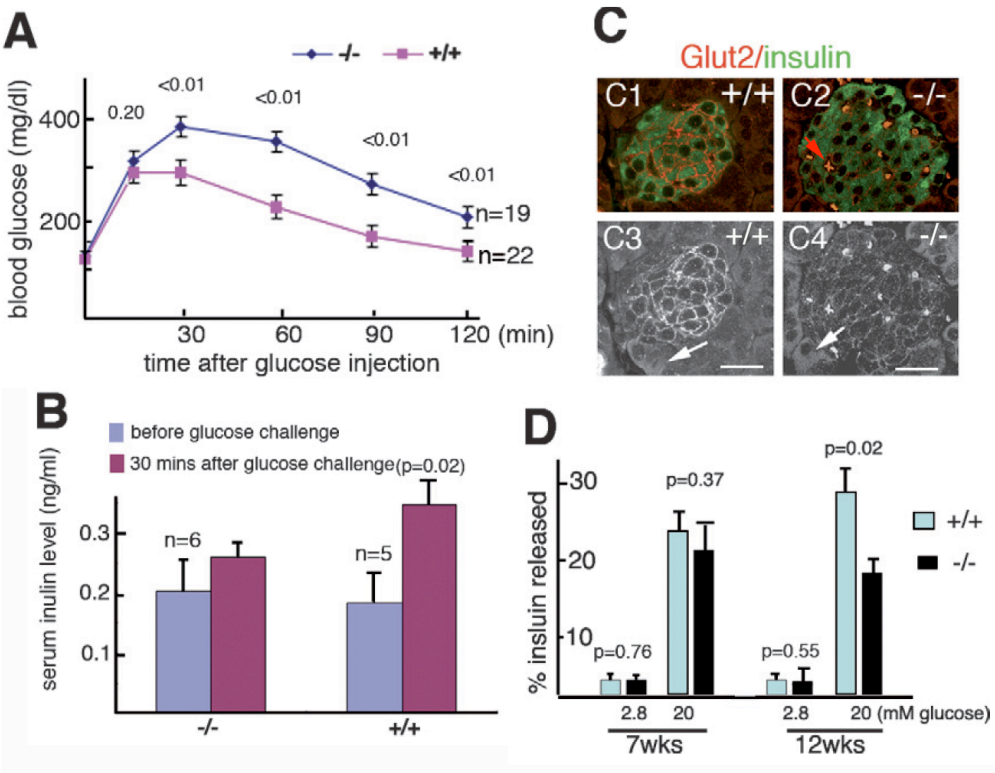


Figure 2.10. Adult *Myt1*$^{fl/-}$*;Pdx1-Cre* male mice develop impaired glucose tolerance. (A) IPGTT test (Materials and methods). Six to nine-week-old *Myt1*$^{fl/-}$*;Pdx1-Cre* male mice and wild tyte littermates were used in this test. P-values were marked above each data points. (B) Glucose-induced insulin release assay (Materials and methods). Ten-week-old *Myt1*$^{fl/-}$*;Pdx1-Cre* males and wild type littermates were used in this assay. The p-value was calculated based on the percentage of serum insulin increase of each animal, before and after glucose challenge. (C) Glut2 expression in 3-month-old *Myt1*$^{fl/-}$*;Pdx1-Cre* and wild type islets. Insulin staining was used to locate the islets. C3 and C4: red Glut2 channel in C1 and C2, respectively. White arrows: acinar cells that do not express Glut2. Red arrow in C2 and C4: blood cells. (D) GSIS in isolated islets from *Myt1*$^{fl/-}$*;Pdx1-Cre* and wild type males at 7 and 12 weeks of age. P-values were marked above each data set. Scale bars: 20μm.

Myt1L and Myt3 are up regulated in the absence of Myt1

Myt1$^{-/-}$*(or Myt1*$^{fl/-}$*;Pdx1-Cre)* pancreata with loss of *Myt1* function, displayed mild endocrine differentiation defects during pancreatic development, which is a weaker phenotype than the previous dominant negative based studies (Gu et al., 2004). We therefore examined whether the expression of *Myt1L* and *Myt3* is induced in the absence of *Myt1*, which may compensate for the loss of *Myt1* function. In fact, both *Myt1L* and *Myt3* mRNA were detected in *Myt1*$^{-/-}$, but not in wild type pancreata at E13.5, E14.5 and E15.5 (Figure 2.11). Interestingly, only Myt3 but not Myt1L protein could be detected in *Myt1*$^{-/-}$ pancreata, suggesting that either *Myt1L* is post-transcriptionally regulated in the pancreas or the Myt1L protein level is too low to be detected by our current antibodies. Nevertheless, since Myt1, Myt1L and Myt3 share high similarities regarding their protein structures, it is highly possible that Myt1L and/or Myt3 activity compensate for the loss of *Myt1* in the developing pancreas.

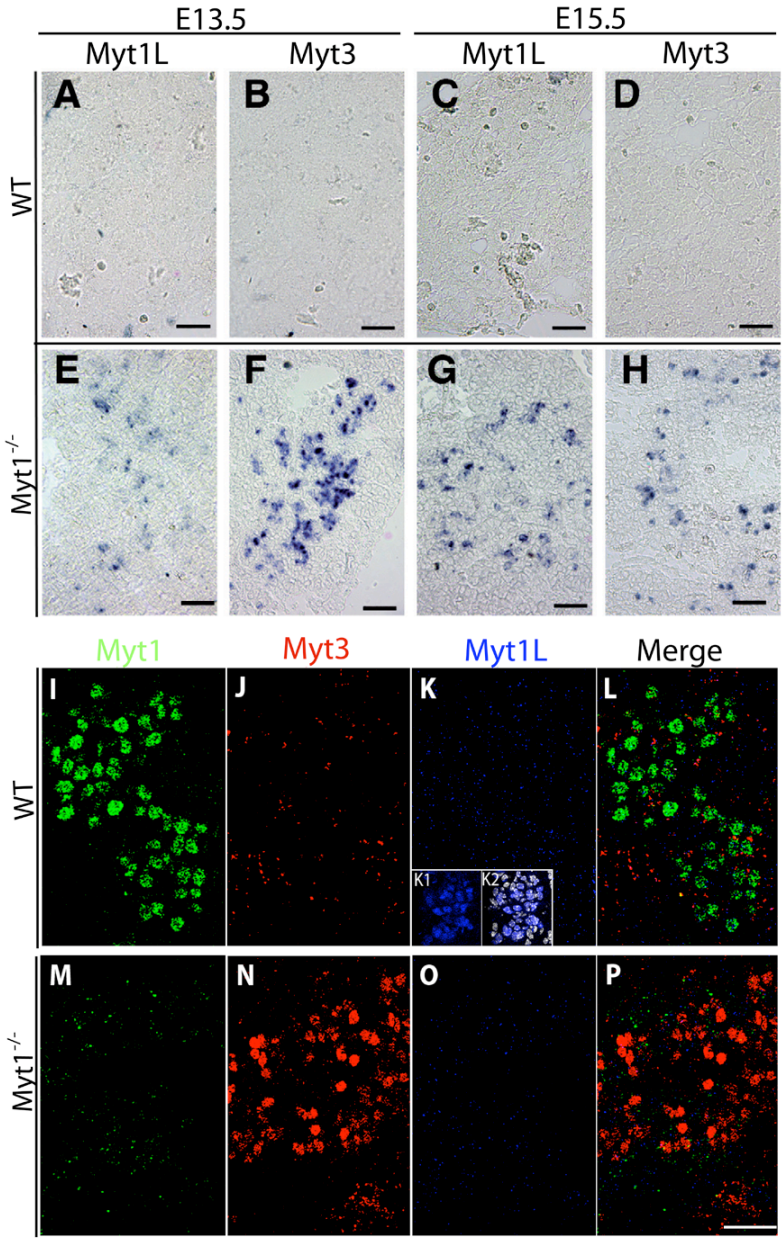


Figure 2.11. *Mty1L* and *Myt3* are expressed in the absence of *Myt1*. (A-H) *Myt1L* and *Myt3* transcripts were detected by *in situ* hybridization at E13.5 and E15.5. A-D: wild type pancreata; E-H: *Myt1*$^{-/-}$ pancreata. (I-P) Myt1L and Myt3 protein levels were examined by immunofluorescent staining at E17.5. I-L: wild type pancreata; M-P: *Myt1*$^{-/-}$ pancreata. K1 & K2: retina tissues of the same embryo were collected and stained at the same time. Myt1L was detected in the retina. White: nuclei; Green: Myt1, Blue: Myt1L; Red: Myt3. Scale bar: 20μm.

Discussion

The differentiation of endocrine progenitors into mature functional islet cells requires many signaling pathways and transcription factors. The zinc finger transcription factor Myt1 was previously identified as a factor that is specifically enriched and functioned in the endocrine pancreas (Gu et al., 2004). In this study, we investigated the roles of *Myt1* in the pancreas via loss-of-function analyses. Inactivation of *Myt1* partially compromises endocrine differentiation and islet function in mice, suggesting that *Myt1* plays important roles during endocrine differentiation and function.

Myt1 is involved in promoting monotypic production of endocrine cell types during pancreatic development

Myt1 is detected in $Pdx1^+$ pancreatic progenitor cells from the beginning of pancreatic development. Later, a portion of $Ngn3^+$ endocrine progenitors, as well as most of the hormone-expressing endocrine cells, expresses high levels of *Myt1*. These results suggest that *Myt1* may work in $Ngn3^+$ cells to promote endocrine differentiation. However, during embryogenesis, neither the number of $Ngn3^+$ endocrine progenitors nor the endocrine cell mass is significantly altered in $Myt1^{-/-}$ (*Myt1*$^{fl/-}$*;Pdx1-Cre*) pancreata compared with that of wild type controls. Instead, significant number of endocrine cell co-expressing insulin and PP (ins^+PP^+) or somatostatin and PP (ss^+PP^+) were found in the pancreas upon *Myt1* inactivation, indicating *Myt1* might help to maintain the cellular identity of

differentiated endocrine cells. Two hypotheses would help to explain the appearance of the multi-hormone-expressing cells.

First, it is possible that a transient PP^+ endocrine progenitor pool, which can give rise to ins^+ β cells or ss^+ δ cells in adult islets, exists during endocrine differentiation. Ins^+PP^+ and ss^+PP^+ cells could be intermediate states of these PP^+ endocrine progenitors while they undergo differentiation. When *Myt1* is inactivated, the differentiation process of PP^+ progenitors might be slowed down. In consistent with this explanation, it had been suggested that at least a portion of adult β cells is derived from PP-expressing progenitors (Herrera, 2000). Thus, *Myt1* may be involved in promoting PP^+ progenitors to quickly undergo differentiation.

Another possible explanation is that *Myt1* may be required in ins^+ β or ss^+ δ cells to suppress PP expression, or required in PP^+ cells to suppress insulin and somatostatin expression. Because the total number of PP^+ cells is not significantly altered between $Myt1^{-/-}$ and wild type pancreata, the latter situation is more likely the case. A temporally controlled lineage tracing of PP^+ cells may help to clarify these possibilities.

Myt1 is important for maintaining proper islet function

In the adult pancreas, *Myt1* is highly expressed in most of the mature islet cells. The glucose intolerance phenotype in the $Myt1^{fl/-}$*;Pdx1-Cre* mice was consistent with this hypothesis. Two reasons may contribute to these diabetic-related phenotypes in $Myt1^{fl/-}$*;Pdx1-Cre* animals.

First, the ins^{+} cells co-expressing PP or glucagon may interfere with the normal function of β cells in the adult *Myt1$^{fl/-}$;Pdx1-Cre* pancreas. Our results showed that these ins^{+}PP^{+} and ins^{+}glu^{+} cells in adult mutant pancreata express immature β cell marker MafB, instead of mature β cell marker MafA, suggesting they are immature cells and may not function normally. Furthermore, it has been suggested that individual β cells of a single islet are actively communicating and synchronized with their neighboring cells via gap junctions and intercellular signals (Konstantinova et al., 2007). A small number of abnormal performing β cells may dominantly hinder β cell communication and cause insulin secretion defects and glucose intolerance (Konstantinova et al., 2007). Therefore, even though the abnormal insulin expressing cells only represent a small portion of total β cells in *Myt1$^{fl/-}$;Pdx1-Cre* pancreata, they may significantly interfere with β cell function. In addition, while these ins^{+}PP^{+} cells were derived during embryogenesis, ins^{+}glu^{+} cells were only observed in significant numbers in adults. This result suggests that *Myt1* may also function to maintain at least the proper identity of a portion of β cells during adulthood.

Another possibility is that *Myt1* may actively regulate the expression of some factors that are essential for β cell function, such as Glut2. In adult *Myt1$^{fl/-}$; Pdx1-Cre* pancreata, Glut2 expression is significantly reduced compared with controls, suggesting that *Myt1* may be required to maintain Glut2 levels in the adult pancreas. Alternatively, it is also possible that Glut2 reduction is just a consequence but not a cause of hyperglycemia in *Myt1$^{fl/-}$;Pdx1-Cre* animals, as had been suggested by other groups (Thorens et al., 1990). Other factors, which

may potentially work downstream of *Myt1* in adult islets, still remain to be identified.

Gene compensation between Myt gene family members

An interesting finding of our studies is the gene compensatory effects between three *Myt* gene family members. When *Myt1* was deleted, *Myt1L* and *Myt3*, which are normally absent in the developing pancreas, were up-regulated. The underlying mechanisms of this phenomenon require further investigation. It is possible that *Myt1* may negatively regulate *Myt1L* and *Myt3* expression in a direct or indirect manner in wild type situation. In the absence of *Myt1*, the expression of *Myt1L* and *Myt3* is induced. A detailed examination of *Myt1* downstream targets and *Myt1L/Myt3* upstream regulators may shed light on this issue. Nevertheless, whether up-regulation of *Myt1L* or *Myt3* actually compensates for the loss of *Myt1* function remains unclear.

In summary, we showed that loss of *Myt1* partially compromises both the endocrine differentiation and islet function in the mouse pancreas. Two paralogs of *Myt1*, *Myt1L* and *Myt3*, which are not expressed in the developing pancreas, are induced upon *Myt1* deletion, and possibly compensate for *Myt1*'s function. Results from this study not only establish the basic role of *Myt1* in the pancreas, but also encourage us to further investigate how *Myt1* regulates endocrine differentiation and islet function mechanistically at the molecular level.

CHAPTER III

Myt1 AND Ngn3 FORM A FEED-FORWARD EXPRESSION LOOP TO PROMOTE ENDOCRINE ISLET CELL DIFFERENTIATION

This paper is published under the same title in *Developmental Biology*, 2008 (Wang et al., 2008b).

Abstract

The bHLH transcription factor Neurogenin 3 (Ngn3), which marks pancreatic endocrine progenitors, is essential for endocrine differentiation in the pancreas. Our previous studies showed that *Myt1 (Myelin transcription factor 1)* is expressed in a significant portion of $Ngn3^+$ endocrine progenitors during pancreatic development, suggesting it may play important roles in these endocrine progenitors. Here, we report that Myt1 can induce endocrine differentiation through enhancing *Ngn3* expression in the developing mouse pancreas. Vice Versa, Ngn3 also induces *Myt1* expression. Furthermore, a small number of cells expressing *Myt1* and endocrine hormone glucagon were found in *Ngn3*$^{-/-}$ pancreata, suggesting that *Myt1* can also function in an *Ngn3*-independent manner. Based on these results, we propose that Myt1 can help $Ngn3^+$ endocrine progenitor cells to enter endocrine differentiation pathways by forming a feed-forward expression loop with Ngn3 in the developing mouse pancreas.

Introduction

The bHLH transcription factor Ngn3 is both necessary and sufficient to initiate endocrine differentiation, and *Ngn3* expression marks the endocrine progenitors (Apelqvist et al., 1997; Gradwohl et al., 2000; Gu et al., 2002). Previously, we reported that a significant portion of $Ngn3^+$ endocrine progenitors expresses *Myt1* (Wang et al., 2007). This result encouraged us to further explore how *Myt1* functions in endocrine progenitors.

In *Xenopus* embryos, *xMyt1* (homolog of *Myt1* in *Xenopus*) promotes ectopic neuronal differentiation in an *xNgnr1* (homolog of *Ngn3* in *Xenopus*) dependent fashion during neurogenesis (Bellefroid et al., 1996). Intriguingly, while *xNgnr1* over-expression alone cannot reverse the inhibitory effects of activated Notch signaling on neuronal differentiation, *xMyt1* and *xNgnr1* together can induce ectopic neural differentiation even in the presence of activated Notch signaling (Bellefroid et al., 1996). This result suggests that xMyt1 may help to modify the competence of $xNgnr1^+$ cells to overcome Notch-mediated lateral inhibition and undergo differentiation.

In chicken studies, *Myt1* (specifically *Myt1b*) can induce the ectopic differentiation of glucagon and somatostatin-expressing endocrine cells in the hindgut epithelium (Gu et al., 2004). A dominant negative Myt1 (without the transactivation domain) significantly impairs the ability of Ngn3 to induce ectopic endocrine differentiation (Gu et al., 2004). Moreover, Myt1 is able to partially antagonize the repressive effects of activated Notch signaling on Ngn3 function (Ahnfelt-Ronne et al., 2007).

These data indicate that Myt1 alone can promote the differentiation of various progenitor cell types, and probably does so by interacting with Ngn3 either directly or indirectly. However, the difficulty to precisely control the timing and levels of ectopic gene expression, as well as the lack of loss-of-function assays in chicken embryos, strongly hinders our ability to understand the function of *Myt1* in $Ngn3^+$ endocrine progenitors. Mouse models were used here to determine the underlying mechanisms regarding Myt1's ability to drive ectopic endocrine differentiation and its relationship with Ngn3.

We found that over-expression of *Myt1b*, which is the predominantly expressed *Myt1* isoform in the mouse pancreas, significantly induces precocious endocrine differentiation during embryogenesis. Moreover, the ability of Myt1b to induce endocrine differentiation is *Ngn3*-dependent. Increased Myt1b levels can enhance *Ngn3* expression. Vise Versa, Ngn3 also induces *Myt1* expression. Furthermore, glucagon-expressing cells were detected in *Ngn3* deficient embryonic pancreata, most of which co-express *Myt1*. These findings suggest that Myt1 and Ngn3 form a feed-forward expression loop to promote endocrine differentiation in the pancreas.

Materials and Methods

1. Mouse strains and care

Mouse production and care follow standard protocols approved by the Vanderbilt Medical Center IACUC. For embryonic staging, the noon of vaginal

plug appearance was counted as embryonic day 0.5 (E0.5). For routine mouse embryo production, the CD1 (ICR) mouse strain was utilized (Charles River Laboratories, Inc., Wilmington, MA). For transgenic mouse production, B6/D2 mice were used (Charles River Laboratories, Inc. Wilmington, MA). Subsequent strain maintenance and crosses utilized CD1 mice. The *Pdx1*$^{tTA/+}$ mouse strain was described previously (Holland et al., 2002). Genotyping followed published methods.

2. DNA construct and transgenic animal derivation

To over-express *Myt1b*, a mini-gene containing intron # 6 of the *Myt1b* transcript was constructed using PCR and conventional molecular cloning. The final construct included the full *Myt1b* open reading frame with minimal 5′UTR and 3′UTR. *TetO-CMV* promoter was PCR-amplified from *pTRE2* (Clontech, Paolo Alto, CA) and inserted in front of the *Myt1b* minigene. A *SV40 PolyA* signal was then inserted to the 3′ end of the *Myt1b* minigene to complete the construction. A similar approach was utilized for construction of *tetO-Ngn3*. Transgenic animal production followed standard pronuclear injection method. Genotyping utilized PCR-based technique. For *tetO-Myt1b* mouse lines: 5′-GCGTGTACGGTGGGAGGCCTATAT-3′ and 5′-ACTCTGTAAGCTTCGATGTCT GGA-3′. The expected fragment is 360 bp. For *tetO-Ngn3* mouse lines: 5′-GCGTGTACGGTGGGAGGCCTATAT-3′ and 5′-GGGTGGAATTGGAACTGAGCACT-3′. The expected fragment is 300 bp.

3. Immunohistochemistry/immunofluorescence (IHC/IF)

Immunofluorescence/immunohistochemistry followed established protocols. Tissues were stained either as frozen sections or paraffin sections. For frozen sections, dissected tissues were immediately frozen in OCT and then sectioned. Sectioned tissues were collected onto Superfrost-plus slides, left at room temperature for 30 min, and fixed in 4% paraformaldehyde at room temperature for 20 min. Slides were washed in PBS, permealized in 0.2% triton X-100, and immunostained. For paraffin sections, tissues were fixed in 4% paraformaldehyde overnight at 4 °C or 4 h at room temperature. Dehydration and section followed routine procedures. Primary antibodies used were: guinea pig anti-glucagon (Dako, Carpinteria, CA), Rabbit anti-MafB, gifts from R. Stein (Matsuoka et al., 2004), goat anti-Pdx1, a gift from C. V. Wright, guinea pig anti-Ngn3, a gift from M. Sander (Seymour et al., 2007), rabbit anti-Ngn3 (Gu et al., 2002), rabbit anti-Myt1 (Wang et al., 2007), mouse anti-Nkx6.1, mouse anti-Pax6 (Development Hybridoma Bank, University of Iowa, Iowa, IA) and rabbit anti-PC1/3 (Chemicon, Temetula, CA). Secondary antibodies used were: FITC-conjugated donkey anti-rabbit IgG; Cy3-conjugated donkey anti-rabbit IgG, Cy3-conjugated donkey anti-mouse IgG, Cy3-conjugated donkey anti-guinea pig IgG, Cy3-conjugated donkey anti-goat, and Cy5-conjugated donkey anti-rabbit (Jackson Immunoresearch, West Grove, PA). All antibodies utilized 1:500–1:2000 dilutions.

4. Microscopy and statistical analysis

All fluorescent images were obtained by confocal microscopy. For quantification of glucagon expression and *Myt1b* ectopic expression, samples were frozen, sectioned at 15μm intervals, and stained for hormone and transcription factor expression. Confocal optical sections were taken at 0.4–0.6μm intervals on all stained tissues. For *Myt1b* level estimation in each cell, all optical sections for individual nucleus are projected to one picture. The fluorescence intensity within each nucleus was then compared using BIOQUANT true color windows system (R&M Biometrics, Nashville, TN). For quantification of Nkx6.1 and MafB expression, paraffin-sections at 6μm intervals were utilized. In this case, only one optical section of each tissue section was counted. Statistical analyses utilized standard student t-test. P-values of 0.05 or less were considered as significant. All quantification data are presented as the mean ± standard error over the mean.

Results

Derivation of transgenic mouse lines that express Myt1b in a tTA-dependent manner

To address whether *Myt1* is sufficient to induce pancreatic endocrine differentiation, well-established inducible Tet-Off system was used to over-express the predominant *Myt1* isoform *Myt1b* in pancreatic progenitor cells (Berens and Hillen, 2003; Gossen et al., 1994). Specifically, transgenic mouse

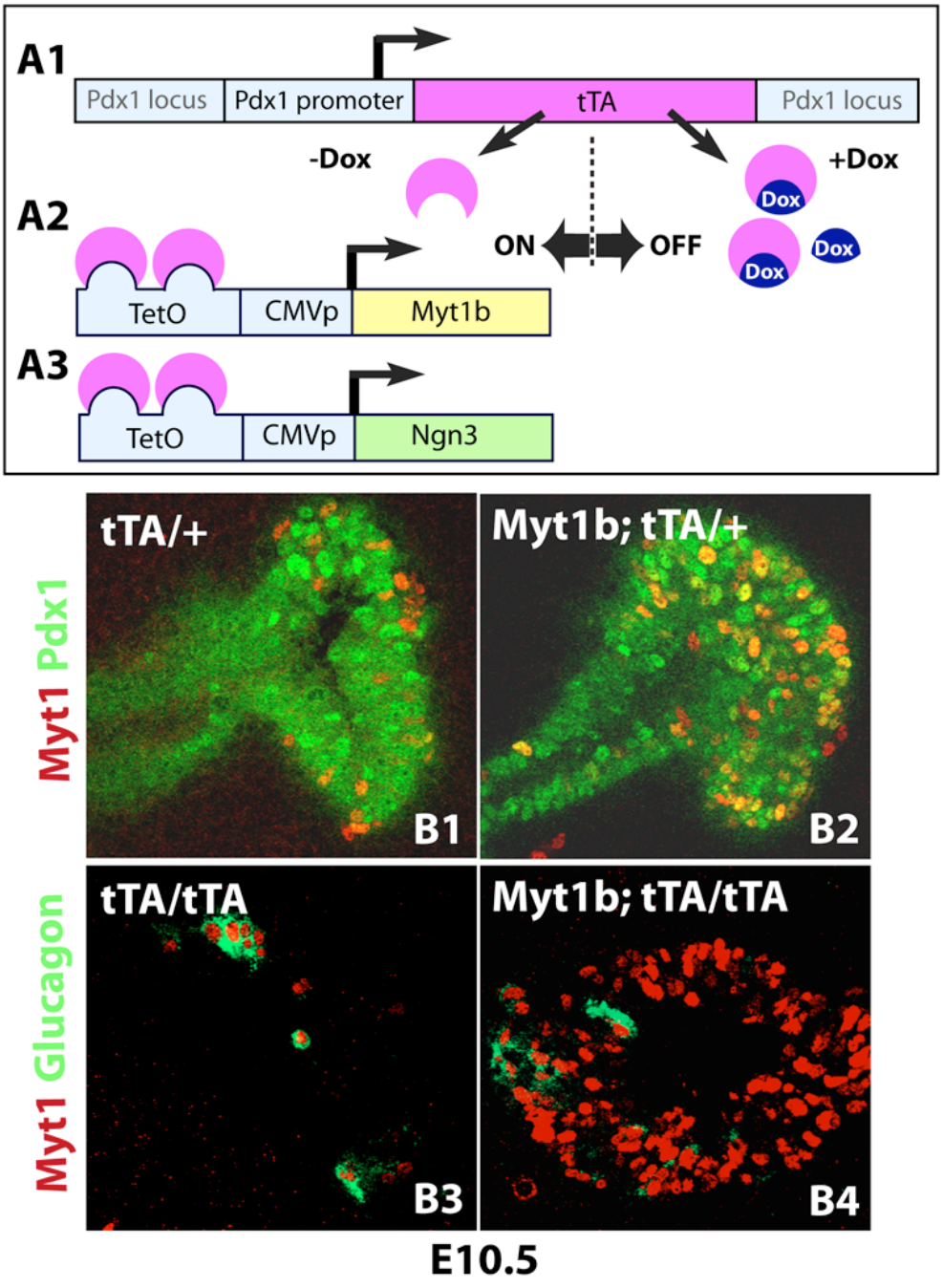
lines that express *Myt1b* under a *TetO-CMV* synthetic promoter was generated, and termed *Myt1*^{tet} for simplicity (Figure 3.1 A2). *Myt1*^{tet} can only be activated in the presence of the transactivator tTA when tetracycline or doxycycline (Dox) is not provided (Gossen et al., 1994). The advantage of using this approach is that it can avoid the possible lethality caused by ectopic *Myt1b* over-expression in pancreatic cells and allow for the derivation of stable mouse lines. A knock in mouse line expressing *tTA* under the *Pdx1* promoter (*Pdx1*$^{tTA/+}$) was utilized to induce *Myt1b* expression in pancreatic progenitor cells (Figure 3.1A1) (Holland et al., 2002). Compound *Myt1*^{tet}*;Pdx1*$^{tTA/+}$ animals were expected to ectopically express *Myt1b* in most, if not all, of the Pdx1^{+} pancreatic progenitors .

Seven *Myt1*^{tet} independent transgenic mouse lines were derived through pronuclear injection. As expected, none of these lines ectopically expressed the transgene in the absence of tTA proteins. One of these *Myt1*^{tet} lines, *Myt1*^{tet2}*,* when combined with *Pdx1*$^{tTA/+}$ (*Myt1*^{tet2}*;Pdx1*$^{tTA/+}$, without Dox), produced Myt1 protein in 20.5±9.5% (n=6) of Pdx1^{+} cells in E10.5 mouse pancreata, whereas only 7.3±0.8% (n=5) of Pdx1^{+} cells expressed detectable levels of Myt1 in *Pdx1*$^{tTA/+}$ controls, which showed no difference in comparison with wild type (WT) (Figure 3.1 B1-2). Moreover, Myt1 was produced at comparable levels in *Myt1*^{tet2}*;Pdx1*$^{tTA/+}$ and *Pdx1*$^{tTA/+}$ pancreata on per cell basis, as judged by fluorescence intensity within each nuclei during side-by-side immunofluorescence staining (Figure 3.1 B1-2 & Materials and Methods). The *Myt1*^{tet2} line, which showed the highest over-expression efficiency compared with the other six lines, was therefore used in all the following experiments.

Myt1^{tet2} was also crossed into a homozygous *Pdx1*$^{tTA/tTA}$ background, in which no functional Pdx1 protein exists, but higher levels of tTA were expected. In the *Myt1*^{tet2}*;Pdx1*$^{tTA/tTA}$ pancreas, *Myt1* was ectopically expressed in most, if not all, of the cells in the pancreatic epithelium, suggesting that a higher Myt1 production can be achieved by increasing tTA levels (Figure 3.1 B3-4).

In addition, we examined whether the *Myt1*^{tet2} transgene could be activated at different developmental stages by controlling the availability of Dox. *Myt1*^{tet2}*;Pdx1*$^{tTA/+}$ embryos were exposed to Dox (Dox was added into the drinking water of pregnant females) starting from E7.5, right before the initiation of pancreatic development to repress tTA function. Dox was then withdrawn from the embryos to allow for the activation of the *Myt1*^{tet2} transgene at E12.5 or E18.5. The expression of *Myt1* in *Myt1*^{tet2}*;Pdx1*$^{tTA/+}$ and *Pdx1*$^{tTA/+}$ control pancreata was examined and compared at E15.5 or postnatal day 1 (P1), respectively. Other than a few differentiated islet cells expressing higher levels of *Myt1*, there was no significant difference between *Myt1*^{tet2}*;Pdx1*$^{tTA/+}$ and *Pdx1*$^{tTA/+}$ controls regarding the percentage of Pdx1^{+} cells that express *Myt1*. Thus, the low efficiency of our over-expression system prevented us from studying the effects of *Myt1b* over-expression in a temporally controlled manner.

Figure 3.1 Derivation of transgenic mouse lines that ectopically express *Myt1b*. (A1) The published *Pdx1*^{tTA} allele (Holland et al., 2002). *tTA* was inserted into the *Pdx1* locus. This allele therefore equaled to a *Pdx1*$^{-}$ allele. In the absence of Dox (Doxycycline), tTA can bind the *teto* operator sequence, and activate gene transcription. When Dox is added into the system, it bind to tTA and prevents tTA from behaving as a transactivator. (A2) The structure of the *Myt1*^{tet} transgene. The expression of *Myt1b* was driven by the *CMV* promoter, which is under the control of the tetO operator sequence. (A3) The structure of the *Ngn3*^{tet} transgene. (B) Pancreatic *Myt1b* expression at E10.5. *Pdx1*$^{tTA/+}$ pancreata (label as tTA/+, B1), which showed identical *Myt1* expression pattern as *WT*, and *Pdx1*$^{tTA/tTA}$ (label as tTA/tTA, B3) pancreata were used as controls. The presence of *Myt1*^{tet2} (label as Myt1b, B2&B4) led to *Myt1b* ectopic expression in the absence of Dox. Pdx1 (green) or glucagon staining (green) were used to mark the pancreatic regions. Note that Myt1 antibodies only stain frozen, lightly fixed sections, on which the Pdx1 signal appeared diffuse.


A1
Pdx1 locus
Pdx1 promoter
tTA
Pdx1 locus
-Dox
+Dox
Dox
Dox
Dox
A2
ON
OFF
TetO
CMVp
Myt1b
A3
TetO
CMVp
Ngn3
Myt1 Pdx1
tTA/+
Myt1b; tTA/+
B1
B2
Myt1 Glucagon
tTA/tTA
Myt1b; tTA/tTA
B3
B4
E10.5

Myt1b is sufficient to induce endocrine differentiation in pancreatic cells

Myt1^{tet2}*;Pdx1*$^{tTA/+}$ animals were derived via standard genetic crosses in the absence of Dox, which allows for the ectopic expression of *Myt1b* in pancreatic progenitors as soon as *Pdx1* expression is activated at around E8.5 (Holland et al., 2002). Major hormone expression (insulin, glucagon, somatostatin and PP) was examined to determine whether *Myt1b* is sufficient to induce endocrine differentiation at early stages (E10.5 and E11.5), when glucagon-expressing cells are the dominant endocrine subtypes produced under normal conditions. In *Myt1*^{tet2}*;Pdx1*$^{tTA/+}$ pancreata, the number of glucagon^{+} cells, but not other endocrine cell types, was significantly increased compared with that of *Pdx1*$^{tTA/+}$ pancreata at E11.5 (Figure 3.2 A). This phenotype caused by ectopic *Myt1b* expression is similar to that induced by ectopic *Ngn3* over-expression, suggesting that additional factors other than Myt1b and Ngn3 are necessary for the induction of other endocrine cell types (Schwitzgebel et al., 2000).

At later stages (E15.5 & E18.5), the number of hormone expressing endocrine cells produced in *Myt1*^{tet2}*;Pdx1*$^{tTA/+}$ pancreata was comparable with that in *Pdx1*$^{tTA/+}$ controls. This is consistent with the finding that ectopic *Myt1b* expression can not be achieved at stages later than E13.5, which makes it impossible to determine whether *Myt1b* is sufficient to induce endocrine differentiation at later developmental stages.

We also examined whether *Myt1b* can induce endocrine differentiation in a *Pdx1*-independent manner by examining *Myt1*^{tet2}*;Pdx1*$^{tTA/tTA}$ and control *Pdx1*$^{tTA/tTA}$ pancreata. The number of glucagon^{+} cells increased about 1.8 fold in

Myt1^{tet2};*Pdx1*$^{tTA/tTA}$ pancreata compared with *Pdx1*$^{tTA/tTA}$ controls at E11.5 (Figure 3.2 A, D&E), suggesting that *Myt1b* is sufficient to induce endocrine differentiation in the absence of *Pdx1*.

Unlike endodermal cells that ectopically expressing *Ngn3*, which largely differentiated into endocrine cells, only about half of the Myt1^{+} cells (n=6) in the *Myt1*^{tet2};*Pdx1*$^{tTA/tTA}$ pancreas activated glucagon expression (Figure 3.2 B-E) (Apelqvist et al., 1999) (Schwitzgebel et al., 2000). By E18.5, glucagon^{+} cells could rarely be detected in *Myt1*^{tet2};*Pdx1*$^{tTA/tTA}$ pancreata. This finding indicates that without *Pdx1*-dependent cell expansion or survival, early glucagon^{+} cells may have died or have trans-differentiated into other cell types, possibly due to the lack of paracrine signals produced by Pdx1^{+} cells or other unknown reasons (Jonsson et al., 1994; Offield et al., 1996).

Myt1b-induced glucagon-expressing cells express immature α cell markers

During pancreatic development, the endocrine cells produced from E9.0 to E12.5 are mostly glucagon-expressing α cells (Jensen et al., 2000a). It had been suggested that these cells might not significantly contribute to the adult islet pool, in that their production does not require *Pdx1* activity (Herrera, 2000) (Offield et al., 1996). Moreover, they express Prohormone Convertase 1/3 (PC1/3), which is not maintained in mature adult α cells (Wilson et al., 2002). These early glucagon-expressing cells are therefore generally considered as immature α cells.

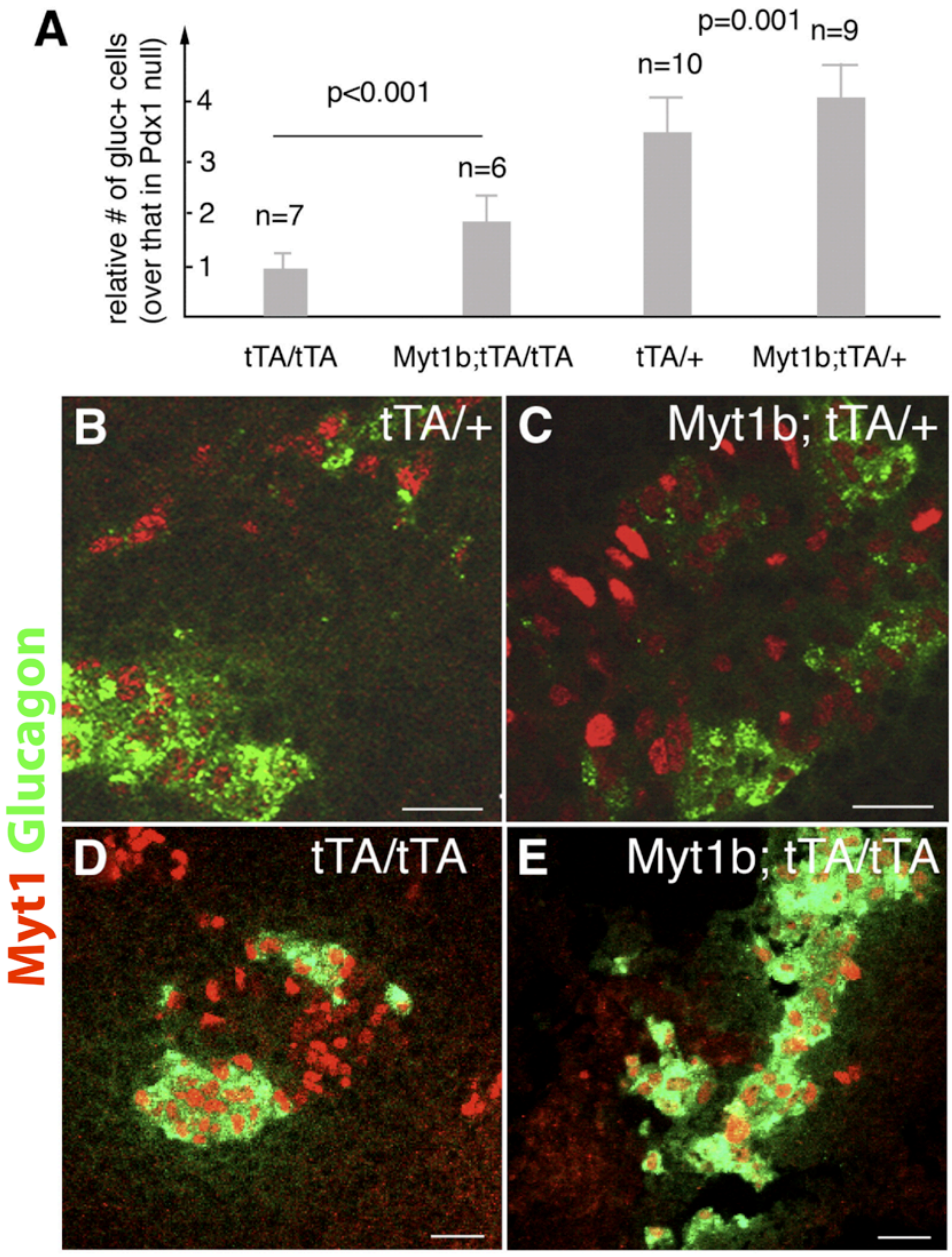


Figure 3.2 Ectopic *Myt1b* expression induces glucagon expression. (A) The relative number (mean ± S.E.M.) of glucagon-expressing cells in E11.5 pancreata (tTA/+: *Pdx1*$^{tTA/+}$. Myt1b;tTA/+: *Myt1*^{tet2};*Pdx1*$^{tTA/+}$. tTA/tTA: *Pdx1*$^{tTA/tTA}$. Myt1b;tTA/tTA: *Myt1*^{tet2};*Pdx1*$^{tTA/tTA}$). (B–E) Myt1 and glucagon production in representative sections of E11.5 pancreata. Scale bars: 20μm.

To determine whether *Myt1b*-induced glucagon-expressing cells are mature α cells, the expression of *PC1/3* and other known α cell markers, including *MafB* and *Nkx6.1*, was carefully examined (Artner et al., 2007; Henseleit et al., 2005; Wilson et al., 2002). Most, if not all, glucagon-expressing cells in the $Myt1^{tet2};Pdx1^{tTA/tTA}$ or $Myt1^{tet2};Pdx1^{tTA/+}$ pancreas expressed PC1/3 and MafB at E11.5 (Figure 3.3), suggesting that *Myt1b* over-expression is sufficient to activate MafB expression but fails to induce mature α cells. Interestingly, there was a 273±160% increase of the number of $Nkx6.1^{+}$ cells in the $Myt1^{tet};Pdx1^{tTA/tTA}$ pancreas over that of $Pdx1^{tTA/tTA}$ controls (Figure 3.4). Nkx6.1/Nkx6.2 were reported to be required for strong *Myt1* expression and α cell differentiation (Henseleit et al., 2005). Thus, it is highly possible that a feedback activation link between Myt1 and Nkx6.1/Nkx6.2 transcription factors exists in the developing pancreas. This hypothesis is also consistent with the finding that *Nkx6.1* expression is maintained in glucagon expressing cells in $Pdx1^{-/-}$ pancreata (Pedersen et al., 2005).

Myt1b induces endocrine differentiation through Ngn3

Ngn3, which is well accepted as a pancreatic endocrine progenitor marker, is both necessary and sufficient for endocrine differentiation in the mouse pancreas. We examined whether *Myt1b* induced endocrine differentiation is dependent on *Ngn3*.

A targeted conditional *Ngn3* allele, which has the *Ngn3* coding region flanked by two LoxP sites to potentially produce an *Ngn3* null allele, had been generated in the lab (Figure 3.5 A-C). $Myt1^{tet2};Pdx1^{tTA/tTA};Ngn3^{-/-}$ embryos were

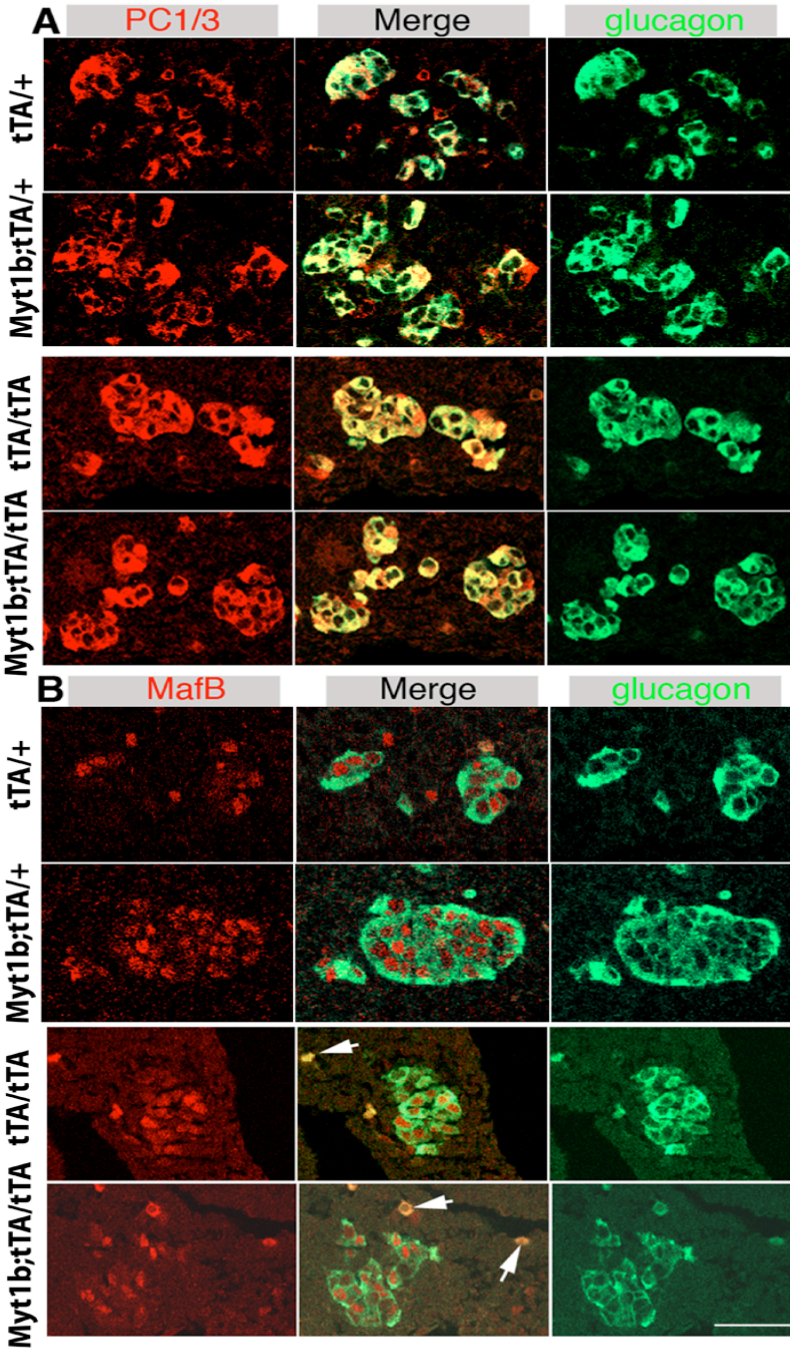


Figure 3.3 *Myt1b* induced glucagon-expressing cells express PC1/3 and MafB. (A) Co-expression of glucagon and PC1/3 in E12.5 pancreata. (B) Co-expression of MafB and glucagon in E12.5 pancreata. tTA/+: *Pdx1*$^{tTA/+}$. Myt1b;tTA/+: *Myt1*^{tet2};*Pdx1*$^{tTA/+}$. tTA/tTA: *Pdx1*$^{tTA/tTA}$. Myt1b;tTA/tTA: *Myt1*^{tet2};*Pdx1*$^{tTA/tTA}$. White arrows: blood cells. Scales bar: 20μm.

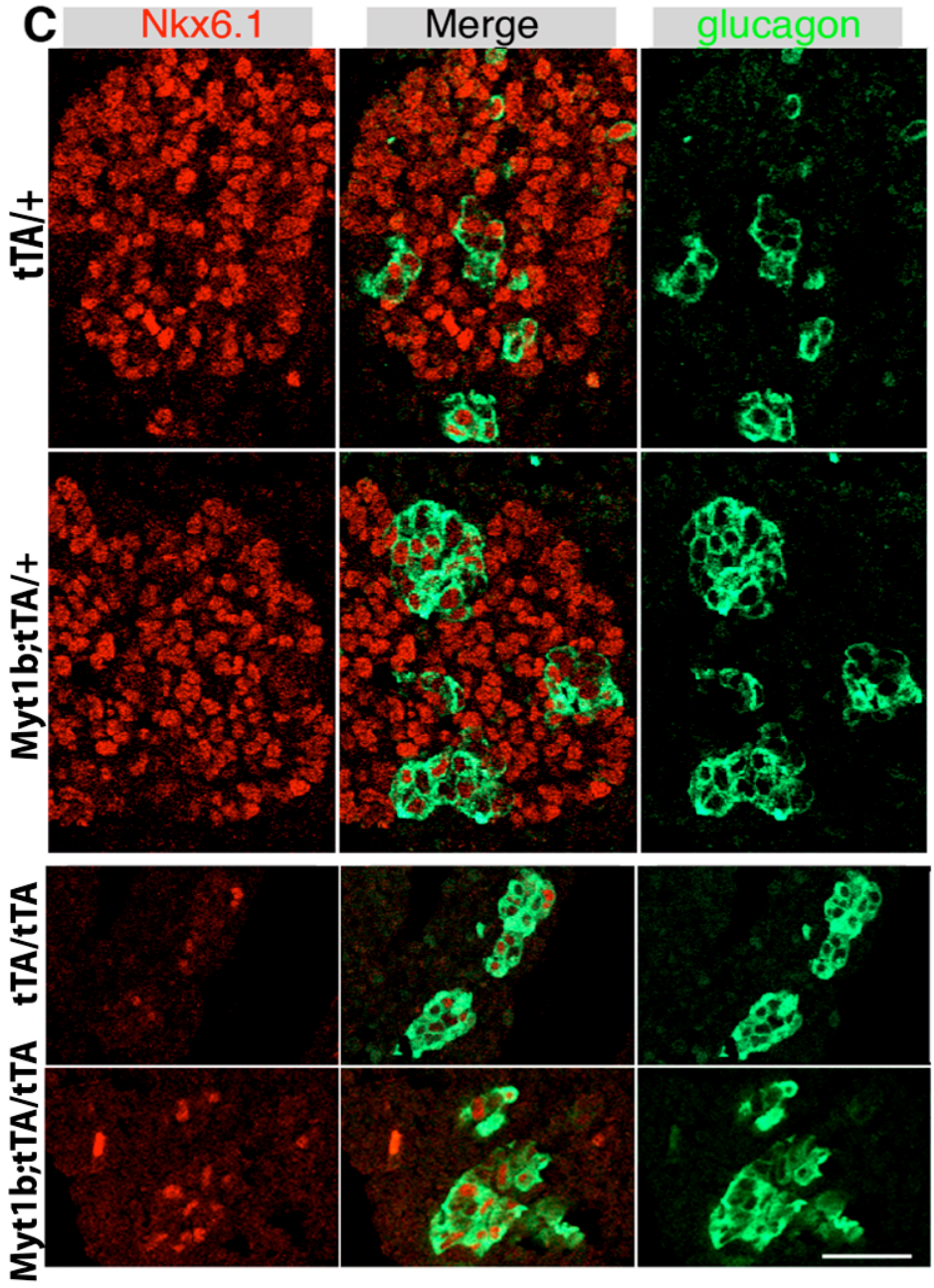


Figure 3.4 *Myt1b* induced glucagon-expressing cells express Nkx6.1. tTA/+: *Pdx1*$^{tTA/+}$. Myt1b;tTA/+: *Myt1*^{tet2};*Pdx1*$^{tTA/+}$. tTA/tTA: *Pdx1*$^{tTA/tTA}$. Myt1b;tTA/tTA: *Myt1*^{tet2};*Pdx1*$^{tTA/tTA}$. Scales bar: 20 μm.

obtained, in which *Myt1b* was ectopically expressed in the *Ngn3* nullizygous background. Compared with *Myt1*^{tet2};*Pdx1*$^{tTA/tTA}$ controls, almost no glucagon-expressing cells were induced by *Myt1b* in *Myt1*^{tet2};*Pdx1*$^{tTA/tTA}$;*Ngn3*$^{-/-}$ pancreata (Figure 3.5 D1-2). This result not only provided strong genetic evidence that *Ngn3* is required for *Myt1b*-induced endocrine differentiation, but also encouraged us to investigate whether *Myt1* induces endocrine differentiation by activating *Ngn3* expression.

Ngn3 expression was examined in *Myt1*^{tet2};*Pdx1*$^{tTA/tTA}$, *Pdx1*$^{tTA/tTA}$, *Myt1*^{tet2};*Pdx1*$^{tTA/+}$ and *Pdx1*$^{tTA/+}$ pancreata. At E10.25, the number of Ngn3^{+} cells in *Myt1*^{tet2};*Pdx1*$^{tTA/+}$ pancreata increased by 136±22% (n=3) over that of *Pdx1*$^{tTA/+}$ controls. Enhanced *Ngn3* expression was observed in *Myt1*^{tet2};*Pdx1*$^{tTA/tTA}$ pancreata as well. At E9.5, *Myt1*^{tet2};*Pdx1*$^{tTA/tTA}$ pancreata contained visibly more Ngn3^{+} cells than *Pdx1*$^{tTA/tTA}$ controls (Figure 3.5 E1-4). At E10.25, *Pdx1*$^{tTA/tTA}$ pancreata produced no more than two Ngn3^{+} cells (n=10), yet *Myt1*^{tet2};*Pdx1*$^{tTA/tTA}$ pancreata produced an average of 12 Ngn3^{+} cells per pancreas (n=10). These data suggest that *Myt1b* promotes endocrine differentiation by enhancing *Ngn3* expression, even in the absence of *Pdx1*.

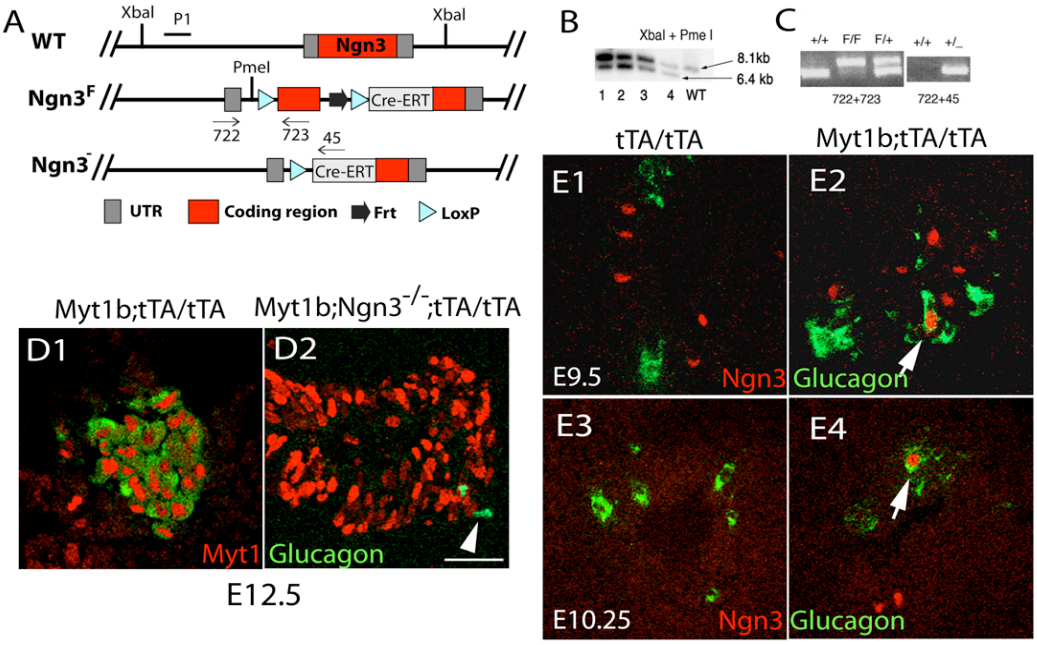


Figure 3.5 *Myt1b* induces glucagon expression through *Ngn3*. (A, B&C) The production and genotyping strategies of a novel *Ngn3* null allele (*Ngn3*$^{-}$). (A) The structure of the *Ngn3* wild type, floxed (*Ngn3*^{F}) and null alleles. A *PmeI* site was introduced into the 5′ end of the *Ngn3* coding cDNA in the *Ngn3*^{F} allele. Note that the *Ngn3*$^{-}$ allele produced CreER™, a feature that was not used in this study. The numbers (45, 722, and 723) and small arrows indicated the position of oligos used for PCR-based genotyping. (B) Southern blot identified four targeted ES cell clones, producing a 8.1 kb wild type and a 6.4 kb mutant band when digested with *XbaI* + *PmeI* and blotted with P1 probe. (C) Genotyping results with 722+723 and 722+45 primers. (D1-2). *Myt1b*-induced glucagon expression in pancreata with or without *Ngn3* at E12.5. Note the rare glucagon^{+} cells (green, white arrowhead) in the *Ngn3*$^{-/-}$ background. Red: Myt1; green: glucagon. (E1-4) Ngn3 production in pancreata ectopically expressing *Myt1b*. Two stages, E9.5 (E1-2) and E10.25 (E3-4), were shown. Note the presence of glucagon^{+}Ngn3^{+} cells (white arrows) in pancreata with ectopic *Myt1b* expression. Red: Ngn3; green: glucagon. tTA/tTA: *Pdx1*$^{tTA/tTA}$. Myt1b;tTA/tTA: *Myt1*^{tet2};*Pdx1*$^{tTA/tTA}$. Scales bar: 20μm.

Ngn3 induces Myt1 expression

Previous *Xenopus* studies have suggested that *xNgnr1* (homolog of *Ngn3*) positively regulates *Myt1* expression (Bellefroid et al., 1996). We therefore checked whether Ngn3 could activate *Myt1* expression in the mouse pancreas.

The same strategy was used to ectopically over-expressing *Ngn3* in $Pdx1^{+}$ pancreatic progenitor cells (Figure 3.1 A3). Seven $Ngn3^{tet}$ transgenic lines were obtained and showed wide spread ectopic *Ngn3* expression when combined with the $Pdx1^{tTA}$ allele. Consistent with published data, ectopic *Ngn3* expression induced precocious differentiation of glucagon-expressing cells after E11.5 in $Ngn3^{tet};Pdx1^{tTA/+}$ pancreata (Schwitzgebel et al., 2000). Moreover, most of the $Ngn3^{+}$ cells in the $Ngn3^{tet};Pdx1^{tTA/+}$ pancreas appeared to have delaminated from the epithelium, as had been reported in previous findings that Ngn3 could induce epithelial-to-mesenchymal transition (Ahnfelt-Ronne et al., 2007; Apelqvist et al., 1999; Grapin-Botton et al., 2001; Schwitzgebel et al., 2000). One of the transgenic lines, $Ngn3^{tet8}$, that produced Ngn3 protein in a comparable level to that of wild type littermates on a per cell basis, was used in the following studies (Figure 3.6 A&B).

In the $Ngn3^{tet8};Pdx1^{tTA/+}$ pancreas, where *Ngn3* was ectopically expressed in most, if not all, of the $Pdx1^{+}$ pancreatic progenitors at early embryonic stages, Myt1 production was dramatically increased (Figure 3.6 C&D). Consistent with this observation, we found that *Ngn3* can also directly activate *Myt1* promoter in different cell lines *in vitro* (Wang et al., 2008b). These results suggest that Ngn3 induces *Myt1* expression in the developing mouse pancreas.

Myt1b expression is largely but not totally dependent on Ngn3

We examined whether endogenous *Myt1* expression depends on *Ngn3*. Myt1$^+$ cells were detected at various developmental stages (E10.5, E12.5, E13.5, and E15.5) in *Ngn3*$^{-/-}$ pancreata (Figure 3.7). A portion of these Myt1$^+$cells co-expressed glucagon and MafB, indicating they are pancreatic rather than neuronal cells. After E15.5, Pdx1$^+$Myt1$^+$ cells could still be detected, even though no glucagon$^+$ cells was found in *Ngn3*$^{-/-}$ pancreata.

At all stages examined, the number of Myt1$^+$ cells represented less than 5% of that in *WT* littermates. Thus, *Myt1* expression is largely, but not totally, dependent on *Ngn3*. At present, we do not know whether the emergence of these Myt1$^+$ cells depends on the activation of *Ngn3* homologs, *Ngn1* and *Ngn2*, in the absence of *Ngn3* (Sommer et al., 1996).

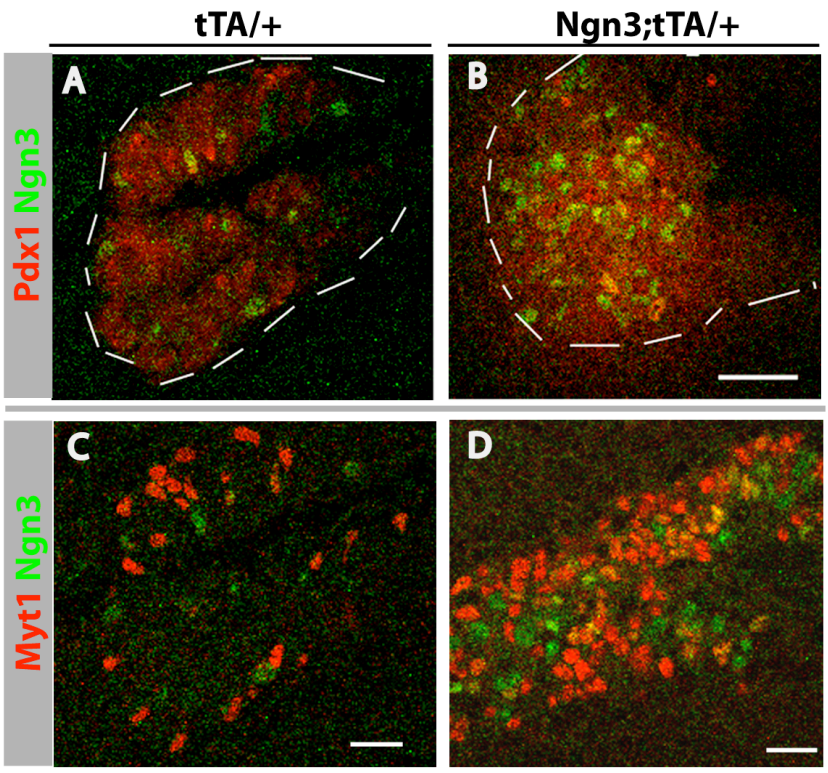


Figure 3.6 *Ngn3* induces *Myt1* expression. (A, B) *Ngn3* expression in *Pdx1*$^{tTA/+}$ (A, labeled as tTA/+) and *Ngn3*^{tet8}*;Pdx1*$^{tTA/+}$ (B, labeled as Ngn3;tTA/+) pancreata at E9.5. White broken lines highlighted the pancreatic region. Green: Ngn3; Red: Pdx1. Note the apparent thickening of pancreatic epithelium in B. Scales bar: 20 μm. (C, D) *Myt1* and *Ngn3* expression in *Pdx1*$^{tTA/+}$ (C, labeled as tTA/+) and *Ngn3*^{tet8}*;Pdx1*$^{tTA/+}$ (D, labeled as Ngn3;tTA/+) pancreata at E10.5. Red: Myt1; Green: Ngn3. Scale bars: 10μm.

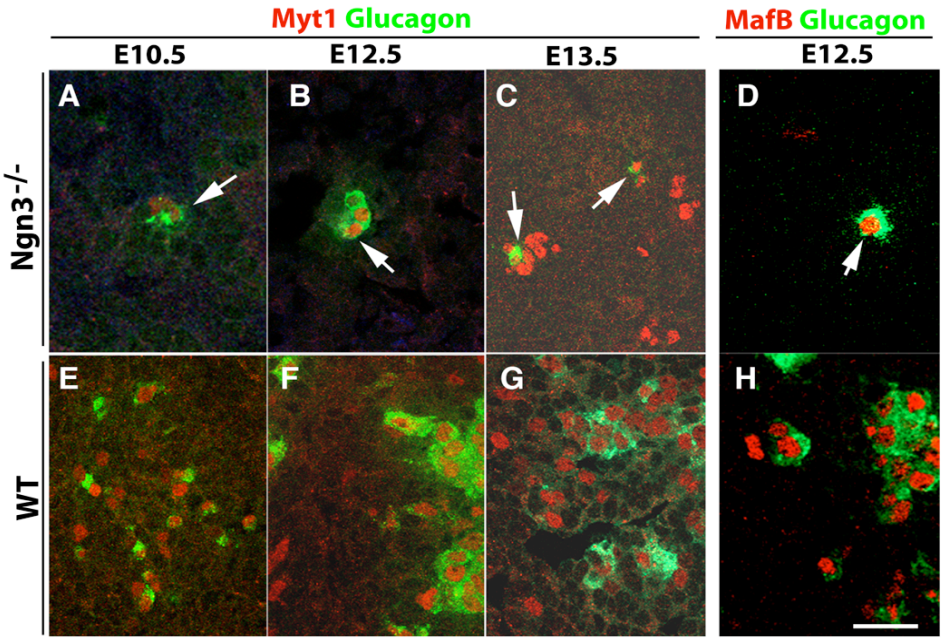


Figure 3.7 The *Ngn3*$^{-/-}$ pancreas maintains Myt1 and glucagon expression. (A–C, E-G) Myt1 and glucagon expression in *Ngn3*$^{-/-}$ and *WT* pancreata, respectively, at E10.5, E12.5 and E13.5. (D, H) *MafB* and glucagon expression in *Ngn3*$^{-/-}$ and *WT* pancreata at E12.5. White arrows: glucagon^{+}Myt1^{+} cells in *Ngn3*$^{-/-}$ pancreata. Scales bars: 10µm.

Discussion

It is well established that activated Notch signaling pathway directly represses *Ngn3* expression in pancreatic progenitors (Chapter 1). However, it is less clear how Ngn3 levels are up regulated to allow for endocrine differentiation after the pancreatic cells are released from Notch repression. The finding that Ngn3 can repress its own transcription makes the situation even more complicated (Smith et al., 2004). While this self-inhibitory loop helps to explain the transient nature of *Ngn3* expression, it prevents the cells from accumulating high levels of Ngn3. Additional signals or factors are necessary to increase Ngn3 levels in endocrine progenitors. Several signaling pathways and transcription factors have been revealed to promote Ngn3 expression and activity in the pancreas (Chapter 1).

Here, we report that Myt1 helps to promote endocrine differentiation by forming a feed-forward expression loop with Ngn3. It is likely that a slight stochastic difference in Notch activity initiates *Myt1* and *Ngn3* expression in putative endocrine progenitors. These two factors then positively promote each other's expression to ensure robust *Ngn3* production and endocrine differentiation.

This model is largely but not completely consistent with previous *Xenopus* studies. In *Xenopus*, *xMyt1* (homolog of *Myt1* in *Xenopus*) and *xNgnr1* (homolog of *Ngn3* in *Xenopus*) can induce neuronal differentiation individually. However, even though *xNgnr1* positively regulates *xMyt1* expression, the expression of *xNgnr1* is not noticeably affected by *xMyt1* ectopic expression. It seems that

xMyt1 works downstream of *xNgnr1* to interfere with Notch-mediated repression and promote neuronal differentiation in *Xenopus* (Bellefroid et al., 1996). More interestingly, even though over-expression of *xNgnr1* under an exogenous promoter cannot antagonize the inhibitory effects of activated Notch signaling, xMyt1 and xNgnr1 together can overcome Notch-mediated repression on neuronal differentiation (Bellefroid et al., 1996). These results suggest that *xNgnr1* is not the only Notch target required for neuronal differentiation. *xMyt1* may be directly regulated by Notch in an *xNgnr1* independent way, which is consistent with our finding that *Myt1* expression is not totally dependent on *Ngn3* in the mouse pancreas. It would be intriguing to examine whether Myt1 and Ngn3 together can bypass Notch-mediated inhibition in the mouse pancreas.

Taken together, these studies suggest that the feed-forward expression loop formed by Myt1 and Ngn3 may play essential roles during development. The mechanism underlying this feed-forward expression loop is still unclear. It is highly possible that Ngn3 directly binds to the *Myt1* promoter and increases *Myt1* expression (Wang et al., 2008b). *Myt1* may enhance *Ngn3* expression in an indirect manner in that *Myt1b* ectopic expression only moderately increases the number of $Ngn3^+$ cells. Careful examination of *Myt1* and *Ngn3* promoters for consensus binding sites may be necessary to finally address this question.

Only a portion of ectopic $Myt1^+$ cells becomes hormone positive cells in the developing mouse pancreas. Therefore, *Myt1* is not as efficient as *Ngn3* in promoting endocrine differentiation, since ectopic *Ngn3* expression drives most, if not all, of pancreatic progenitor cells to an endocrine fate. Combining the fact that

endocrine cells with moderate Ngn3 levels often express high levels of Myt1, it is highly possible that Myt1 promotes the cells with low levels of Ngn3 to undergo endocrine differentiation. It would be interesting to examine whether ectopic expression of *Myt1* could further promote endocrine differentiation in a background with reduced *Ngn3* dosage, for example in *Ngn3*$^{+/-}$ pancreata.

In summary, our studies suggest that Myt1 and Ngn3 form a feed-forward expression loop to promote endocrine differentiation. This mechanism may act to facilitate the *Ngn3*-mediated endocrine differentiation during embryogenesis and ensure the proper function of islets in adults.

CHAPTER IV

REDUCED *Ngn3* DOSAGE IMPAIRS ENDOCRINE ISLET DIFFERENTIATION AND FUNCTION IN THE PANCREAS

Abstract

The Basic Helix-loop-Helix transcription factor Neurogenin3 (Ngn3) actively initiates the endocrine differentiation program in the mouse pancreas. Many signaling pathways and transcription factors are involved in regulating Ngn3 levels and activities. However, it remains largely unknown whether the level of Ngn3 in endocrine progenitor cells affects endocrine differentiation. To directly address this issue, we manipulated *Ngn3* expression levels in endocrine progenitor cells without altering its expression pattern using heterozygosity and a hypomorphic *Ngn3* allele. Reduced Ngn3 levels significantly decreased the number of endocrine cells generated in the pancreas during embryogenesis, resulting in poor glucose tolerance during adulthood. Detailed gene expression analysis showed that in the progenitor cells with low Ngn3 levels, a small portion of *Ngn3*-dependent endocrine genes could be activated, but the endocrine differentiation program failed to complete. As a result, a significant portion of $Ngn3^{+}$ cells adopted the exocrine fate with reduced *Ngn3* dosage. These results demonstrate that sufficient levels of Ngn3 are necessary for endocrine progenitors to accomplish the endocrine differentiation program and become functional hormone-expressing endocrine islet cells.

Introduction

Ngn3, among many other signals/factors, is the core regulator of endocrine differentiation. To this end, *Ngn3* is both necessary and sufficient for initiating endocrine differentiation in pancreatic progenitor cells by controlling the expression of many genes that are essential for endocrine differentiation, such as *Nkx2.2*, *Pax4, IA-1* and *NeuroD* (Gierl et al., 2006; Gradwohl et al., 2000; Mellitzer et al., 2006; Naya et al., 1997; Oliver-Krasinski and Stoffers, 2008; Schwitzgebel et al., 2000; Sosa-Pineda et al., 1997; Sussel et al., 1998; Wang et al., 2004; Watada, 2004). Moreover, ectopic expression of *Ngn3*, alone or together with other genes, can convert differentiated pancreatic exocrine cells or determined liver cells into insulin-producing cells, indicating the potential application of *Ngn3* in diabetes therapies (Yechoor et al., 2009; Zhou et al., 2008). These findings highlight the importance of understanding how *Ngn3* expression is regulated in the pancreas.

$Ngn3^+$ cells emerge from the seemingly equivalent pancreatic epithelium in a scattered fashion (Murtaugh, 2007). Previous studies have suggested that $Ngn3^+$ endocrine progenitors are selected by Notch-mediated lateral inhibition. Similar to neurogenesis, stochastic events or cell division induced asymmetries may cause variations in Notch signaling strength and Ngn3 levels. The latter is directly repressed by activated Notch signaling in a portion of pancreatic progenitors. Possibly by up-regulating Notch ligand levels, the pancreatic cells with slightly higher levels of Ngn3 inhibit their neighbors from up-regulating Ngn3, and consequently receive less Notch signaling and further increase their own

Ngn3 levels (Beatus and Lendahl, 1998; Skipper and Lewis, 2000). Eventually, the cells that accumulated sufficient levels of Ngn3 in a restricted developing time window adopt endocrine fates and initiate endocrine differentiation. Even though many details of the lateral inhibition model remain to be verified in the developing pancreas, it suggests that Ngn3 levels in pancreatic progenitors change dynamically. The cells with sufficient Ngn3 proteins may enter the endocrine lineage.

In addition to being repressed by Notch signaling, *Ngn3* expression is also repressed by itself (Smith et al., 2004). On the other hand, activin and FGF signaling from the mesenchyme adjacent to the pancreatic epithelium, and many intrinsic transcription factors, such as Myt1, Hnf6, Pdx1, Hnf1a and Foxa2, positively regulate *Ngn3* expression (Jacquemin et al., 2000; Lee et al., 2001; Murtaugh, 2007; Wang et al., 2008b; Watada, 2004). It is therefore intriguing to understand whether the level of Ngn3 in endocrine progenitor cells is important for endocrine differentiation.

To directly address this issue, we reduced Ngn3 levels in the mouse pancreas by manipulating *Ngn3* gene dosage. A lower level of Ngn3 in pancreatic cells could activate many Ngn3 downstream targets to low levels, but was not sufficient to produce end-stage hormone-expressing endocrine cells. As a result, a subset of $Ngn3^{+}$ cells, probably those expressing very low levels of Ngn3, were allocated to exocrine rather than endocrine fates. Animals with reduced Ngn3 levels contained less endocrine mass in postnatal life and developed glucose intolerance during adulthood. These results suggest that

Ngn3 levels are important for pancreatic cells to enter the endocrine lineage and differentiate into functional hormone-expressing islet cells.

Materials and Methods

1. Mouse strains and care

Mouse production and care followed protocols approved by the Vanderbilt Medical Center IACUC. The noon of vaginal plug appearance was counted as embryonic day 0.5 (E0.5). Routine mouse embryo production utilized CD1 mice (Charles River Laboratories, Inc. Wilmington, MA). The $Ngn3^{F}$ and $Ngn3^{-}$ mouse strains were described in Chapter 3. $Ngn3^{TgBAC\text{-}Cre}$ is a generous gift from A. Leiter (Schonhoff et al., 2004). $R26R^{EYFP}$ is a gift from F. Costantini (Srinivas et al., 2001). Genotyping followed published methods with minor modification.

2. Immunohistochemistry/immunofluorescence (IHC/IF)

Immunofluorescence/immunohistochemistry followed established protocols. Tissues were stained either as frozen sections or paraffin sections. For frozen sections, dissected tissues were immediately frozen in OCT and sectioned on the day of staining. Primary antibodies used were: guinea pig anti-glucagon, guinea pig anti-insulin, guinea pig anti-PP, and rabbit anti-SS (Dako, Carpinteria, CA), rabbit anti-MafB, a gift from R. Stein (Matsuoka et al., 2004), goat anti-Pdx1, a gift from C. Wright, rabbit anti-Ngn3 (Gu et al., 2002), mouse anti-Nkx6.1 (Development Hybridoma Bank, University of Iowa, Iowa, IA), rat

anti-BrdU (BU1/75) (ABCam, Cambridge, MA), mouse anti-mouse alpha tubulin and Dolichos Biflorus Agglutinin (DBA) (Sigma Aldrich, St Louis, MI). Secondary antibodies used were: Cy3- conjugated donkey anti-rabbit IgG, Cy3-conjugated donkey anti-mouse IgG, FITC-conjugated donkey anti-guinea pig IgG, Cy3-conjugated donkey anti-goat, Cy5- conjugated donkey anti-guinea pig IgG and Cy5-conjugated donkey anti-rabbit (Jackson Immunoresearch, West Grove, PA). All antibodies utilized 1:500-1:2000 dilutions, depending on the amount of tissue on each slide. Antigen retrieval (pH6.0 citrate buffer) was used for recognition of Nkx6.1 and MafB (only for paraffin sections).

3. Assays and quantification

Intraperitoneal glucose tolerance test (IPGTT), serum insulin assays, and Insulin secretion from isolated islets were performed as described in Chapter 2 (Wang et al., 2007). Western blot utilized α-tubulin as loading control. Twenty or 10 μg of total protein was loaded for each lane.

In order to assay islet mass, pancreata of each stage were weighted, fixed and prepared as 6μm paraffin sections. One of every 10 sections was collected and stained with a mix of antibodies against insulin, glucagon, PP, and SS respectively as HRP signals. Slides were counter-stained with hematoxylin. Sections were then scanned by Ariol SL-50 (Genetix, Hampshire, UK) for quantitative IHC Analysis to calculate the ratio of the stained endocrine areas over that of the total pancreas. Islet mass was calculated based on the

endocrine/exocrine ratio and the pancreas weight. For each genotype and stage, at least four individual pancreata were analyzed.

In order to examine the mitotic index, animals were injected IP with multiple doses of BrdU (at 50mg/Kg body weight). For P1 animals, two doses at 2-hour interval were injected. For P10 animals, 4 doses at 2-hour interval were injected. Pancreata were then collected 2 hours after the last BrdU injection, and prepared as paraffin sections to assay for $BrdU^{+}$ cells, which were visualized by immunofluorescence and confocal imaging. Multiple images/sections from each pancreas were counted to obtain representative labeling index.

To count the number of pancreatic cells derived from $Ngn3^{TgBAC\text{-}Cre}$-expressing progenitors, pancreata were fixed in 4% paraformaldehyde overnight (at 4 °C). The pancreata were then prepared as frozen sections and stained with antibodies against all hormones (as blue fluorescence) and DBA (as red fluorescence). Confocal images were captured for cell counting. For each pancreas, ¼ of all sections were examined.

All statistical analyses utilized standard student t-test. P-values less than 0.05 were considered significant. Quantification data were presented as the mean ± standard error over the mean.

4. Microscopy

Confocal microscopy was utilized to visualize fluorescent signals. When protein production levels were compared, samples were processed side by side

and images were captured using identical confocal parameters. In order to estimate the *Ngn3* expression level in individual nuclei, Z-sections (1 μm apart) were projected onto a single plane for side-by-side comparison.

5. Real time QRT-PCR

For assays of mRNA levels, whole pancreata were used for RNA preparation. Two duplicate cDNA preparations and four QRT-PCR reactions were analyzed for each RNA preparation. For each genotype, three independent batches of embryos were processed independently. Reverse-transcription PCR (RT-PCR) followed standard protocol. cDNA synthesis and RT-PCR were done by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). QRT-PCR was performed using mouse-specific primers (Table1) with iQ SYBR Green Supermix on an iCycler Thermal Cycler. The abundance of most transcripts was normalized versus the abundance of GAPDH or amylase transcripts.

Results

Ngn3 production is reduced in Ngn3$^{+/-}$ and Ngn3$^{F/-}$ pancreata

In order to investigate whether Ngn3 levels in pancreatic progenitor cells are important for them to undergo endocrine differentiation, we reduced *Ngn3*

dosage in the mouse pancreas, with the hope to obtain reduced Ngn3 production.

We utilized three *Ngn3* alleles, $Ngn3^{+}$ *(wild type allele)*, $Ngn3^{-}$ and $Ngn3^{F}$. In the $Ngn3^{-}$ allele, the *Ngn3* coding region was replaced by a $CreER^{T}$ cDNA. This allele does not produce any *Ngn3* products and equals to a null allele. In the $Ngn3^{F}$ allele, LoxP sites flanked the *Ngn3* coding region. A $CreER^{T}$ cDNA lies farther 3' (Chapter 3, Figure 3.5 A). Because no DNA element of the *Ngn3* locus was deleted in the $Ngn3^{F}$ allele, we expected the $Ngn3^{F}$ allele to transcribe floxed *Ngn3* mRNA in a pattern and level similar to the $Ngn3^{+}$ allele.

Ngn3 production in $Ngn3^{+/-}$, $Ngn3^{F/-}$ and *WT* ($Ngn3^{+/+}$) mouse pancreata were examined at E14.5, E15.5 and E16.5 by semi-quantitative immunofluorescence (IF), western blotting and quantitative real time QT-PCR. For IF staining-based assays, pancreata were dissected, processed and imaged side by side (Materials and Methods). The average Ngn3 levels, judged by fluorescent intensities, in individual $Ngn3^{+}$ cells were visibly reduced in $Ngn3^{+/-}$ and $Ngn3^{F/-}$ pancreata (Figure 4.1 A), as were the number of $Ngn3^{+}$ cells. Noticeably, the $Ngn3^{F/-}$ pancreas had an even lower level of Ngn3 production compared with the $Ngn3^{+/-}$ pancreas, suggesting that the $Ngn3^{F}$ allele is a hypomorphic allele. This is possibly caused by the insertion of the hair-pinned LoxP site in front of the *Ngn3* ATG, which may significantly interfere with translation initiation. Consistent with this result, western blot and QRT-PCR analyses estimated that the total amounts of Ngn3 protein and mRNA in $Ngn3^{+/-}$

and *Ngn3*$^{F/-}$ pancreata were significantly reduced compared with *WT* controls at E15.5 (Figure 4.1 B&C). We therefore used *Ngn3*$^{+/-}$ and *Ngn3*$^{F/-}$ mice to examine the effects of reduced Ngn3 levels on endocrine differentiation.

Reduced Ngn3 dosage impairs endocrine islet function

We first examined whether reduced *Ngn3* dosage affects proper endocrine islet function. The resting blood glucose levels of the adult *Ngn3*$^{+/-}$, *Ngn3*$^{F/-}$ and *WT* mice did not vary significantly. Yet intraperitoneal glucose tolerance test (IPGTT), a more sensitive endocrine functional assay, revealed that both male and female *Ngn3*$^{+/-}$ and *Ngn3*$^{F/-}$ animals displayed significantly compromised glucose-clearance capacities at 4 and 8 weeks of age (Figure 4.2 A). Despite the overt glucose intolerance problem, *Ngn3*$^{+/-}$ and *Ngn3*$^{F/-}$ animals did not develop frank diabetes (random feeding blood glucose over 300 mg/dl) even at one year of age.

To investigate whether the glucose intolerance in *Ngn3*$^{+/-}$ and *Ngn3*$^{F/-}$ animals was due to compromised insulin secretion or reduced responses of peripheral tissues to insulin, insulin sensitivity and insulin secretion of these animals were examined. All three groups of animals (*Ngn3*$^{+/-}$, *Ngn3*$^{F/-}$ and *WT* mice) displayed similar profiles of blood glucose reduction in response to insulin administration (Figure 4. 2 B), demonstrating that their insulin sensitivities were not altered with reduced *Ngn3* dosage. Yet *Ngn3*$^{+/-}$ and *Ngn3*$^{F/-}$ animals showed reduced serum insulin levels in response to glucose challenge, compared with *WT* control animals (Figure 4.2 C). These data suggest that a high level of Ngn3

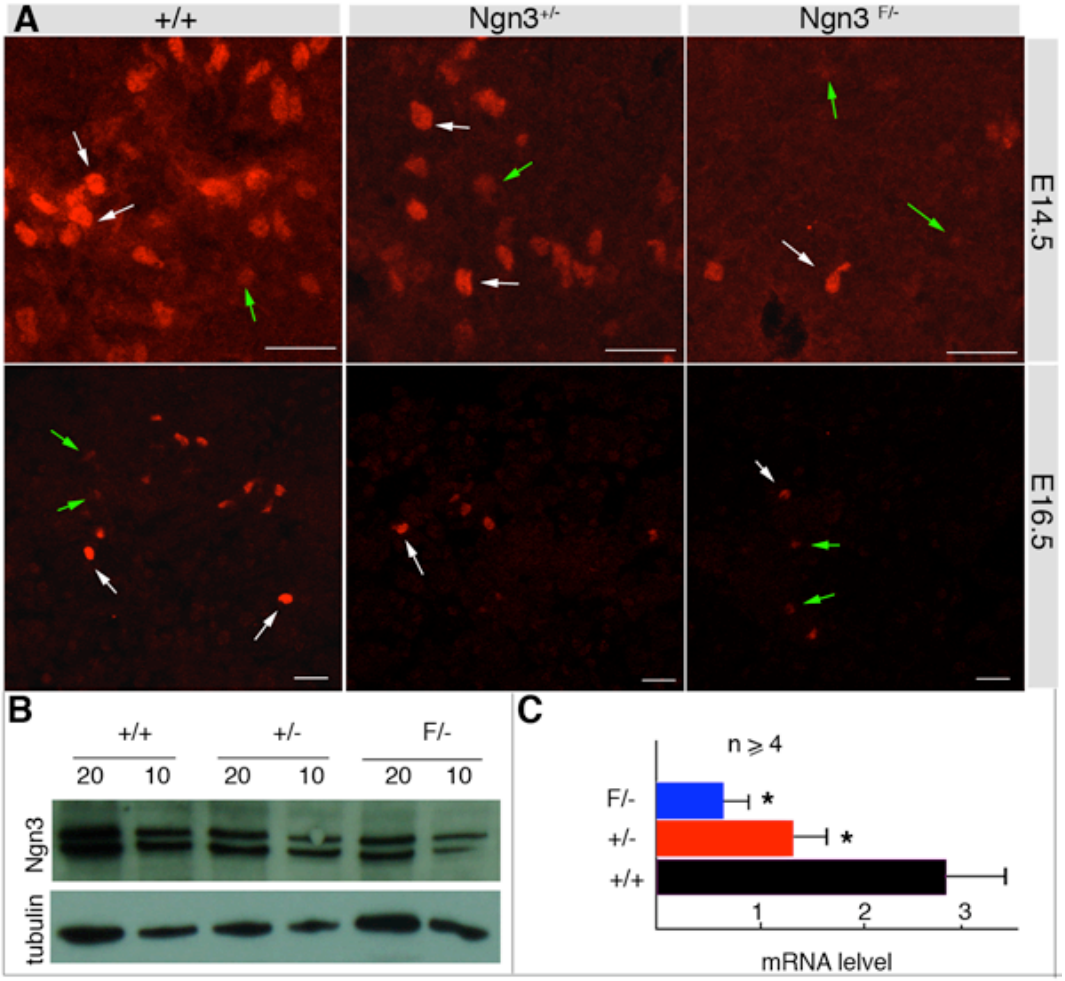


Figure 4.1 Ngn3 production is reduced in *Ngn3*$^{+/-}$ and *Ngn3*$^{F/-}$ pancreata. (A) Immunofluorescence detection of Ngn3 at E14.5 and E16.5. Shown were Z-stacked images captured at identical confocal parameters (methods and materials). Green arrows: Ngn3^{low} cells. White arrows: Ngn3^{high} cells. Scale bars: 20μm. (B) Western blot detection of Ngn3 protein in E15.5 pancreata. Each sample was loaded into two lanes, with different amount of total protein loaded. α-tubulin was used as loading controls. Note that Ngn3 protein migrated as two visible bands under this condition. (C) Real time RT-PCR detection of *Ngn3* mRNA levels in E15.5 pancreata. Presented were relative mRNA levels normalized against amylase mRNA. "*": $P<0.05$.

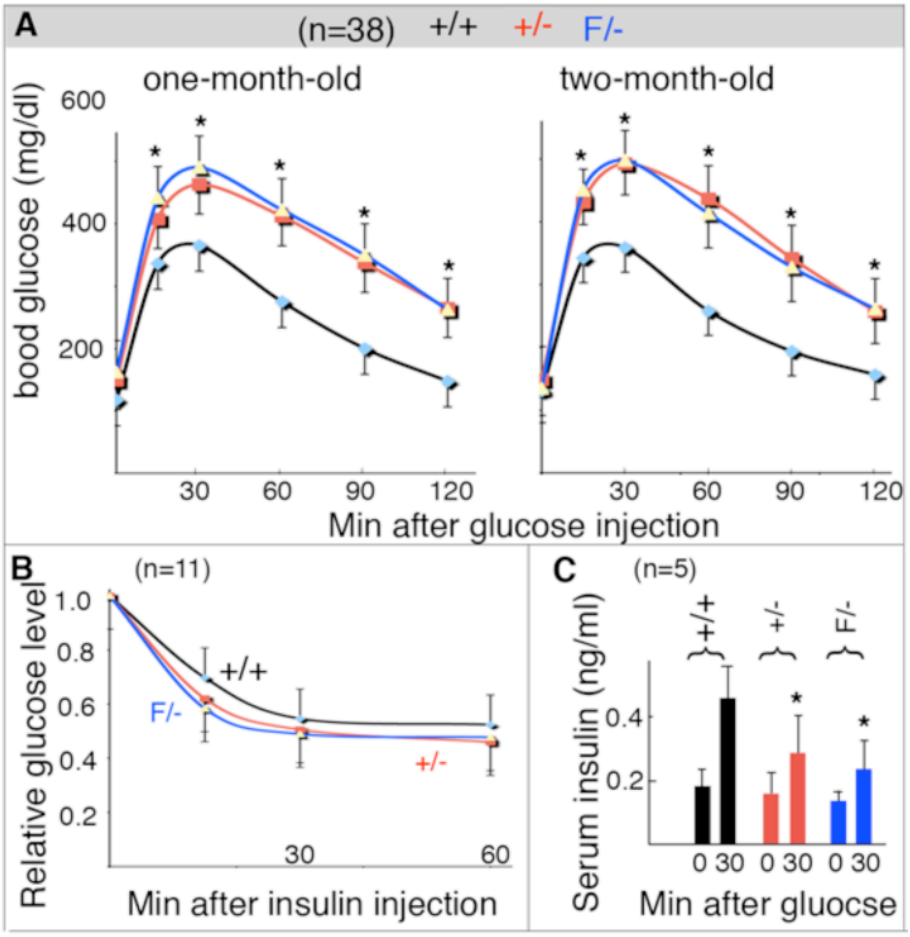


Figure 4.2 Reduced *Ngn3* dosage impairs endocrine islet function. (A) IPGTT results of one and two-month-old animals (both male and female were grouped together). (B) Insulin sensitivity in two-month-old mice (both male and female mice). The "Y" axis represented the ratio of glucose level at points of assay over that at pre-insulin injection. (C) Serum insulin levels before and 30 minutes after glucose challenge. Only male mice were utilized here. "*": P<0.05. +/+: WT; +/-: *Ngn3*$^{+/-}$; F/-: *Ngn3*$^{F/-}$.

is required for proper islet function.

Reduced Ngn3 dosage decreases endocrine islet mass

We next examined how reduced Ngn3 dosage affects islet function. In the adult pancreas, overall islet architecture did not vary in *Ngn3*$^{+/-}$, *Ngn3*$^{F/-}$ and *WT* animals. However, total islet mass, as measured by islet area, was substantially reduced at P1 (postnatal day 1) and P28 in *Ngn3*$^{+/-}$ and *Ngn3*$^{F/-}$ animals over that of the *WT* controls (Figure 4.3 A-D). This reduced endocrine islet mass may contribute to the impaired glucose tolerance in *Ngn3*$^{+/-}$ and *Ngn3*$^{F/-}$ animals. Furthermore, islets were isolated from 6-week-old *Ngn3*$^{+/-}$, *Ngn3*$^{F/-}$ and *WT* pancreata to examine their insulin secretion capabilities. *Ngn3*$^{+/-}$ and *Ngn3*$^{F/-}$ islets displayed a trend of increased insulin secretion over that of *WT* islets in response to glucose (Figure 4.3 E). This observation can explain why *Ngn3*$^{F/-}$ animals with reduced islet mass, had similar GTT profiles compared with *Ngn3*$^{+/-}$ animals. The underlying mechanism of this phenomenon is not clear at present. It may represent an adaptive behavior of the islets which up-regulate glucose-induced insulin secretion to compensate for endocrine mass loss, a process that occurs prior to phenotypic manifestation of Type 2 Diabetes in human patients.

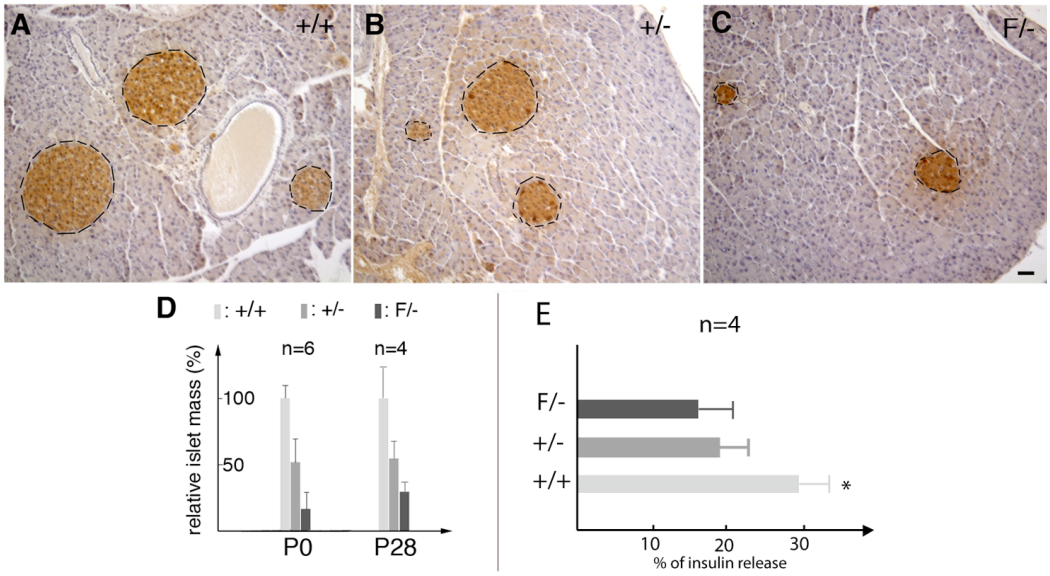


Figure 4.3 Reduced Ngn3 levels decrease pancreatic islet cell production. (A-C) Representative images of islets in 4-week-old *Ngn3*$^{+/-}$; *Ngn3*$^{f/-}$ and *WT* pancreata. Islets (brown) were stained with a combination of insulin, glucagon, SS and PP antibodies. Scale bar: 50μm. (D) Quantification of islet mass at one day (P1) and 4 weeks after birth (P28). The islet mass in *WT* pancreata was artificially set to 100%. (E) Insulin secretion in isolated islets of 8-week-old male mice. The X-axis represented the % of insulin released from isolated islets within 1 hour under 20mM glucose stimulation, after pre-incubated in 3.3 mM glucose. "*": P<0.05. +/+: WT; +/-: *Ngn3*$^{+/-}$; F/-: *Ngn3*$^{f/-}$.

Reduced Ngn3 dosage shunts Ngn3^{+} cells to exocrine fates

To further elucidate the underlying reasons for decreased endocrine islet mass upon *Ngn3* dosage reduction, the proliferation and apoptosis rates of endocrine cells in *Ngn3*$^{+/-}$, *Ngn3*$^{F/-}$ and *WT* pancreata were examined at various stages. No significant difference was discovered (data not shown). Therefore, it is possible that pancreatic progenitor cells with reduced *Ngn3* levels cannot enter the endocrine lineage, which results in reduced endocrine mass. To test this hypothesis, *Ngn3*$^{TGBAC\text{-}Cre}$ transgenic and *R26R*^{EYFP} reporter mice were utilized to trace the fates of Ngn3^{+} cells in both *WT* and *Ngn3*$^{+/-}$ backgrounds (Schonhoff et al., 2004; Srinivas et al., 2001). The advantage of this approach is that activation of the *R26R*^{EYFP} allele can lead to a similar strong level of EYFP expression irrespective of cell types. Thus, we could evenly detect all cells that express *Ngn3* at a level above the threshold for Cre activity.

The EYFP^{+} progeny of Ngn3^{+} cells was monitored at embryonic and postnatal stages. At E14.5, most EYFP^{+} cells in *Ngn3*$^{TGBAC\text{-}Cre}$*;R26R*$^{EYFP/+}$ pancreata, appeared in clusters of endocrine cells, whereas a substantial number of EYFP^{+} cells was in the exocrine compartment, readily recognized by their typical cellular morphology and marker expression in *Ngn3*$^{+/-}$;*Ngn3*$^{TGBAC\text{-}Cre}$*;R26R*$^{EYFP/+}$ pancreata (Figure 4.4 A-C). The similar phenomenon was also observed at newborn and P10. While about 15% of *Ngn3*$^{TGBAC\text{-}Cre}$-labeled cells became exocrine cells in the *WT* background, about 45% of *Ngn3*$^{TGBAC\text{-}Cre}$-

labeled cells became exocrine cells in the *Ngn3*$^{+/-}$ background with reduced Ngn3 production (Figure 4.4 D).

The fates of Ngn3^{+} cells in the *Ngn3*$^{F/-}$ and *Ngn3*$^{-/-}$ backgrounds were also determined. The *Ngn3*$^{F/-}$;*Ngn3*$^{TgBAC\text{-}Cre}$;*R26R*$^{EYFP/+}$ pancreas contained nearly no endocrine cells, due to the deletion of the *Ngn3*^{F} allele by the *Ngn3*$^{TgBAC\text{-}Cre}$ transgene at some time point after sufficient amounts of Cre protein were produced. In this case, most of the EYFP^{+} cells ended up in the exocrine pancreas. In the *Ngn3*$^{-/-}$ background, EYFP^{+} pancreatic cells are all exocrine cells, confirming that *Ngn3* is required for endocrine differentiation (Figure 4.4 C1-C3, D).

In summary, these data suggest that *Ngn3* expression must reach a threshold level in order to allocate the cells to endocrine fates. The Ngn3^{low} cells may maintain the features of multi-potent progenitors, and can be re-allocated to exocrine fates in proper environments.

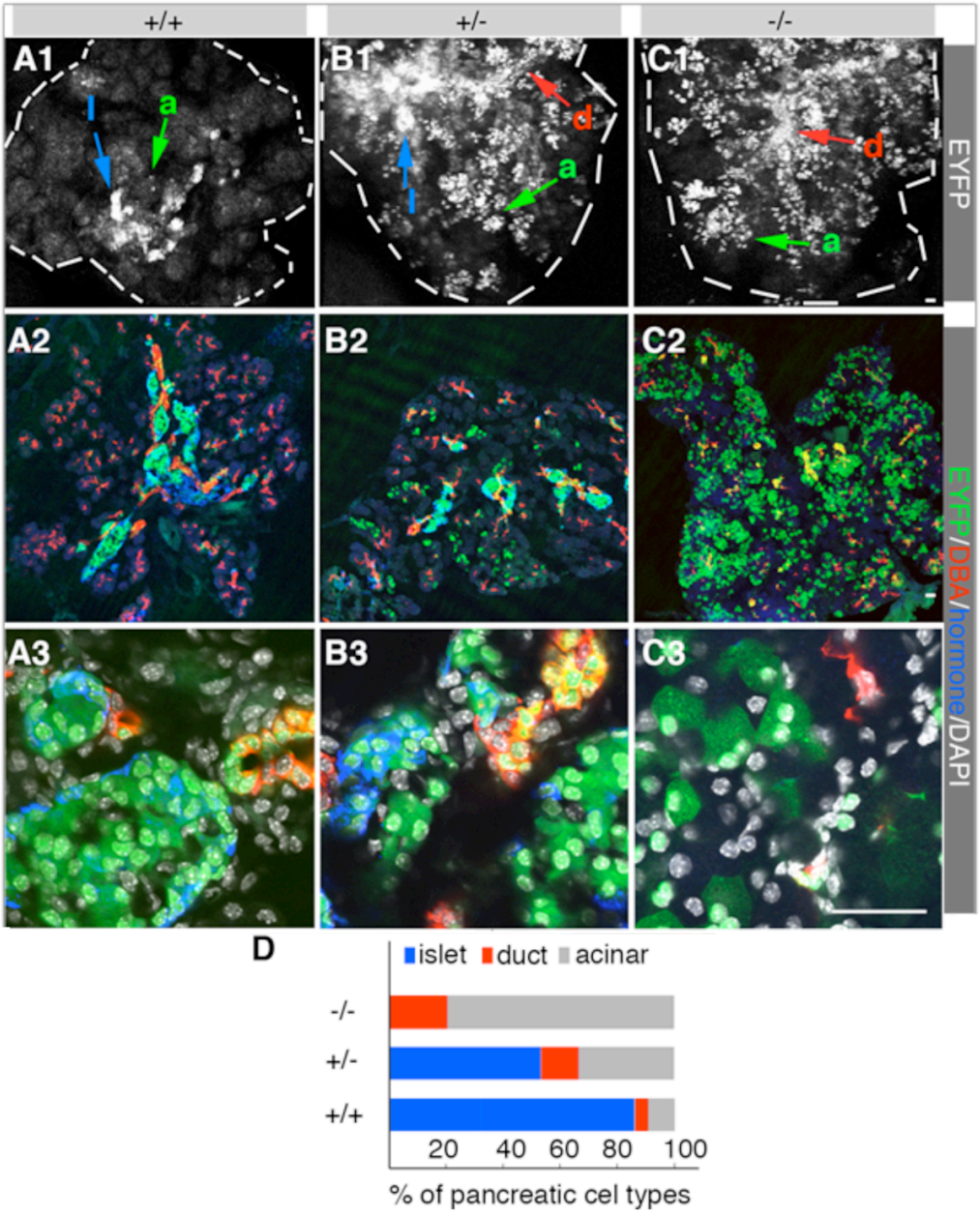


Figure 4.4 Reduced Ngn3 activity shunts more Ngn3^{+} cells to exocrine fates. (A1, B1, and C1) EYFP expression (white) in E14.5 *Ngn3*$^{TgBAC\text{-}Cre}$;*R26R*$^{EYFP/+}$ pancreata under different genetic backgrounds. Whole-mount pictures were showed. Dotted lines circled the pancreatic regions. (A2-C3) The identities of EYFP^{+} pancreatic cells. A2 and A3, B2 and B3, or C1 and C3 showed images of identical pancreata, respectively, with different magnifications. Scale bars: 50µm. (D) Percentage of EYFP^{+} cells that were of islet, acinar or duct cells at P0 (n=6). +/+: WT; +/-: *Ngn3*$^{+/-}$; F/-: *Ngn3*$^{F/-}$.

Discussion

Ngn3 is well known for its essential roles in pancreatic endocrine differentiation. However, it remains unclear whether *Ngn3* levels in individual pancreatic cells affect their ability to adopt endocrine fates. Here, we provide evidence to show that sufficient Ngn3 levels are necessary for the pancreatic progenitors to initiate and fulfill endocrine differentiation. When Ngn3 levels are reduced, significant portion of $Ngn3^{+}$ cells fails to enter the endocrine lineage, but becomes exocrine cells. As a result, animals with reduced *Ngn3* dosage contain less endocrine islet mass postnatally, and fail to secrete enough insulin and maintain glucose homeostasis upon challenge during adulthood. This *Ngn3*-level-dependent regulatory mechanism may act to ensure the proper allocation of the endocrine and exocrine pancreas.

A threshold level of Ngn3 serves as a checkpoint for entry into endocrine lineage.

When *in situ* hybridization or various mono- or polyclonal antibodies was used to detect *Ngn3* expression, $Ngn3^{+}$ cells were always observed to express unequal levels of Ngn3 mRNA/protein as judged by signal intensities. However, it is not clear whether these $Ngn3^{low}$ and $Ngn3^{high}$ cells possess similar features and eventually adopt same fates. It is possible that $Ngn3^{low}$ and $Ngn3^{high}$ cells represent different steps of the Ngn3 protein accumulating process in pancreatic cells. In other words, $Ngn3^{low}$ cells may gradually become $Ngn3^{high}$ cells, and adopt endocrine fates. Alternatively, $Ngn3^{low}$ cells may not necessarily move

towards the Ngn3^{high} state, but instead possess high plasticity to become exocrine cells.

Previous studies suggested that both of these possibilities might exist in the pancreas. While *Ngn3*^{CreERT} line, which can only mark the Ngn3^{high} cells, labels mostly endocrine cells in the presence of Cre-reporter, *Ngn3*$^{TgBAC\text{-}Cre}$ based lineage tracing showed that a small portion of *Ngn3*-expressing precursors gives rise to exocrine cells under WT situation, when a sensitive Cre reporter *R26R* mouse was used (Schonhoff et al., 2004; Wang et al., 2009). This result suggests that a portion of Ngn3^{low} cells marked by the *Ngn3*$^{TgBAC\text{-}Cre}$ line may adopt exocrine fates under normal conditions. Here, we directly show that Ngn3^{low} cells maintain the progenitor cell plasticity and are able to revert to becoming exocrine cells. The threshold level of Ngn3 seems to serve as the checkpoint for initiating endocrine differentiation (Figure 4.5).

Ngn3-level-dependent regulation of Notch mediated lateral inhibition

Except for initiating the endocrine differentiation program, Ngn3 is also an essential component of the Notch-mediated lateral inhibition, which functions to select Ngn3^{+} cells from seemingly equivalent pancreatic progenitors. During neurogenesis, Ngn3 positively regulates the level of Notch ligands (for example, Dll1) in some cells, to activate Notch signaling in their neighboring cells and prevent their neighbors from accumulating Ngn3. If this classic view of lateral inhibition fits the context of the pancreas as proposed, we would expect

decreased expression of Notch ligands and increased number of $Ngn3^{low}$ cells when Ngn3 dosage is reduced.

In fact, there were more $EYFP^+$ cells in $Ngn3^{+/-}$;$Ngn3^{TgBAC\text{-}Cre}$;$R26R^{EYFP/+}$ pancreata compared with $Ngn3^{TgBAC\text{-}Cre}$;$R26R^{EYFP/+}$ controls at E14.5, when no significant difference was observed in proliferation and apoptosis rates between these pancreata. Consistent with the classic lateral inhibition, this result suggests that more pancreatic cells activate *Ngn3* expression, albeit low levels, when Ngn3 dosage is reduced. However, reduced Ngn3 dosage did not significantly reduce the transcription of major Notch ligands in the pancreas, when $EGFP^+$ cells were isolated from E14.5 $Ngn3^{EGFP/+}$ and $Ngn3^{EGFP/F}$ pancreata by Fluorescence-Activated-Cell-Sorting (FACS), and analyzed for their expression of Notch ligands (Dll1 and Dll4) by QRT-PCR (Unpublished data, Jingbo Yan and Guoqiang Gu). It is possible that *Ngn3* utilizes other unknown mechanisms to achieve the lateral inhibition. Alternatively, QRT-PCR may not be sensitive enough to detect a statistically insignificant variation of expression levels. Faithful/sensitive antibodies for Notch ligands may help to solve this problem.

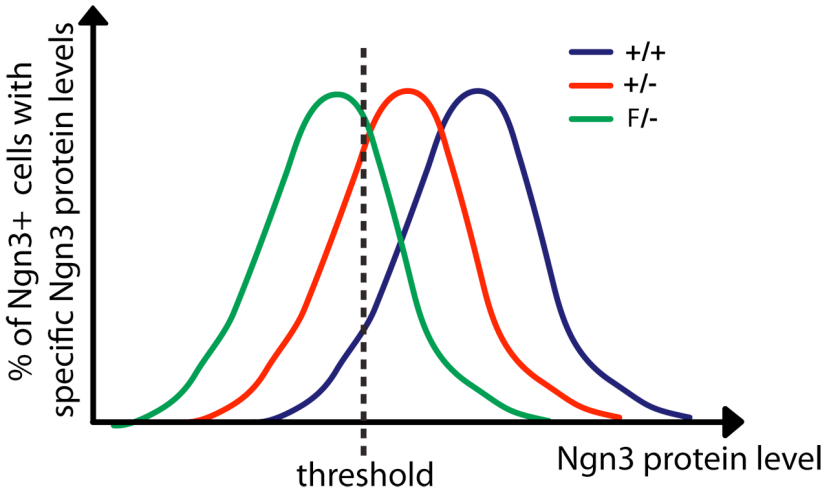


Figure 4.5 Certain levels of Ngn3 are important for pancreatic cells to enter the endocrine lineage. X-axis: Ngn3 protein levels. Y-axis: the percentage of $Ngn3^{+}$ cells with corresponding Ngn3 levels. $Ngn3^{+}$ cells with different Ngn3 levels showed a normal distribution (Gaussian Distribution). Dash line: the suspected threshold Ngn3 level for endocrine differentiation. The cells with an Ngn3 level surpassing this threshold level could undergo endocrine differentiation. With reduced Ngn3 dosage in *Ngn3*$^{+/-}$ and *Ngn3*$^{F/-}$ pancreata, less pancreatic cells could get beyond the threshold level, resulting in decreased endocrine mass. Blue: *WT*; Red: *Ngn3*$^{+/-}$; Green: *Ngn3*$^{F/-}$.

Dosage requirement of transcription factors during development

During development, extrinsic gradients of morphogens (BMP, Wnt, Hh, FGF et al.) trigger different developmental events and specify various cell types. Intrinsically, many studies suggest that the concentration/level of transcriptional factors also affects developmental processes and organ functions. In the pancreas, several transcription factors have been shown to function in a dosage dependent manner.

Mice heterozygous for *Pdx1* showed severe defects in maintaining their glucose homeostasis, which is consistent with the finding that *Pdx1* haploinsufficiency results in maturity-onset diabetes of the young 4 (MODY4) in human patients (Macfarlane et al., 2000; Stoffers et al., 1997a; Stoffers et al., 1998; Stoffers et al., 1997b). More interestingly, Fujitani et al. showed that different gene dosage of *Pdx1* is required for the formation of endocrine and exocrine pancreas, as well as foregut organs, by targeted deletion of a cis-regulatory region in the *Pdx1* promoter (Fujitani et al., 2006). Recently, Oliver-Krasinski also revealed that mice homozygous for a *Pdx1* hypomorphic allele displayed a selective reduction in endocrine differentiation. Similarly, reduced dosage of *Sox9*, which marks the multipotent pancreatic progenitors, results in decreased endocrine mass (Seymour et al., 2008).

These studies suggest that the levels of transcription factors within individual cells are important for their function. It is possible that higher local concentrations of transcription factors in promoter regions can strongly and sustainably activate downstream target genes. Moreover, it appears that

threshold levels are set up for some transcription factors. When the concentration of transcription factors surpasses the threshold level, certain differentiation events could be triggered. It currently remains unclear how the threshold level is determined. Possibly, many events cooperate intensively to make the decision, such as the interaction between transcription factors, chromatins and their binding partners, as well as the mobility and duration of transcription factors.

In summary, we provide direct evidence to show that Ngn3 levels are important for pancreatic cells to undergo proper endocrine differentiation. This mechanism not only helps to ensure the proper balance between different tissue types in the pancreas, but also suggests that Ngn3 levels need to be tightly controlled for the generation of endocrine cells from various cell sources *in vitro* or *in vivo*.

CHAPTER V

SUSTAINED *Ngn3* EXPRESSION IN HORMONE-EXPRESSING ISLET CELLS IS REQUIRED FOR ENDOCRINE MATURATION AND FUNCTION

This chapter is published under the same title in *PNAS*, June 2009 (Wang et al., 2009).

Abstract

Ngn3 is both necessary and sufficient to induce endocrine islet cell differentiation from embryonic pancreatic progenitors. Since robust *Ngn3* expression has not been detected in hormone-expressing cells, Ngn3 is used as an endocrine progenitor marker and regarded as dispensable for the function of mature islet cells. Here we used 3 independent lines of *Ngn3* knock-in reporter mice and mRNA/protein-based assays to examine *Ngn3* expression in hormone-expressing islet cells. *Ngn3* mRNA and protein are detected in hormone-producing cells at both embryonic and adult stages. Significantly, inactivating *Ngn3* in insulin expressing cells at embryonic stages or in Pdx1 expressing islet cells in adults impairs endocrine function, a phenotype that is accompanied by reduced expression of several Ngn3 target genes that are essential for islet cell differentiation, maturation and function. These findings demonstrate that *Ngn3* is required not only for initiating endocrine cell differentiation, but also for promoting islet cell maturation and maintaining islet function.

Introduction

It is well established that the basic helix–loop–helix transcription factor Ngn3 has an essential role in pancreatic endocrine cell differentiation. All such endocrine islet cells are derived from $Ngn3^+$ precursors (Gu et al., 2002; Schonhoff et al., 2004) and *Ngn3* deficiency virtually abolishes islet cell differentiation (Gradwohl et al., 2000; Wang et al., 2008b). Moreover, ectopic *Ngn3* expression converts early endodermal progenitor cells into endocrine islet cells (Apelqvist et al., 1999; Grapin-Botton et al., 2001; Johansson et al., 2007; Schwitzgebel et al., 2000), and *Ngn3* controls the expression of multiple genes that influence both endocrine differentiation and function (Gradwohl et al., 2000; Petri et al., 2006; White et al., 2008). Because *Ngn3* has not been detected in differentiated islet cells, its expression in the adult pancreas is proposed as a marker for putative endocrine progenitors (Gu et al., 2002; Xu et al., 2008). Contradictory findings exist regarding *Ngn3* expression in the adult pancreas. Several reports have shown *Ngn3* expression in *WT* adult islet cells (Dror et al., 2007; Gu et al., 2002; Joglekar et al., 2007; Kodama et al., 2005), and this expression is enhanced by regenerative conditions (Joglekar et al., 2007; Kodama et al., 2005; Xu et al., 2008). Yet these analyses have failed to establish whether *Ngn3* expression is restricted to only a few putative endocrine progenitor cells at a high level, or whether *Ngn3* is also present in differentiated islet cells at a low level. Nor is it clear how the sustained *Ngn3* expression impacts endocrine function. Here we have used a combination of knock-in reporter mice, immunoassays, and loss-of-gene function studies to show that differentiated

hormone-expressing islet cells continue to express *Ngn3*, and that *Ngn3* is important for both islet cell production and function.

Materials and Methods

1. Mouse strains and care

Routine mouse crosses used ICR or CD1 mice (Charles River Laboratories). The *Ngn3*^{CreERT}, *TetO*^{Lacz}, *Ins2*^{Cre}, and *Pdx1*^{CreERT} alleles were described previously (Gu et al., 2002; Wang et al., 2008b; Yu et al., 2005) (Gannon et al., 2000b; Lee et al., 2006). *Ngn3*^{EYFP} and *R26R*^{EYFP} mice were kind gifts from K. Kaestner (University of Pennsylvania School of Medicine, Philadelphia) and F. Costantini (Columbia University Medical Center, New York), respectively (Lee et al., 2002; Srinivas et al., 2001). All animals used for IPGTT assays were maintained on a mixed genetic background, with roughly 50% 129Sv/Ev + 25% CD1 + 25% CBA/Bl6 composition (estimated from the number of intercrosses). Genotyping and TM administration followed published methods (Gu et al., 2002; Lee et al., 2006; Wang et al., 2008b; Yu et al., 2005).

2. Immunohistochemistry/Immunofluorescence and Western Blot

Immunoassays followed established protocols with minor modifications. For MafA (1:500 dilution) and Ngn3 antibody (preincubated with *Ngn3*$^{F/-;}$*Ins2*^{Cre} adult pancreas, 1:1000 dilution) staining, pancreata were frozen in OCT (optimum cutting temperature compound, Sakura Finetek) after dissection. Air-

dried sections of 10–20 μm thickness were fixed in 4%paraformaldehyde in PBS (with 1mMEGTA and 1.5mM MgCl2) for 15–20 min. Sections were permeated in 0.1% Triton-X100 (in PBS) for 20 min and blocked with antibody solution (0.5% BSA + 5% donkey serum + 0.1% Tween-20 in PBS; pH 7.6 –7.8) for 30 min. Slides were incubated overnight at 4 °C with primary antibodies. After 4 washes with PBS containing 0.1% Tween-20, fluorophore-conjugated secondary antibodies were used to visualize signals. Glut2 staining used paraffin sections. Guinea pig anti-insulin, goat anti-C-peptide, guinea pig anti-glucagon, guinea pig anti-PP, and rabbit anti-SS were obtained from Dako. Rabbit anti-MafA was a gift from R. Stein (Vanderbilt University Medical Center, Nashville, TN) (Matsuoka et al., 2004) and goat anti-Pdx1 was a gift from C. Wright (Vanderbilt University Medical Center, Nashville, TN). Guinea pig anti-Ngn3 was described previously (Seymour et al., 2007), rabbit anti-Glut2 was from Alpha Diagnostics, and Rabbit anti-EGFP was from Clontech. FITC-conjugated donkey anti-rabbit IgG, Cy3-conjugated donkey anti-rabbit IgG, Cy3-conjugated donkey anti-mouse IgG, Cy3-conjugated donkey anti-guinea pig IgG, Cy3-conjugated donkey anti-goat, Cy5-conjugated donkey anti-rabbit, and Cy5-conjugated donkey anti-guinea pig were all from Jackson Immunoresearch. All transcription factor antibodies (except Ngn3) were used at a 1:500 dilution. Hormone antibodies were diluted at 1:1,000. Secondary antibodies were used at 1:2,000 dilutions. For Western assays, islets from 6–8 adult animals of desired genotypes were hand-picked (Wang et al., 2007) and used for nuclear extract preparation by using NuPer following

manufacturer's protocol (Pierce). For blot, nuclear extract from ≈50–100 islets was loaded into each well (≈5–10μg protein). E16.5 pancreatic nuclear extract was prepared as individual samples from each bud. After genotyping, extract from 3 buds of identical genotype were pooled and loaded for Western (≈5 μg protein per lane). Recombinant Ngn3 protein from whole 293HEK cell lysate was used as positive control. Acrylamide gels (18%) were used to resolve the protein for blotting.

3. Glucose Tolerance Test, Microscopy, QRT-PCR, and Statistical analysis

IPGTT followed published procedures (Wang et al., 2007). All fluorescent images were obtained by using confocal microscopy. QRT-PCR used the Bio-Rad I-cycler. For PCR analysis of embryonic tissues, 2 or 3 embryos of the same genotype were prepared as a single RNA sample. Four individual RNA samples of control and 4 samples of experimental animals were prepared. For adult islets, 12 controls or 12 experimental pancreata were perfused. Four or five islet pools were handpicked from both groups and were used to extract total RNA. For each RNA preparation, duplicated reverse transcriptase reactions were used to prepare cDNA. Each cDNA was used to assay the abundance of each transcript as duplicated PCRs. DNA oligos used are listed in Table 1. Statistical analyses used the standard student's 2-tailed t test. A P value of <0.05 was considered significant. Data are presented as the mean $\pm$ SEM.

Table 1. Oligos utilized for realtime RT-PCR

Gene	Sequence 5'-3'	Gene	Sequence 5'-3'
GAPDH	AACTTTGGCATTGTGGAAGG	MafA	CTCCTCCAAGCGCACGTGGT
	GGATGCAGGGATGATGTTCT		TTCAGCAAGGAGGAGGTCAT
amylase	TGCAGACACTACTTGTGGCA	MafB	CAGGGTTTCTGAGCTTCTCC
	GCAACCATGTTCCTGATTTG		CAGGGTTTCTGAGCTTCTCC
insulin	CAGCAAGCAGGTCATTGTTT	NeuroD1	CAGCATCAATGGCAACTTCT
	GGGACCACAAAGATGCTGTT		GAAGATTGATCCGTGGCTTT
glucagon	ACATCTCGTGCCAGTCACTT	Nkx6.1	ACTTGGCAGGACCAGAGAG
	CGTTGGGTTACACAATGCT		GCGTGCTTCTTTCTCCACTT
SS	CCCAGACTCCGTCAGTTTCT	Ngn3	CAGGGTTTCTGAGCTTCTCC
	ACTTGGCCAGTTCCTGTTTC		GGGAAGGGTAACGACTTGAA
PP	ATGACCCAGGCGACTATGC	Myt1	GGGAGCTACAGACCCAATGT
	TGTATCTGCGGAGCTGAGTT		GAACTTCTCCAGCTCCTTGG
Glut2	AGCAACTGGGTCTGCAATTT	Pax4	AGTGTACCCTCAGCTGCCTT
	AAGAACACGTAAGGCCCAAG		GGTGTCACTGGAACATCTGC

Results

Knock-in reporter mice reveal Ngn3 expression in adult islet cells

Three independent *Ngn3* knock-in mice were used to examine *Ngn3* expression in the adult pancreas (Figure 5.1A-B). The $Ngn3^{tTA}$ line, in which an *IRES-tTA-PolyA* cassette replaced the endogenous Ngn3 coding sequences, was generated by our collaborator (Wang et al., 2009). They found that in combination with a tTA reporter line $TetO^{LacZ}$, the pancreata of 9-week-old $Ngn3^{tTA}$;$TetO^{LacZ}$ animals (without Doxycycline) contained large proportions of islet cells expressed robust levels of β-Gal, suggesting *Ngn3* expression is maintained in adult islet cells (Wang et al., 2009). We obtained the same findings by using an $Ngn3^{CreERT}$ allele in which the 5' 150 base pairs of the *Ngn3* coding region were replaced by $CreER^{T}$ cDNA (Wang et al., 2008b). $CreER^{T}$ remains cytoplasmic and inactive, and unable to recombine LoxP sites in the absence of tamoxifen (TM). The conditional $R26R^{EYFP}$ (Srinivas et al., 2001) reporter line was used to monitor for the presence of $CreER^{T}$. In $R26R^{EYFP/+}$ mice, enhanced yellow fluorescent protein (EYFP) is ubiquitously expressed under *Rosa26* promoter control, but in a strictly Cre-dependent manner. In $Ngn3^{CreERT};R26R^{EYFP}$ adult mice, no pancreatic cells expressed EYFP without TM (Johansson et al., 2007). Seven days after the administration of TM to 7-week-old adult $Ngn3^{CreERT};R26R^{EYFP}$ mice, up to 4.5% of the 4 major islet cell types expressed EYFP (Figure 5.1 C1-4).

The above findings led us to examine enhanced green fluorescent protein

(EGFP) expression in the pancreata of *Ngn3*^{EGFP} knock-in mice (Lee et al., 2002), a line in which EGFP expression was reportedly absent in the adult pancreas (Joglekar et al., 2007; Lee et al., 2002). By using confocal microscopy, weak yet visible EGFP fluorescence (enriched in nuclei) was seen in a large number of islet cells from *Ngn3*^{EGFP} animals at 2, 4, and 6 months of age. A rabbit anti-EGFP antibody further verified the presence of EGFP in a large portion of adult islet cells (Figure 5.1 D1-2). Notably, no exocrine cells were found to express EGFP (Figure 5.1 D1-2). Not all islet cells exhibited EGFP signals. It is not clear whether this lack of EGFP signal in all islet cells is due to limited EGFP detection sensitivity or variation in *Ngn3* expression between different islet cells.

Ngn3 mRNA and protein can be detected in hormone-expressing islet cells.

The above analyses demonstrate that *Ngn3* expression is maintained in the adult pancreas, albeit at low levels, and with the caveat that all 3 knock-in alleles studied inactivate *Ngn3* and thus may exhibit an *Ngn3* haploinsufficiency phenotype. Additionally, because there could be a time lag between *Ngn3* activation (as represented by CreER^{T} or tTA expression) and the EYFP and β-Gal production, it is not clear whether *Ngn3* expression is restricted to differentiated islet cells or putative islet progenitors (which express *Ngn3* and quickly relocalize to the islets) or both. For this reason, we sought to directly examine the expression of *Ngn3* in *WT* adult islet cells by using RT-PCR, protein blot, and immunofluorescence (IF) methods in 2-month-old mice.

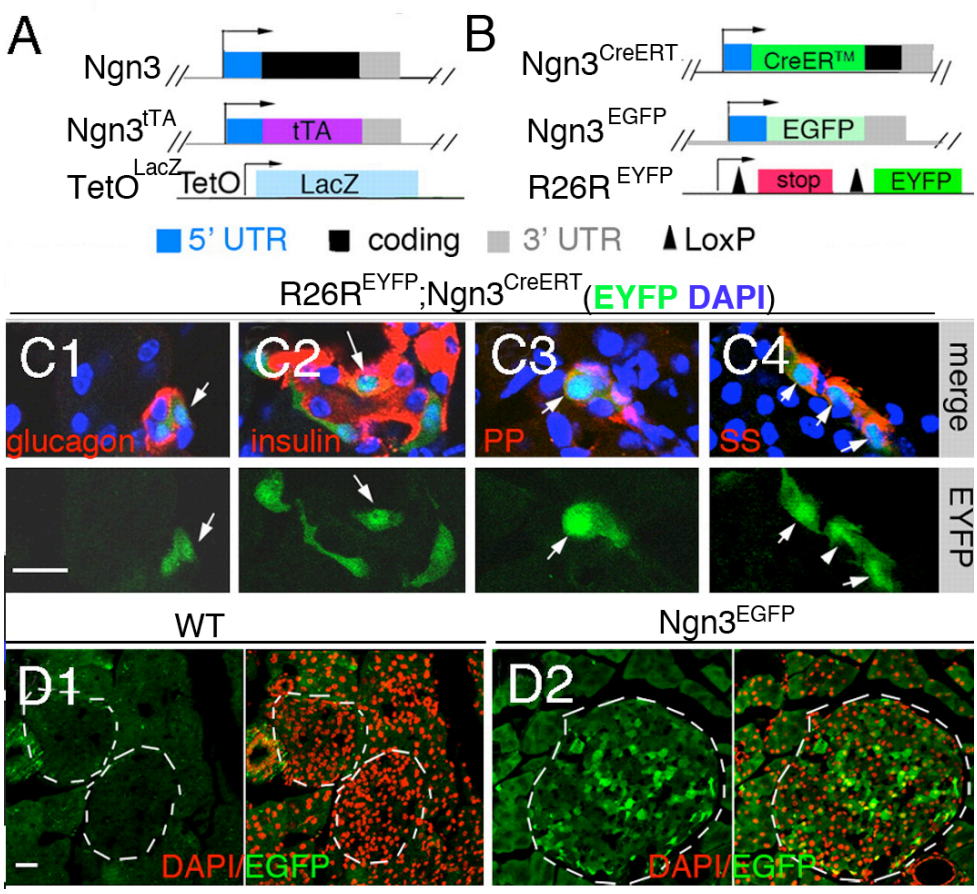


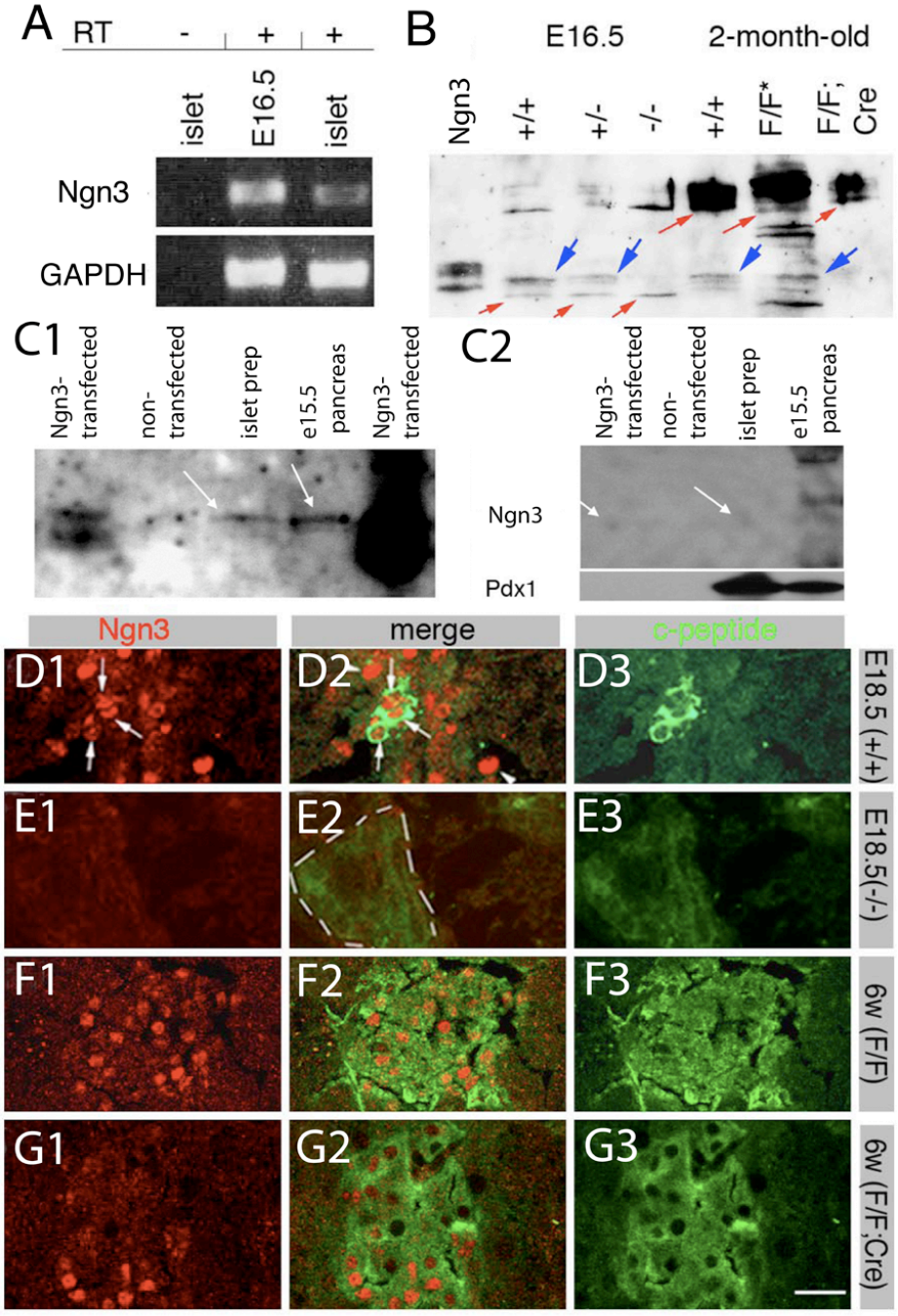
Figure 5.1 Knock-in reporter mice reveal Ngn3 expression in adult islet cells. (A&B) Knock-in and reporter alleles. *TetO*^{LacZ} and *R26*^{REYFP} were reporter alleles of tTA and Cre, respectively. (B) Coexpression of endocrine hormones (red) with EYFP in 8-week-old *R26R*^{EYFP}*;Ngn3*^{CreERT} males that received TM at 7 weeks of age. Two panels (as a column) for each staining were shown: a merged image of EYFP (green), hormone (red), and DAPI (blue) signal, and a single channel of EYFP. Arrows indicated EYFP^{+}hormone^{+} cells. (D) EGFP recognized by a rabbit anti-EGFP antibody in the 6-month-old *Ngn3*^{EGFP} pancreas. Only EGFP alone and EGFP-DAPI merged images were shown. Scale bars: 20μm.

Adult islets were isolated and analyzed for *Ngn3* transcription. Consistent with published findings (Johansson et al., 2007; Kodama et al., 2005), *Ngn3* transcripts were readily detected by RT-PCR in the *WT* adult islet cells (Figure 5.2A). When blotted with a guinea pig anti-Ngn3 or a rabbit anti-Ngn3 antibody (Gu et al., 2002; Seymour et al., 2007), multiple protein bands were detected in islet nuclear extract (Figure 5.2 B). The mobility of the protein closely matched that of recombinant Ngn3 produced in HEK293 cells and Ngn3 in E15.5 or E16.5 *WT* pancreatic buds (Figure 5.2 C1). Importantly, the putative Ngn3 bands were absent in the $Ngn3^{-/-}$ pancreas and substantially reduced in the islets of $Ngn3^{F/F};Ins2^{Cre}$ adults, where *Ngn3* should be inactivated in most, if not all, β cells (see below). Consequently, preabsorbing the Ngn3 antibodies by using Ngn3 produced in HEK293 cells caused a specific block in the detection of the Ngn3 signal (Figure 5.2 C2). Interestingly, Ngn3 produced in transfected cells resolved into 4 major bands, whereas the putative Ngn3 in isolated islets and in embryonic pancreases appeared as 3 species (Figure 5.2 B). Although the presence of multiple Ngn3 species suggests the possibility of posttranslational modification of Ngn3, neither the basis nor implications of this finding are currently known.

IF-based assays on mildly fixed pancreatic cryosections (see Materials and Methods) using a guinea pig anti-Ngn3 antibody showed that a substantial number of hormone-expressing cells expressed Ngn3 at E18.5 and in the adult pancreas (Figure 5.2 D&F). Notably, 2 types of $Ngn3^{+}$ cells were found in the E18.5 pancreas. A small population of cells (≈2–5% of all endocrine clusters)

produced a relatively higher level of Ngn3 based on their brighter IF signals (Figure 5.2 D). These cells did not express detectable insulin C-peptide or other islet hormones. A majority of cells expressed a low level of Ngn3 and co-expressed hormones. As expected, the *Ngn3*$^{-/-}$ pancreas showed no Ngn3 signal in any cells, verifying the specificity of the guinea pig anti-Ngn3 antibody (Figure 5.2 E). Similar to E18.5, large portions of islet cells in adult *WT, Ngn3*$^{F/F}$, and *Ins2*^{Cre} pancreata expressed *Ngn3* (Figure 5.2 F). In some cells the Ngn3 nuclear signal appeared higher than in others, yet we could not find any cells expressing Ngn3 at a level as high as that in embryonic endocrine progenitors. We verified the Ngn3 signal by inactivating *Ngn3* in *Ngn3*$^{F/F}$*;Ins2*^{Cre} animals. This arrangement allows β cells to be generated, yet not produce *Ngn3*. Indeed, the number of Ngn3^{+} islet cells was largely reduced, although some Ngn3^{+} cells remained at the periphery of islets (presumably non-β cells, or β cells escaping recombination) (Figure 5.2 G). Because of the light fixation, endocrine hormone signals appeared diffuse, and we could not identify the exact islet cell type(s) that express Ngn3 by using the guinea pig antibody. We also examined Ngn3 production in the adult islet cells by using a mouse monoclonal Ngn3 antibody (Zahn et al., 2004) and a rabbit anti-Ngn3 antibody (Gu et al., 2002). Both antibodies produced high background in lightly fixed postnatal islets, making it impossible to discern whether a true Ngn3 signal existed.

Figure 5.2 Differentiated islet cells express *Ngn3*. (A) RT-PCR detection of *Ngn3* mRNA in 2-month-old *WT* (+/+) islets and E16.5 embryonic pancreata. GAPDH expression was used as RT control. (B) Western blot detection of Ngn3. Recombinant Ngn3 produced in HEK293 cells, *WT* (+/+), and $Ngn3^{+/-}$ (+/−) E16.5 embryonic pancreata were used as positive controls. E16.5 $Ngn3^{-/-}$ (−/−) total pancreata and 2-month-old $Ngn3^{F/F};Ins2^{Cre}$ (F/F; Cre) islets were used to verify the specificity of the Ngn3 antibodies. All pancreatic samples except the $Ngn3^{F/F}$ lane (F/F, labeled with *) were nuclear extract. Note the presence of the nonspecific bands (red arrows), which served as internal loading controls of total proteins. The Ngn3 proteins were marked with blue arrows. (C1) Western blot of two-month-old islet cells with rabbit anti-Ngn3. Nuclear extracts from HEK293 cells transfected or nontransfected with Ngn3-producing plasmids and *WT* E15.5 pancreatic tissues were used as controls. White arrows pointed to the putative Ngn3 protein bands. (C2) Western blot using guinea pig anti-Ngn3 that was preabsorbed with Ngn3 expressed in HEK293 cells. Note the lack of Ngn3 signal in the positive control (HEK293 expressing Ngn3) and islet cells samples (at the position pointed by the arrows). Also note that after prolonged exposure, the lane containing the whole E15.5 pancreatic bud lysate produced nonspecific signals. The blot used Pdx1 antibody as a loading control. (D-G) IF detection of Ngn3 in embryonic (E18.5) and 6-week-old pancreata. C-peptide co-staining localized the endocrine compartment. Also note that the $Ngn3^{High}$ cells in D2 (arrowheads) did not express insulin c-peptide, but some $Ngn3^{low}$ cells did (white arrows). Broken lines in E2 showed a duct. Scale bars: 20μm.


A
RT
-
+
+
islet
E16.5
islet
Ngn3
GAPDH
B
E16.5
2-month-old
Ngn3
+/+
+/-
-/-
+/+
F/F*
F/F; Cre
C1
Ngn3-transfected
non-transfected
islet prep
e15.5 pancreas
Ngn3-transfected
C2
Ngn3-transfected
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Pdx1
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c-peptide
D1
D2
D3
E18.5 (+/+)
E1
E2
E3
E18.5(-/-)
F1
F2
F3
6w (F/F)
G1
G2
G3
6w (F/F;Cre)

Ngn3 expression is maintained in all islet cell types

To understand the potential functional significance of *Ngn3* expression in hormone^{+} cells, we determined which islet cell type(s) maintains *Ngn3* expression. We chose EGFP production as a marker for *Ngn3* expression. Because EGFP has a half-life of ≈26 h in mouse cells (Corish and Tyler-Smith, 1999), detectable EGFP is unlikely to survive beyond 2 months after birth if *Ngn3^{EGFP}* expression is silenced after birth. EGFP can survive harsh fixation, which is required for clear cellular resolution detection of hormones. In 2- and 6-month-old pancreata, double immuno-IF assays showed that all islet cell types maintained *Ngn3* expression (Figure 5.3 A), suggesting that *Ngn3* may have a general role for endocrine cell maintenance, either for survival or function. We also examined whether any hormone^{-} (negative) cells expressed Ngn3, which may potentially represent adult islet progenitor cells. Only rarely did we find EGFP^{+} cells remain hormone^{-} (≈1% of total EGFP^{+} cells; Figure 5.3 B). At present, we do not know whether these EGFP^{+}hormone^{-} cells are putative endocrine progenitors.

Ngn3 plays a functional role in newly born β Cells.

We next determined whether the expression of *Ngn3* in differentiated β cells is essential for islet functions. *Ngn3* was efficiently inactivated in newly born insulin-expressing cells in *Ngn3$^{F/F}$;Ins2^{Cre}* animals (Figure 5.2 G) via Cre recombination, which we verified to be restricted to insulin-producing β cells (Gannon et al., 2000b) (Data not shown). *WT, Ins2^{Cre}*, and *Ngn3$^{F/F}$* animals were

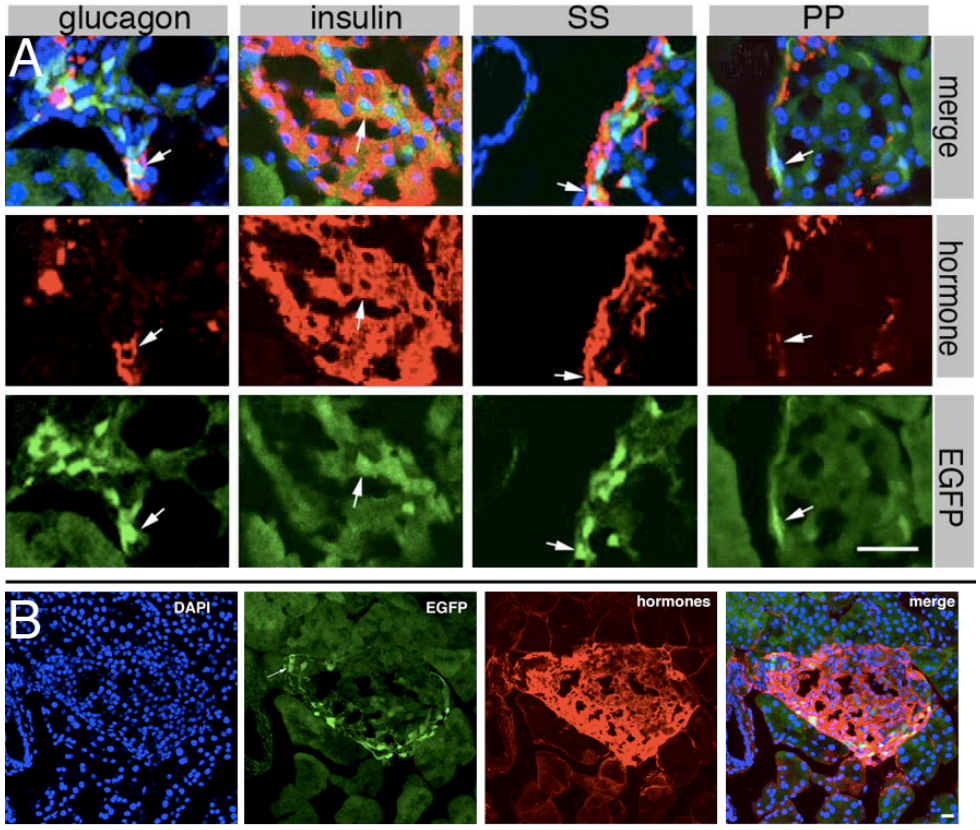


Figure 5.3 *Ngn3* expression is maintained in all islet cell types. (A) Co-expression of EGFP with endocrine hormones in three-month-old *Ngn3*^{EGFP} pancreata. Note that all 4 major islet cell types expressed detectable EGFP. White arrows pointed to examples of EGFP^{+}hormone^{+} cells. In the "merge" channel, DAPI signal (blue) was included to show the enriched EGFP presence in nuclei. Scale bars: 20μm. (B) Most EGFP^{+} cells in *Ngn3*^{EGFP} adult pancreata expressed endocrine hormones. Immunofluorescence (IF) staining in 2-month-old *Ngn3*^{EGFP} pancreata. Red fluorescence was a combination of insulin, glucagon, somatostatin and pancreatic polypeptide signals. Note that most EGFP^{+} cells expressed hormone. The white arrow showed an EGFP^{+}hormone^{-} cell. Also note the lack of EGFP^{+} cells outside the islet. Scale bar: 20μm.

used as controls. The fasting blood glucose levels of these 4 groups of animals showed no significant variation at 6 weeks of age (Figure 5.4 A). By 13 weeks after birth, male *Ngn3*$^{F/F}$*;Ins2*^{Cre} animals displayed significantly higher fasting blood glucose levels than the control groups (Figure 5.4 A), suggesting that loss of *Ngn3* in β cells compromises islet function. These findings demonstrate that *Ngn3* expression in insulin-producing β cells affects overall/global glucose homeostasis, which was most clearly evident in male animals.

During our analysis of *Ngn3* dosage (Chapter 4) on islet cell differentiation, we found that although glucose homeostasis in *Ngn3*$^{F/F}$ animals was normal, *Ngn3*$^{+/-}$and *Ngn3*$^{F/-}$adult animals displayed significantly impaired glucose tolerance. Thus, *Ngn3*$^{F/-}$ animals provided a sensitized background to examine the consequences of *Ngn3* deficiency in β cells. Indeed, six-week-old *Ngn3*$^{F/-}$*;Ins2*^{Cre} animals, in this case both males and females, displayed significantly higher fasting blood glucose levels compared with *Ngn3*$^{+/-}$*;Ins2*^{Cre} and *Ngn3*$^{F/-}$ control animals (Figure 5.3B). These findings further demonstrate that *Ngn3* expression in β cells contributes to endocrine function.

We also examined whether *Ngn3* deficiency in insulin^{+} cells affects the expression of *insulin, Glut2, Myt1, MafA, MafB*, *NeuroD1, Nkx6.1, Pax4* and *Pdx1* in E18.5 embryonic pancreata. These genes were chosen because their expression depends on *Ngn3* and they are known to play important roles in endocrine cell differentiation and function. E18.5 embryos were selected to examine the primary effect of *Ngn3* inactivation before the pancreatic function was required for postnatal life, which may feed back to affect gene expression.

We first scrutinized possible *Ins2*^{Cre} toxicity to β cells. E18.5 pancreata of *Ngn3*$^{+/-}$ and *Ngn3*$^{+/-}$*;Ins2*^{Cre} animals were collected for section-based immunoassays or quantitative RTPCR (QRT-PCR). IF staining and QRT-PCR results showed that the presence of Cre did not significantly affect the transcription of the above genes (Figure 5.5 A, B&J), demonstrating the lack of overt Cre toxicity on gene expression in β cells at this stage.

Protein production of the above genes in E18.5 *Ngn3*$^{F/-}$ and *Ngn3*$^{F/-}$*;Ins2*^{Cre} pancreata was analyzed. Qualitative evaluation from side-by-side IF staining suggested that the levels of insulin, glucagon, Glut2, MafB, Myt1, Nkx6.1 and Pdx1, on a per-cell basis, were not appreciably affected by loss of *Ngn3* in insulin-expressing β cells (Figure 5.5 C–H), whereas MafA expression was considerably reduced in the *Ngn3*$^{F/-}$*;Ins2*^{Cre} pancreas compared with controls (Figure 5.4 C&D). NeuroD1 and Pax4 were not examined because of the lack of suitable, well-characterized antibodies.

QRT-PCR assays showed that *Glut2* and *MafB* mRNA levels were not significantly affected by *Ngn3* inactivation in β cells. Yet the mRNA levels of *insulin, MafA, Myt1, NeuroD1,* and *Pax4,* all of which are required for β cell differentiation/maturation, in the *Ngn3*$^{F/-}$*;Ins2*^{Cre} pancreas, were significantly reduced compared with the *Ngn3*$^{F/-}$ pancreas (Figure 5.4 E). We could not detect *Ngn3* mRNA reduction at this stage (Figure 5.4 E), possibly due to the presence of a significant number of Ngn3^{+} progenitor cells whose high *Ngn3* expression could not be inactivated by *Ins2*^{Cre}. Because we did not detect significant β cell mass variation between *Ngn3*$^{F/-}$*;Ins2*^{Cre} and *Ngn3*$^{F/-}$ animals (Figure 5.5 I), this

reduction in gene expression indicated that the loss of *Ngn3* in insulin-producing cells delayed the maturation of the β cells (Nishimura et al., 2006). One notable finding here is that, although we detected significant reduction in *insulin* and *Myt1* mRNA levels in the $Ngn3^{F/-};Ins2^{Cre}$ animals over that of the controls, we did not observe any apparent decrease in either insulin or Myt1 protein by using confocal microscopy. The underlying reasons for this discrepancy are not known.

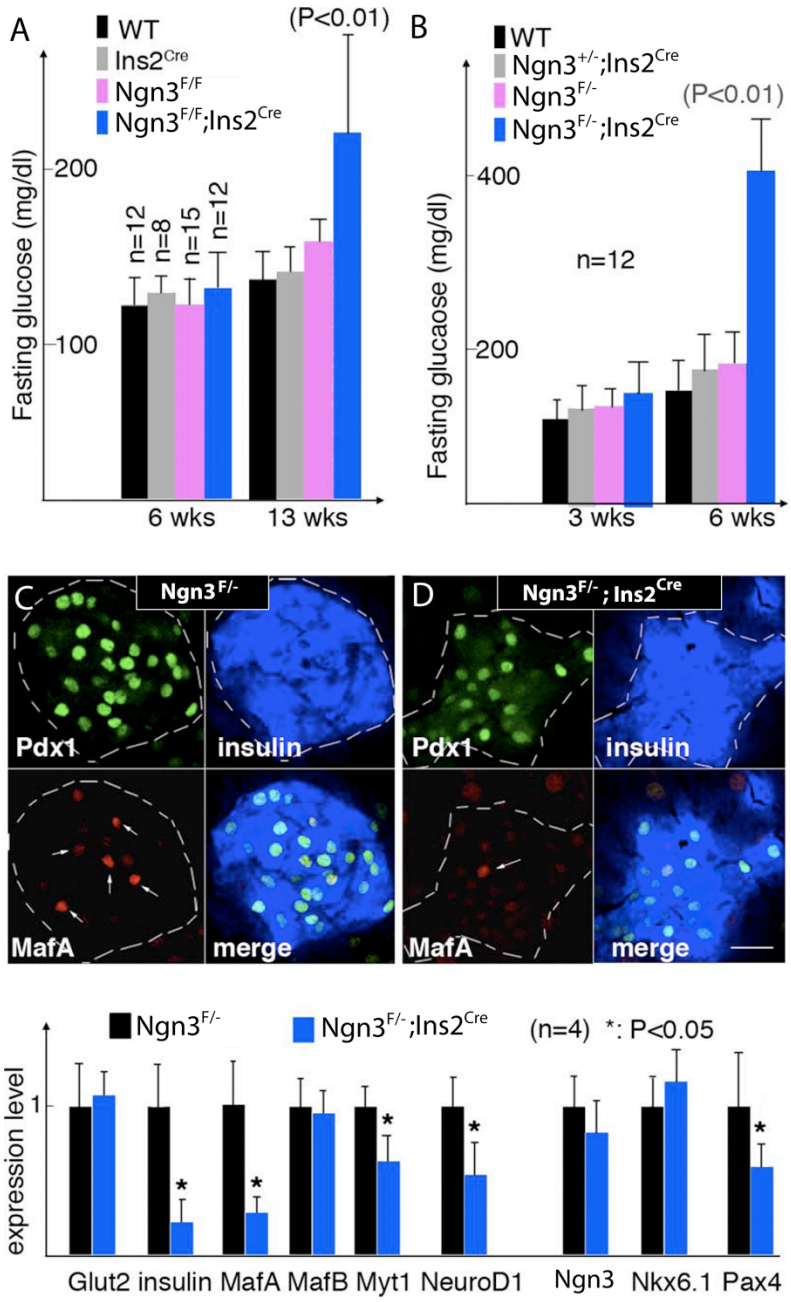


Figure 5.4 β-cell-specific *Ngn3* inactivation impairs endocrine function and endocrine gene expression. (A and B) Fasting blood glucose levels in males. The genotypes and ages of animals were labeled. "P" was calculated between the *Ngn3*$^{F/F}$ and *Ngn3*$^{F/F}$*;Ins2*^{Cre} mice in A and *Ngn3*$^{F/-}$ and *Ngn3*$^{F/-}$*;Ins2*^{Cre} animals in B. (C and D) Insulin, MafA, and Pdx1 expression in E18.5 *Ngn3*$^{F/-}$ and *Ngn3*$^{F/-}$*;Ins2*^{Cre} pancreata. Three single channels and a merged image were presented. Note the percentage of islet cells (Pdx1^{+}) expressing MafA (arrows). Scale bar: 20μm. (E) QRT-PCR analysis of the expression of several genes in *Ngn3*$^{F/-}$ and *Ngn3*$^{F/F}$*;Ins2*^{Cre}.

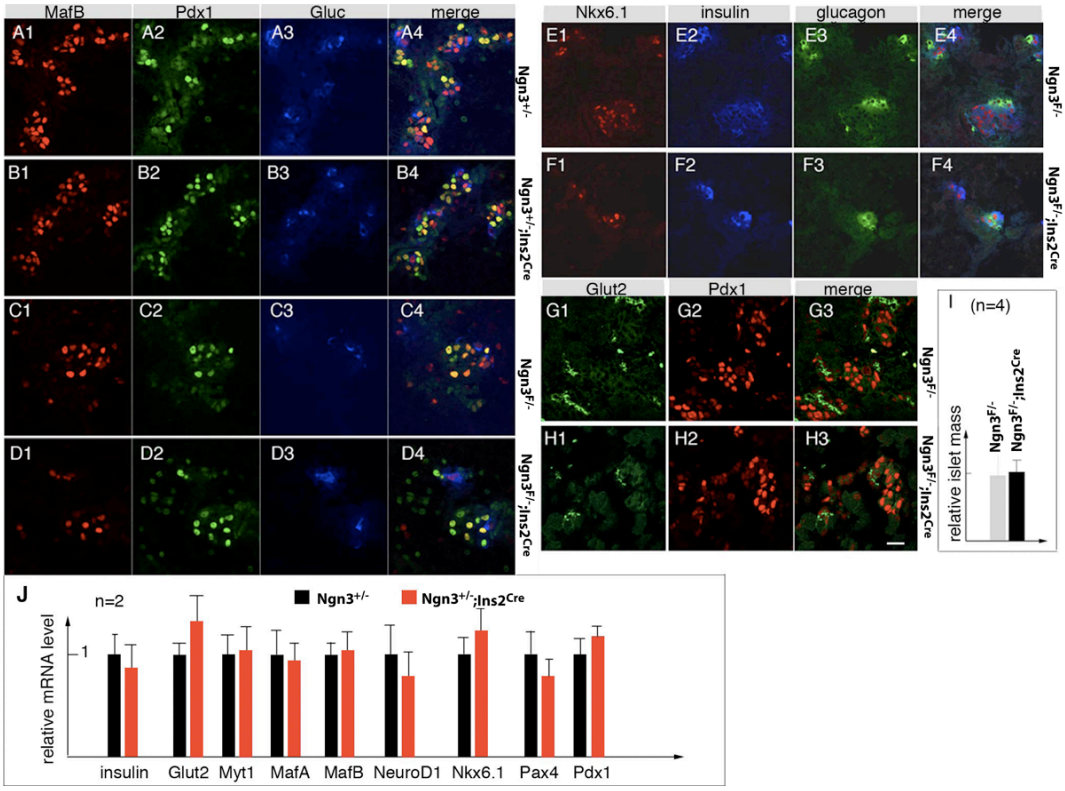


Figure 5.5 Loss of *Ngn3* activity in β cells does not affect the expression of most islet-specific genes (all data at E18.5, except in I)**.** (A and B) The expression of MafB and Pdx1 was not changed in the presence or absence of *Ins2*^{Cre}. (C–H) The expression of several factors (MafB, Pdx1, Glucagon, Nkx6.1, insulin) after *Ngn3* inactivation in β cells. (I) Islet mass in newly born *Ngn3*$^{F/-}$ and *Ngn3*$^{F/-}$;*Ins2*^{Cre} mice. (J) QRT-PCR results of *Ngn3*$^{+/-}$ and *Ngn3*$^{+/-}$;*Ins2*^{Cre} pancreata. Scale bar: 20μm.

Sustained Ngn3 expression in young adult islets contributes to endocrine maintenance

We next examined whether sustained *Ngn3* expression in mature islet cells contributes to endocrine function/maintenance. We used the TM-inducible Cre deleter, $Pdx1^{CreERT}$ (Gu et al., 2002) for this study. One-month-old $Ngn3^{F/F};Pdx1^{CreERT}$ animals were administered with TM. At this stage, $Pdx1^{CreERT}$ based recombination was mostly restricted to insulin-expressing β cells (Gu et al., 2002). As controls, $Ngn3^{F/F};Pdx1^{CreERT}$ mice were administered with vehicle, and $Ngn3^{F/F}$ animals were administered with TM. Six weeks after TM administration, TM-administered male $Ngn3^{F/F};Pdx1^{CreERT}$ animals showed a trend of compromised glucose tolerance capability, although this glucose tolerance reduction did not attain statistical significance (Figure 5.6 A). Ten weeks after TM administration, TM-administered males, but not females, showed a significant decrease in glucose tolerance (Figure 5.6 A). We also tested the effect of *Ngn3* inactivation in $Ngn3^{F/-}$ animals, in which *Ngn3* haploinsufficiency renders them more sensitive to a loss of endocrine function. One-month-old $Ngn3^{F/-};Pdx1^{CreERT}$ animals were administered with TM. Four weeks later, TM-treated male animals displayed a significant ($P < 0.01$) increase in their fasting blood glucose levels compared with control littermates (Figure 5.6 B). The differences between the experimental and control groups increased with time, so that by 12 weeks after TM injection, hyperglycemia (a blood glucose level higher than 250 mg/dL) occurred in most of the TM-treated males but rarely in controls (Figure 5.6 B). Fasting blood glucose levels of female $Ngn3^{F/-};Pdx1^{CreERT}$ animals

did not significantly vary with or without TM administration (Figure 5.7 A, data at "0" min). However, intraperitoneal glucose tolerance testing (IPGTT) showed that 2 and 3 months after TM administration, TM-treated *Ngn3*$^{F/F}$;*Pdx1*^{CreERT} females displayed significantly decreased glucose tolerance compared with control animals (Figure 5.7 A). Consistent with this phenotype, post-glucose-challenge-serum-insulin levels in TM-treated *Ngn3*$^{F/-}$;*Pdx1*^{CreERT} males were significantly reduced 12 weeks after treatment compared with control animals (Figure 5.7 B).

Next we examined whether sustained *Ngn3* expression in the adult β cells regulates cell division and cell survival. Ten days after TM administration, both mitotic and cell-death indices remained similar in islets of *Ngn3*$^{F/F}$ and *Ngn3*$^{F/F}$;*Pdx1*^{CreERT} animals (Figure 5.7 D-F). These data suggest that *Ngn3* in mature islet cells regulates β cell function, but not cell division or cell death.

Finally, we investigated whether *Ngn3* regulates the same set of genes in adult β cells as in newly born insulin^{+} cells (see above). Ten days after TM administration in 1-month-old *Ngn3*$^{F/F}$;*Pdx1*^{CreERT} animals, a time at which the animals still showed normal glucose homeostasis, gene expression in the islets was examined by QRT-PCR and IF. As expected, QRT-PCR showed that *Ngn3* mRNA in TM-treated *Ngn3*$^{F/F}$;*Pdx1*^{CreERT} islets was substantially reduced (Figure 5.8 A). The mRNA level of *insulin, Glut2,* and *MafA*, but not of *MafB, Myt1, NeuroD1, Nkx6.1, Pax4,* and *Pdx1,* was significantly down regulated (Figure 5.8 A). Accordingly, MafA and Glut2 protein levels in the TM-treated islets were significantly reduced (Figure 5.8 B&C). To our surprise, insulin IF staining did not appear reduced, despite a >3-fold reduction in insulin mRNA levels (Figure 5.8 B

&C). Because we could not detect statistically significant variation in insulin secretion in isolated islets of TM-treated and control *Ngn3*$^{F/F}$*;Pdx1*^{CreERT} animals 10 days after TM administration, we do not know the underlying reason for the discrepancy. Notwithstanding, these combined studies suggest that sustained *Ngn3* expression in adult islet cells is required to maintain a high level expression of some endocrine genes, which are necessary for proper endocrine function.

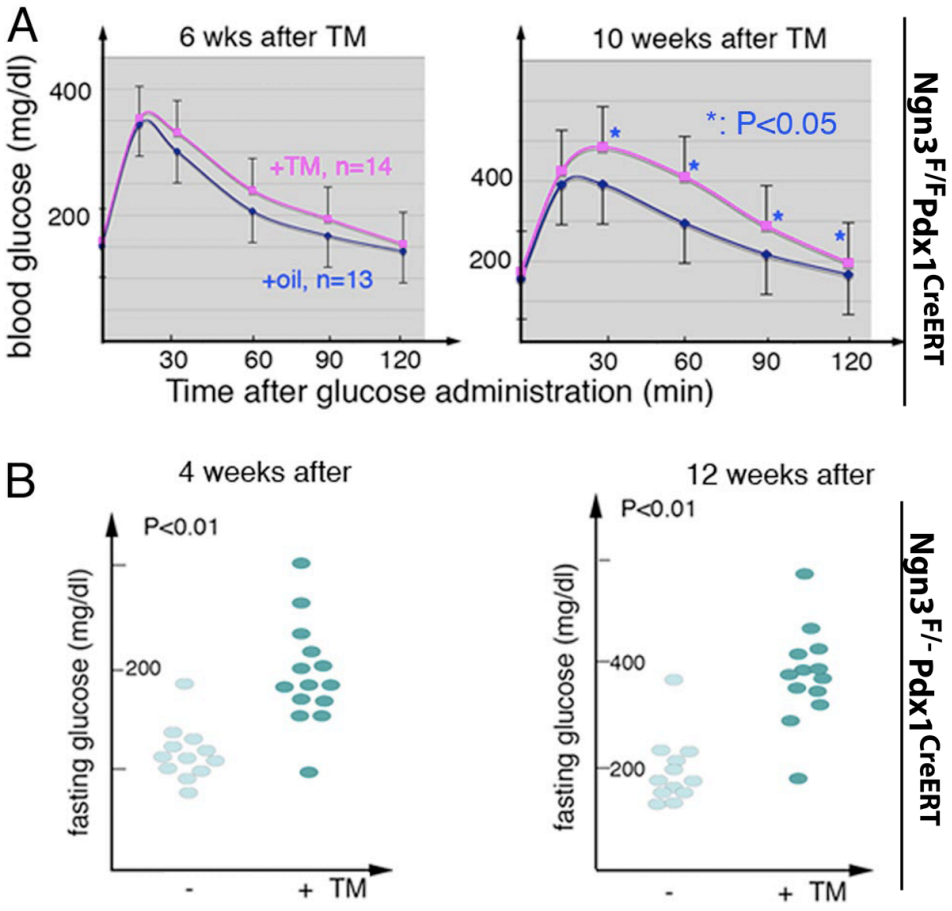


Figure 5.6 *Ngn3* inactivation in adult islet cells compromises endocrine function. (*A*) IPGTT assays after *Ngn3* inactivation in *Ngn3*$^{F/F}$;*Pdx1*^{CreERT} female animals. (*B*) Fasting blood glucose levels in male *Ngn3*$^{F/-}$;*Pdx1*^{CreERT} animals 4 and 12 weeks after *Ngn3* inactivation. Each dot represented one animal. Control animals received corn oil vehicle instead of TM.

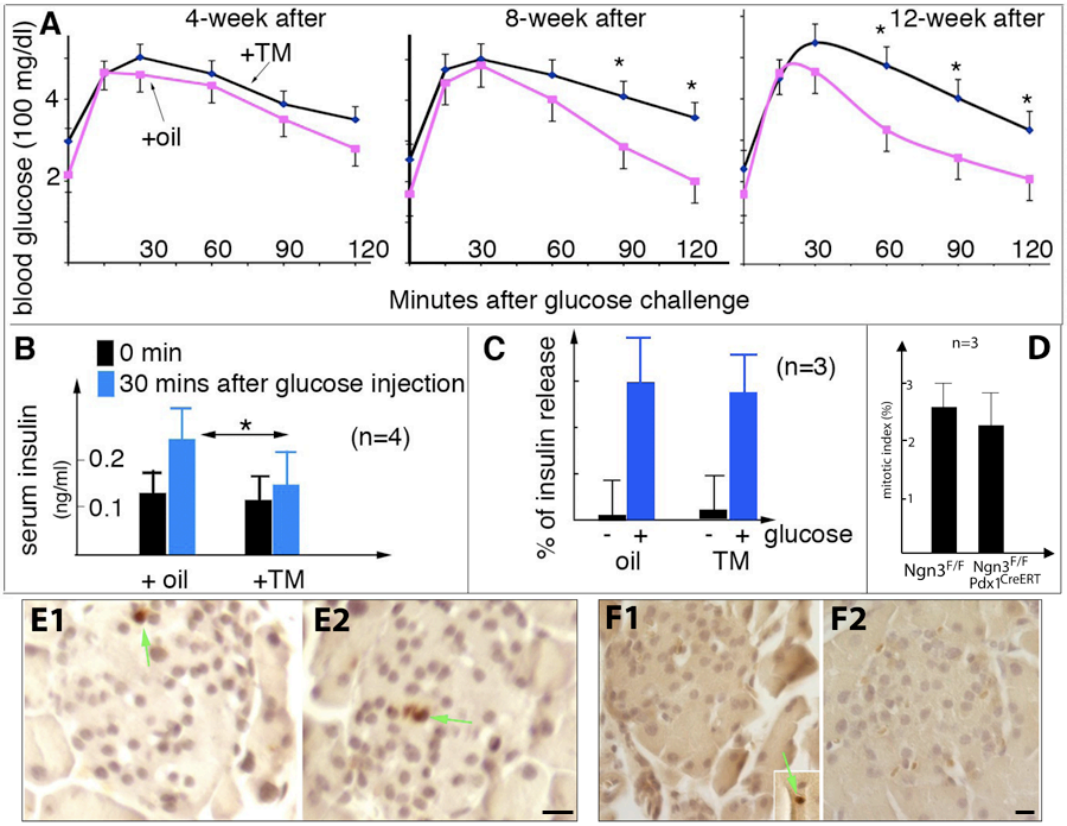


Figure 5.7 Loss of *Ngn3* in mature islet cells compromises endocrine function, but does not affect cell division or cell death. (A) Female *Ngn3*$^{F/-}$;*Pdx1*^{CreERT} animals were administered with TM or corn oil at 1 month of age. Intraperitoneal glucose tolerance testing (IPGTT) was performed 4, 8, and 12 weeks after TM administration (n=8). (B) Serum insulin assays were performed 12 weeks after TM administration on *Ngn3*$^{F/-}$;*Pdx1*^{CreERT} female animals. Serum was collected before and 30 min after glucose challenge. *, P<0.05, calculated based on the ratio of insulin increase in each individual animal between the 2 groups (+oil and +TM). (C) Glucose-induced insulin secretion (GSIS) in isolated islets ten days after TM administration in *Ngn3*$^{F/F}$;*Pdx1*^{CreERT} animals male animals. Data were presented as % of insulin release in one hour of glucose stimulation at 20 mM. (D) Quantification of mitotic index (n=3) of *Ngn3*$^{F/F}$ and *Ngn3*$^{F/F}$;*Pdx1*^{CreERT} animals administered with TM (by BrdU labeling). (E1-2) BrdU labeling in *Ngn3*$^{F/F}$ (E1) or *Ngn3*$^{F/F}$;*Pdx1*^{CreERT} (E2) animals administered with TM. Green arrows pointed to BrdU^{+} cells. (F1-2) Assay for apoptotic cell death. F1 and F2 used the same pancreatic tissues as in E1 and E2, respectively. Inset in F1 showed a dying cell (green arrow), positive for DNA fragmentation (stained brown). Scale bar: 10μm.

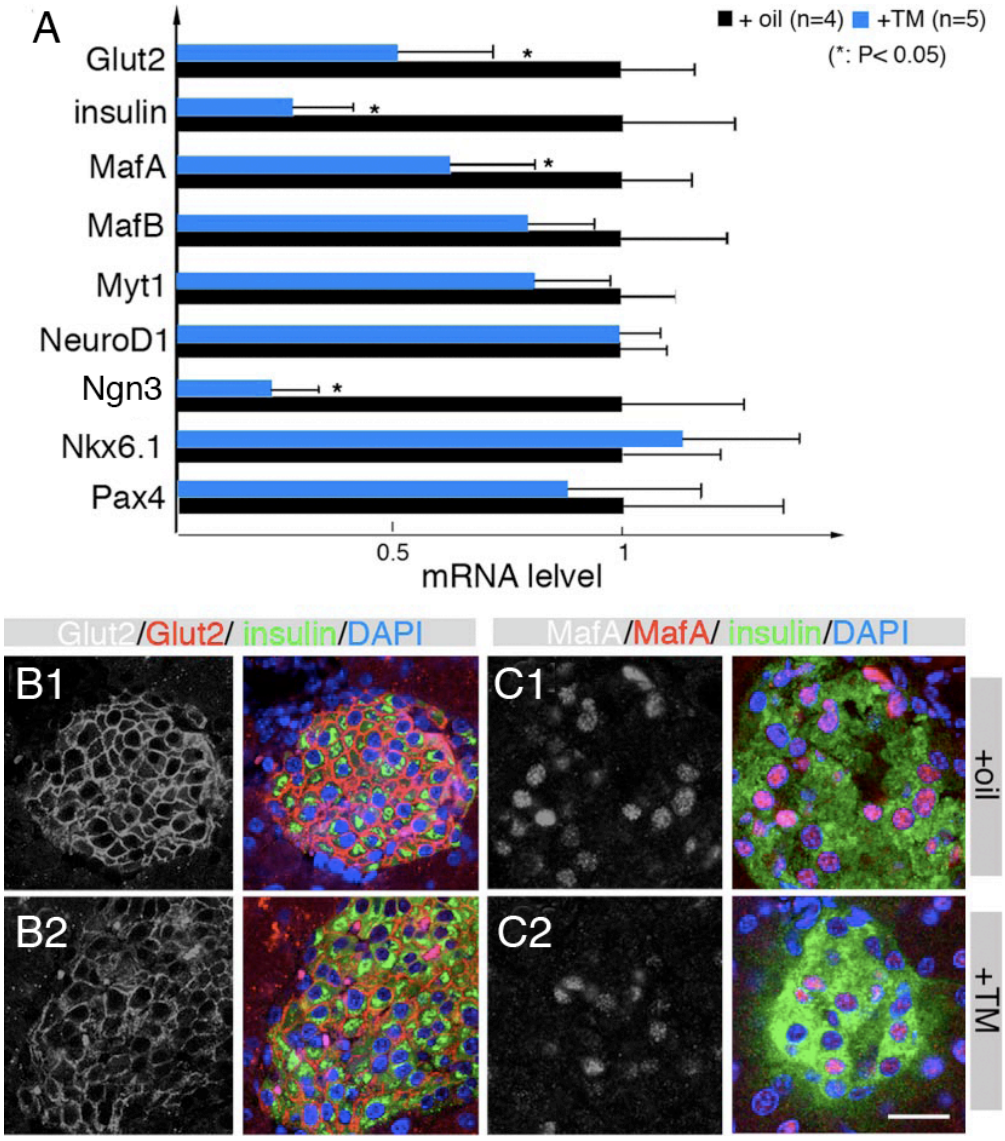


Figure 5.8 *Ngn3* inactivation in mature islet cells compromises insulin, Glut2 and MafA expression. Gene expression 10 days after TM administration in *Ngn3*$^{F/F}$;*Pdx1*^{CreERT} males was shown. (*A*) QRT-PCR analysis of gene expression in handpicked islets. (*B* and *C*) IF staining of Glut2 and MafA. For each tissue section, a single channel (white) was presented to highlight the relative intensity of Glut2 (B) or MafA (C) signals, respectively. A merged image was also presented to show the position of the stained islets (recognized by insulin signals). Scale bar: 20μm.

Discussion

During endocrine islet-cell differentiation, robust *Ngn3* expression was only detected transiently in hormone$^-$ endocrine progenitor cells (Xu et al., 2008). Thus, *Ngn3* expression has been proposed as an exclusive marker for islet progenitors in both embryonic and adult pancreata (Gu et al., 2002; Jensen et al., 2000a; Schwitzgebel et al., 2000). Although *Ngn3* mRNA expression in adult pancreata has been documented consistently (Dror et al., 2007; Gu et al., 2002; Joglekar et al., 2007; Kodama et al., 2005), the presence of Ngn3 protein in adult pancreata was only recently reported in a pancreatic duct ligation regeneration model (Xu et al., 2008). These data have led to the hypothesis that posttranscriptional regulation plays a role in controlling *Ngn3* activity (Joglekar et al., 2007; Villasenor et al., 2008), and only under specific conditions could Ngn3 be produced to facilitate neogenesis in the adult pancreas (Xu et al., 2008).

Here we present several independent lines of evidence to show that *Ngn3* is both transcribed and translated in a large portion of hormone-expressing islet cells at both embryonic and adult stages under normal physiological conditions. We show that *Ngn3* inactivation in β cells of newly born or adult mice results in down-regulation of the expression of several genes required for endocrine differentiation and function. The genes whose expression was most severely affected by *Ngn3* inactivation were *insulin* and *MafA*, 2 genes that are associated with β cell maturation. In contrast, the expression of *NeuroD1* and *Nkx6.1* was reduced only by smaller degrees, which suggests that, unlike in embryonic

progenitors where the expression of *Nkx6.1* and *NeuroD1* depends on Ngn3 activity (Gradwohl et al., 2000), expression of these factors is less dependent on Ngn3 in β cells.

It is presently unclear how *Ngn3* contributes to endocrine function after endocrine differentiation. Although our data clearly demonstrate the presence of Ngn3 transcript and protein in the adult mouse pancreas, the level of the protein is much lower than in endocrine progenitor cells at embryonic stages. It is likely that a transient, high *Ngn3* expression modifies the chromatin structure to allow cells to gain autonomy along the endocrine differentiation pathway (Apelqvist et al., 1999; Jensen et al., 2000b). A sustained low level of *Ngn3*, after full endocrine differentiation, allows cells to maintain their chromatin structure that favors the expression of genes required for islet cell function. By far, the expression of many endocrine-specific genes has been shown to depend on *Ngn3* (Petri et al., 2006; White et al., 2008). However, the absence of a highly specific and high-affinity Ngn3 antibody has prevented the comprehensive study of how Ngn3 interacts with it transcriptional target DNA sequences to regulate gene activity. To fully understand how Ngn3 regulates gene expression and islet cell function will likely require a comprehensive microarray-based expression analysis of sorted β cells, generation of high quality Ngn3 antibodies, and subsequent Ngn3-DNA promoter binding assays.

Overall, our findings reveal a previously unsuspected role for *Ngn3* in islet cell maturation and functional maintenance after initial endocrine differentiation. These results highlight the inherent deficiency when *Ngn3* expression is used as

an endocrine progenitor marker without examining whether the specific $Ngn3^{+}$ cells are mature endocrine islet cells (Gu et al., 2002; Xu et al., 2008). However, the detection of *Ngn3* expression in mature islet cells does not prevent the utilization of *Ngn3* expression as a marker for neogenesis in the adult pancreas, due to low *Ngn3* levels in the hormone-expressing cells. By separating $Ngn3^{high}$ and $Ngn3^{low}$ cells in the adult pancreas, it is possible to distinguish putative endocrine progenitor cells from differentiated islet cells (Xu et al., 2008). Finally, our findings raise the possibility that *Ngn3* over expression in mature islet cells could be used to boost endocrine islet function in diabetic conditions.

CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

Since the endocrine islet malfunction is highly associated with diabetes in humans, the differentiation/function of pancreatic islet cells has been an intense focus of study over the past half of century (Murtaugh and Melton, 2003; Oliver-Krasinski and Stoffers, 2008; Slack, 1995). The goal of these studies is to facilitate the production of functional mature endocrine cells *in vitro* or *in vivo* for transplantation or regeneration-based diabetes therapies respectively. Aided by ever improving technologies, these studies have revealed much about the endocrine islet differentiation, and made islet cell production *in vitro* or *in vivo* a possibility (Baetge, 2008; Bonner-Weir and Weir, 2005; Kroon et al., 2008; Murtaugh, 2007; Oliver-Krasinski and Stoffers, 2008; Yechoor et al., 2009; Zhou et al., 2008).

All pancreatic cells are derived from endodermal cells that are located in the gut epithelium. To differentiate into mature endocrine islet cells, these endodermal cells have to make multiple decisions. They need to go through stages of pancreatic progenitors, endocrine progenitors, hormone-expressing endocrine cells, and finally become functional mature endocrine cells, such as β cells that can secrete insulin in respond to stimuli (Oliver-Krasinski and Stoffers, 2008). A transcriptional network controls different phases of this multi-step process. For instance, *Pdx1*, *Ptf1a* and *Sox9* are required for the establishment

of multipotent pancreatic progenitors (Burlison et al., 2008; Gu et al., 2002; Kawaguchi et al., 2002; Offield et al., 1996; Seymour et al., 2007). *Ngn3* is essential for endocrine differentiation (Gradwohl et al., 2000; Gu et al., 2002; Schwitzgebel et al., 2000). *Pax4, Nkx2.2, Arx1* et al. are important for the specification/differentiation of different endocrine islet cell types (Naya et al., 1997; Oliver-Krasinski and Stoffers, 2008; Sosa-Pineda, 2004; Sussel et al., 1998). *MafA, Nkx6.1, Glut2* and *Pdx1* are necessary for the maturation and maintenance of insulin-expressing β cells (Ahlgren et al., 1998; Gannon et al., 2008; Guillam et al., 1997; Henseleit et al., 2005; Jensen et al., 2000a; Matsuoka et al., 2004; Nishimura et al., 2006; Zhang et al., 2005).

We are particularly interested in understanding how the identity of endocrine progenitors is established and regulated during pancreatic development, specifically, the endocrine progenitors expressing *Ngn3*. Our comprehensive studies showed that the interactions between Myt1 and Ngn3 promote the endocrine differentiation. Specifically, Myt1 and Ngn3 can enhance each other's expression and form a feed-forward expression loop to promote endocrine differentiation from endodermal progenitor cells in mice (Chapter 3 of this thesis work). Centering on this conclusion, we also investigated whether *Myt1* is required for endocrine differentiation and islet function by loss-of-function analyses. In *Myt1*$^{-/-}$ mutant pancreata, significant numbers of endocrine cells co-express multiple hormones during embryogenesis. *Myt1*$^{fl/-}$*;Pdx1-Cre* animals that have lost *Myt1* conditionally in the pancreas, although viable, develop glucose intolerance from 6 weeks of age. Thus, *Myt1* is important for proper endocrine

differentiation and islet function in mice. Interestingly, *Myt1* paralogs, *Myt1L* and *Myt3*, which are not expressed in the *WT* developing pancreas, are induced in the absence of *Myt1,* suggesting that these two genes may compensate for the loss of *Myt1* function (Chapter 2 of this thesis work). Furthermore, we examined the importance of maintaining proper Ngn3 levels within endocrine progenitor cells, and found that reduced *Ngn3* dosage shunts more $Ngn3^{+}$ cells to adopt exocrine fates, which in turn results in reduced endocrine mass by birth and impaired islet function in adults (Chapter 4 of this thesis work). Additionally, we revealed a very interesting but previously unsuspected role of *Ngn3* in islet cell maturation and functional maintenance (Chapter 5 of this thesis work). Taken together, these studies not only have answered some basic biological questions, but also provide important information to improve the generation of mature endocrine cells *in vitro*, for example, β cells, which may greatly favor diabetes therapies.

Future directions

The work outlined in this thesis aimed to understand how endocrine progenitors are established and regulated in the mouse pancreas, with the anticipation to benefit diabetes therapies in the future. Specifically, two transcription factors, Ngn3 and Myt1, were investigated by various approaches. However, we are just beginning to understand how *Myt1* and *Ngn3* function and

how they are regulated in the pancreas. Several important questions remain to be answered (Figure 6.4).

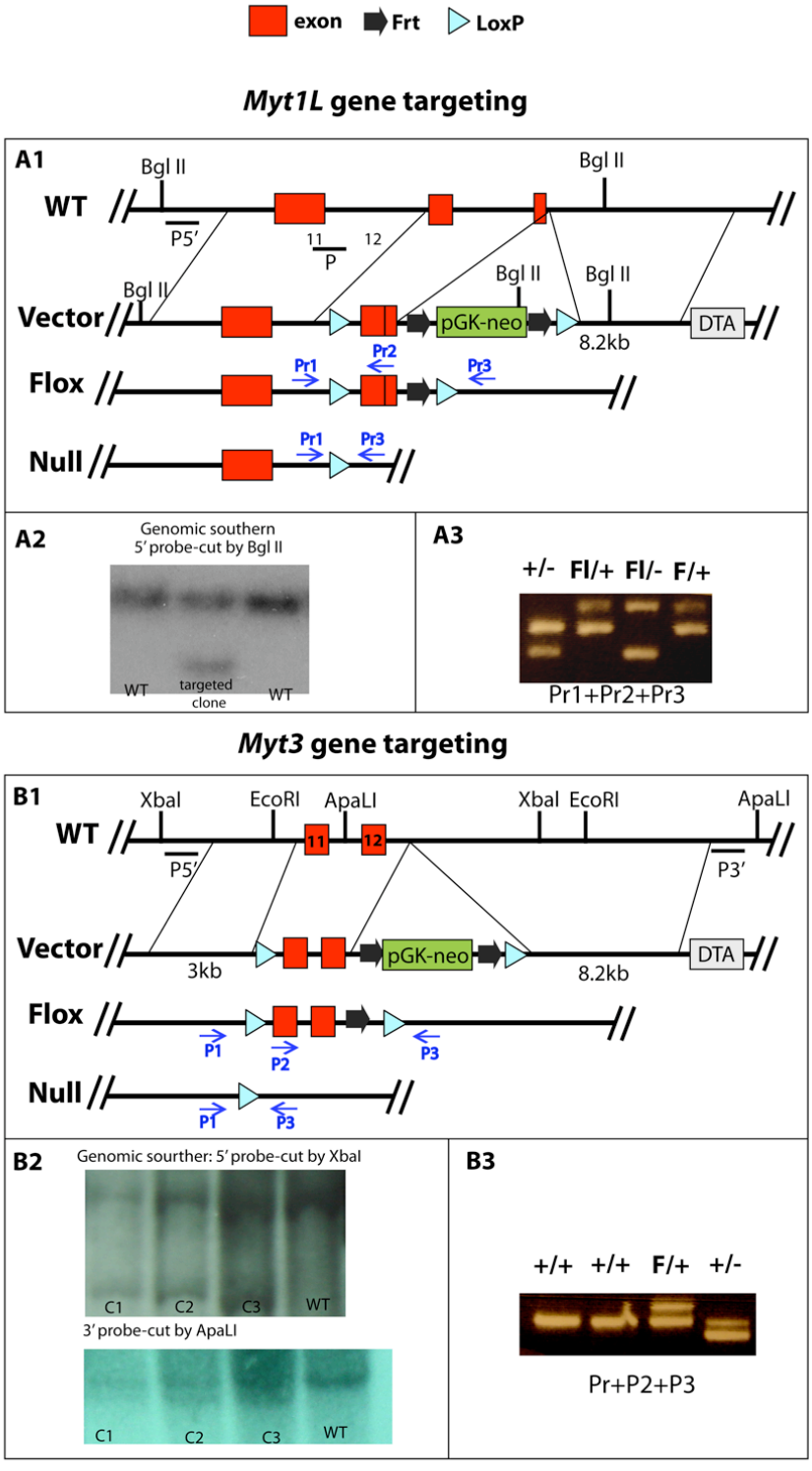
Is the Myt gene family required for endocrine differentiation and function?

Our studies suggest that *Myt1* is important for endocrine differentiation and islet function in the pancreas. However, this study is hampered by the unusual compensation between the *Myt* family members, that is the two paralogs of *Myt1*, *Myt1L* and *Myt3*. They are not normally expressed in the developing mouse pancreas, but induced when *Myt1* is inactivated. Therefore, potential functions of *Myt1* could be compensated by *Myt1L* and *Myt3* in the mouse pancreas.

We have now generated both *Myt1L* and *Myt3* conditional alleles (Figure 6.1). Subsequent crosses of these alleles with *Sox2-Cre* animals led to the production of $Myt1L^{-}$ and $Myt3^{-}$ alleles. As expected, immunofluorescence-based assays showed that both $Myt1L^{-}$ and $Myt3^{-}$ cells produced no functional proteins (Figure 6.2). These alleles will allow us to examine the consequences of inactivating all three Myt factors during pancreatic development and adulthood.

Our preliminary studies from several triple null mutants ($Myt1^{-/-};Myt1^{-/-};Myt3^{-/-}$) showed that although inactivation of Myt factors did not affect the number of endocrine islet cells produced in the developing pancreas, it compromised the expression of endocrine hormones and other well-established endocrine transcription factors (Figure 6.3). These results suggest that Myt factors may regulate endocrine cell maturation and function in the pancreas.

Figure 6.1 *Myt1L* and *Myt3* gene-targeting strategies. (A1) Derivation of *Myt1L*^{F} and *Myt1L*$^{-}$ alleles. Targeted Flox allele had two LoxP sites flanking exon 12 and 13 of *Myt1L*. In this case, the intron 12 was deleted in order to shorten the distance between the LoxP sites, which facilitated ready recombination. P5': DNA southern blot probe. Pr1, Pr2 and Pr3: oligos for genotyping. (A2) Southern blots of the targeted ES cell clone. *Bgl II* digestion and blotting with P5' probe produced a wild-type band of 14kb and a targeted band of 12kb. (A3) PCR-based genotyping using DNA oligos pr1, pr2 and pr3. (B1) Derivation of *Myt3*^{F} and *Myt3*$^{-}$ alleles. Targeted Fl allele had two LoxP sites flanking exon 11 and 12 of *Myt3*. P5' and P3': DNA southern blot probes. P1, P2 and P3: oligos for genotyping. (B2) Southern blots of 3 targeted ES cell clones. *XbaI* digestion and blotting with P5' probe produced a WT band of 6.8kb and a targeted band of 4.1kb. *ApaLI* digestion and blotting with P3' probe resulted in a WT band of 11.2kb and a targeted band of 9.6kb. (B3) PCR-based genotyping using DNA oligos p1, p2 and p3.


exon
Frt
LoxP
Myt1L gene targeting
A1
Bgl II
WT
P5′
11
12
P
Bgl II
Bgl II
Vector
Bgl II
pGK-neo
Bgl II
8.2kb
DTA
Pr2
Pr1
Pr3
Flox
Pr1
Pr3
Null
A2
Genomic southern
5′ probe-cut by Bgl II
WT
targeted clone
WT
A3
+/- Fl/+ Fl/- F/+
Pr1+Pr2+Pr3
Myt3 gene targeting
B1
XbaI
EcoRI
ApaLI
XbaI EcoRI
ApaLI
WT
11
12
P5′
P3′
Vector
3kb
pGK-neo
8.2kb
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P3
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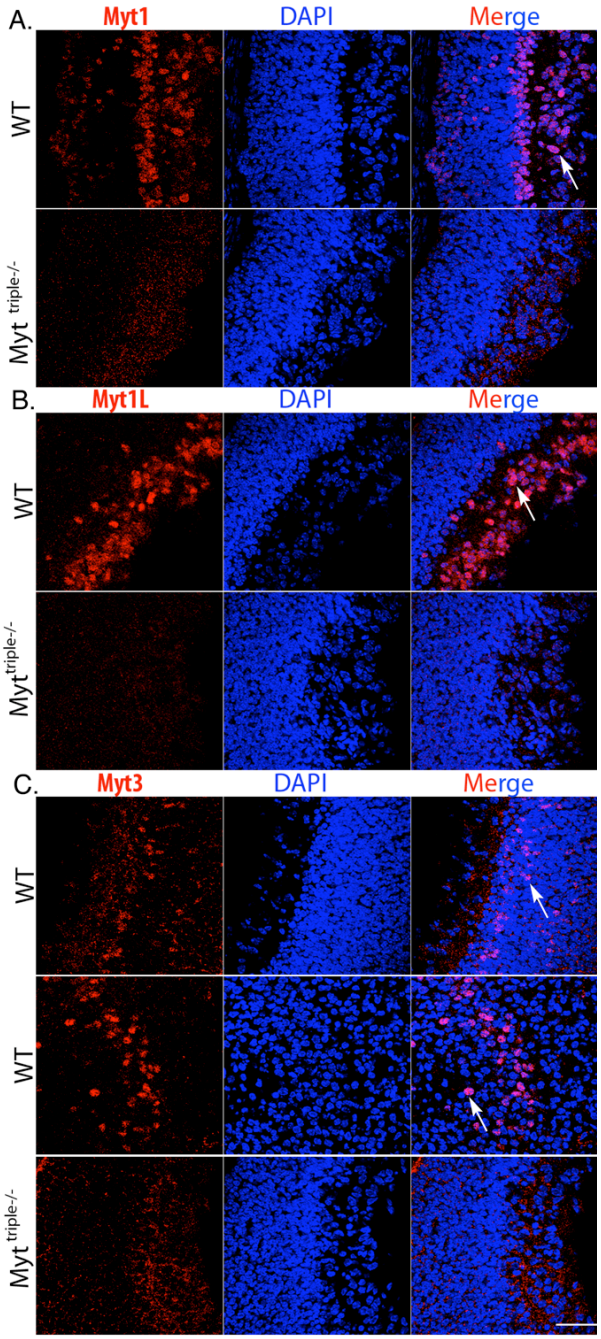

Figure 6.2 No Myt1, Myt1L and Myt3 protein was produced in *Myt*$^{triple-/-}$ embryos. *WT* and *Myt*$^{triple-/-}$ neural retina and brain tissues were stained with Myt1 (A), Myt1L (B) and Myt3 (C) antibodies. Blue: DAPI for nuclei. White arrows: Myt1^{+}, Myt1L^{+} or Myt3^{+} cells. Scale bar: 20μm.

Myt

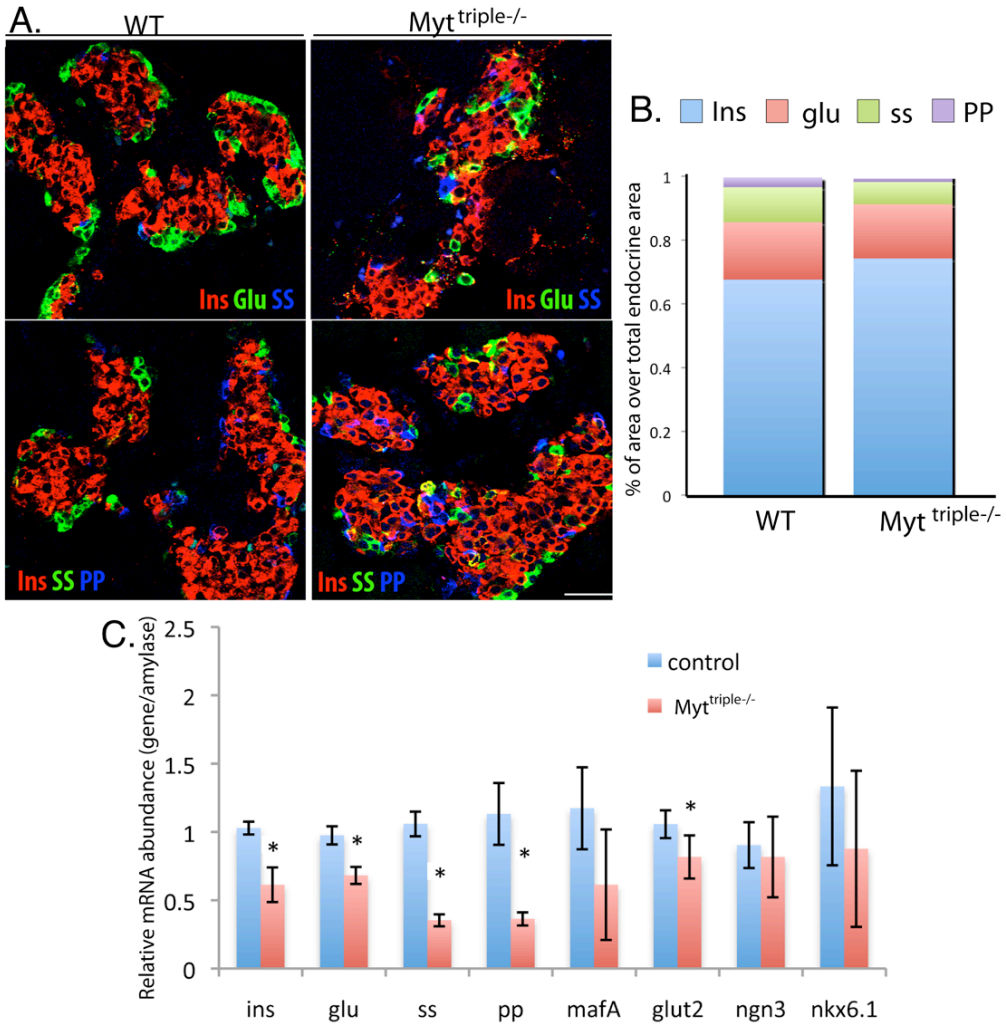

Figure 6.3 Loss of *Myt* gene family members attenuates hormone production in the mouse pancreas. (A) E17.5 *WT* and *Myt*$^{triple-/-}$ pancreata were stained with antibodies against for major endocrine hormones. Scale bar: 20μm. (B) Quantification of the number of each endocrine cell type at E17.5 (materials and methods in Chapter 3). The percentage of stained area for each endocrine cell type over total endocrine areas was calculated (n=2). (C) The mRNA levels of various genes in E17.5 control and *Myt*$^{triple-/-}$ pancreata (n=3, material and methods in Chapter 5). Controls: *Myt1*$^{+/-}$;*Myt1L*$^{+/-}$;*Myt3*$^{+/-}$, *Myt1*$^{+/+}$;*Myt1L*$^{+/+}$;*Myt3*$^{+/-}$ and *Myt1*$^{+/-}$;*Myt1L*$^{+/-}$;*Myt3*$^{-/-}$ (n=3). "*": Significant difference, P<0.01 by student t-test. Ins: insulin; Glu: glucagon; SS: somatostatin; PP: pancreatic polypeptide.

Future studies are needed to examine how losing Myt activity affects islet cell gene expression and endocrine function in a systematic fashion.

How do Myt factors regulate endocrine differentiation/function in the pancreas?

It is possible that Myt factors can directly bind to the promoters of hormone genes and activate their transcription. Alternatively, Myt factors may regulate hormone expression via regulating other transcription factors. The expression levels of some candidate genes, such as *Pdx1*, *IA-1* and *NeuroD*, could be examined in $Myt^{triple-/-}$ ($Myt1^{-/-};Myt1L^{-/-};Myt3^{-/-}$) pancreata to further explore this hypothesis.

It is also worth investigating whether the observed endocrine cells with reduced hormone production during embryogenesis can persist into adulthood, and contribute to adult islet function. Due to the immediate death of $Myt^{triple-/-}$ embryos after birth, pancreatic specific or β cell-specific *Myt* null animals are needed to address this question. If Myt factors could regulate hormone expression, we would expect the conditional null animals to develop diabetic phenotypes, such as impaired glucose tolerance, attenuated insulin secretion and GSIS (glucose stimulated insulin secretion), in their juvenile or adult lives. Additionally, *Myt1* can modulate the proliferation of oligodendrocyte lineage cells (Nielsen et al., 2004). It is possible that endocrine cells fail to proliferate efficiently in the absence of *Myt* genes in the first two weeks after birth, when

endocrine cells normally undergo extensive proliferation, or under conditions with increased insulin demands, i.e. during pregnancy or in obesity.

Furthermore, Myt genes may function differently in newly differentiated endocrine cells and adult mature islet cells. It is also intriguing to understand the role of *Myt* genes specifically in adult islets by deleting *Myt* genes in adult mice using CreERT-mediated recombination.

How do Myt factors function in the pancreas at the molecular level?

In order to understand how Myt factors function in the pancreas at the molecular level, it is important to explore the genes that act downstream of Myt and the factors that can cooperate with Myt to regulate gene transcription.

The downstream targets of Myt factors are largely unknown. Considering the compensatory regulation between *Myt* genes, it is possible that *Myt1* negatively regulates the expression of *Myt1L* and *Myt3* under normal conditions, either directly or indirectly. Therefore, when *Myt1* is inactivated, the expression *Myt1L* and *Myt3* is induced.

At present, Sin3B, which is a well-studied chromatin modifier that can regulate gene transcription by recruiting HDACs, is the only reported direct binding partner of Myt1 or Myt1L (Romm et al., 2005). It has been shown that Myt1-Sin3B (or Myt1L-Sin3B) complexes immunoprecipitated from transfected mammalian cells included HDAC1 and HDAC2. Moreover, Myt1 or Myt1L could bind with both the Sin3B long isoform ($Sin3B_{LF}$), which contains the HDAC-binding domain, and the Sin3B short isoform ($Sin3B_{SF}$) without the HDAC-binding

domain (Romm et al., 2005). Thus, Myt1 or Myt1L may mediate transcription repression or activation by forming complexes with different Sin3B isoforms, which could either recruit or preclude HDAC binding. In fact, Myt1 appears to function as a transcriptional activator during pancreatic endocrine differentiation in chickens (Wang et al., 2008b). It is highly possible that $Sin3B_{SF}$ is the predominant isoform expressed in the chicken/mouse pancreas, and can cooperate with Myt1/Myt1L to activate downstream target genes.

How does Myt1-Ngn3 gene cascade function in the endocrine progenitors?

Myt1 and Ngn3 form a feed-forward expression loop to promote endocrine differentiation during pancreatogenesis (Chapter 3). However, the underlying mechanisms are still unclear (Figure 6.4).

Ngn3 appears to be capable of activating *Myt1* transcription directly, in that Ngn3 significantly enhances *Myt1* promoter activity in cultured cell lines *in vitro* (Wang et al., 2008b). Yet, *Myt1* doesn't appear to be sufficient to directly activate *Ngn3* expression. The number of $Ngn3^+$ cells only moderately increased when *Myt1* is ectopically expressed. It is possible that Myt1 indirectly enhances *Ngn3* expression in endocrine progenitor cells through enhancing the expression of *Hnf6*, *Foxa2* and other unknown factors, which can positively regulate *Ngn3* expression.

In addition of enhancing each other's expression levels, Myt1 and Ngn3 may also cooperate to promote endocrine differentiation. In the chicken gut epithelium, the endocrine differentiation induced by ectopic *Ngn3* expression

under an exogenous promoter can be repressed by constitutively activated Notch signaling, suggesting that *Ngn3* is not the only Notch target required for endocrine differentiation. Additional factors, whose expression is inhibited by activated Notch, are also necessary for the cells to escape Notch repression. Myt1 may be one of these factors, in that ectopic expression of *Myt1 (xMyt1* in *Xenopus)* together with *Ngn3* (*xNgnr1* in *Xenopus*), but not individually, can overcome the repressive effects of activated Notch signaling on differentiation in both *Xenopus* and chicken studies (Ahnfelt-Ronne et al., 2007; Bellefroid et al., 1996). Myt1 and Ngn3 may also work together to antagonize the activated Notch signaling in the developing mouse pancreas. A rescue experiment, in which Myt1 and Ngn3 are ectopically expressed in pancreatic cells with constitutively activated Notch signaling, may help to test this hypothesis.

Does Myt1-Ngn3 gene cascade function in a similar fashion in differentiated islet cells?

In the pancreas, *Myt1* is highly expressed in most of the differentiated endocrine islet cells (Chapter 2). We revealed that *Ngn3* is maintained in most of the differentiated islet cells as well, albeit in a low level, compared with embryonic $Ngn3^{+}$ endocrine progenitors (Chapter 5). It is possible that Myt1 and Ngn3 also interact with each other in differentiated islet cells (Figure 6.4).

When *Ngn3* is inactivated specifically in the insulin-expressing β cells, the expression of *Myt1* is significantly reduced, suggesting that *Ngn3* is important for the maintenance of *Myt1* expression in β cells (Chapter 5). However, it is

unknown whether Ngn3 is sufficient to promote *Myt1* expression, and further enhance islet function in islet cells. We had tried to ectopically express *Ngn3* in adult β cells (*Ngn3*^{teto8};*Pdx1*$^{tTA/+}$, add Dox from E7-P28). Only a small percentage of β cells can achieve high levels of Ngn3. It is possible that Ngn3 levels are kept low in differentiated β cells by some unknown mechanisms. It would be important to understand whether increasing the level of Ngn3 in differentiated islet cells can enhance islet function, and whether it is through the enhancement of *Myt1* expression.

On the other hand, it remains to be clarified whether Myt factors are required for maintaining Ngn3 levels in differentiated islet cells. Examination of pancreatic specific or β cell specific Myt triple null mutants may help to address this question.

Furthermore, enlightened by the embryonic endocrine differentiation, in which activated Notch signaling negatively regulates *Ngn3* expression, we suspect that the Notch signaling pathway may also play important roles in differentiated islet cells. Fujikura et al. revealed that loss of *Rbp-j* (the key mediator of the Notch signaling pathway) specifically in β cells did not affect β cell number and function, suggesting that the Notch/Rbp-j signaling may not be required for maintaining β cell function or mass (Fujikura et al., 2006). Moreover, Murtaugh et al. showed that the constitutive activation of Notch signaling pathway in adult β cells did not perturb mature β cell function two weeks after the Notch activation (Murtaugh et al., 2003). Contrary to these studies, Notch receptors, ligands and targets are expressed in the adult mouse and human

islets, and function to suppress apoptosis in cultured mouse/human islets (Dror et al., 2007). Ectopic expression of the Notch signaling pathway in insulin-expressing β cells for a longer period, probably from embryonic stages to adulthood, may help to address this question, and further understand whether/how the Notch signaling pathway interacts with the *Myt1-Ngn3* gene cascade.

How does Ngn3 control the endocrine differentiation program?

Ngn3 controls the expression of a network of factors that are essential for endocrine differentiation. According to their response to *Ngn3* activation, the factors downstream of *Ngn3* can be divided into two major groups. The factors in the first group are mostly direct targets of *Ngn3*, and respond to *Ngn3* activation quickly, such as Nkx2.2, Pax4, NeuroD and IA-1. The factors in the second group respond to *Ngn3* activation slowly, such as insulin, MafA and Glut2. They help to determine the mature states of endocrine cells, and are mostly under the control of the factors in the first group. Many lines of evidence suggest that there are complicated interactions and crosstalks between these factors downstream of *Ngn3*. However, general views of this network, as well as many key points, remain to be explored.

It is largely unknown how different endocrine islet cell types are specified in the pancreas. Previous studies showed that the specification of islet cell types is not dependent on *Ngn3* (Johansson et al., 2007). It appears that the competence of pancreatic progenitors to generate different islet cell types

changes over time cell-autonomously, but what factors control this competence changing process are still not clear. We observed that the percentage of $Myt1^{+}Ngn3^{+}$ double positive cells over $Myt1^{+}$ and/or $Ngn3^{+}$ cells changes in consistent with the percentage of $insulin^{+}$ cells over total endocrine cells at various stages during pancreatic development (Yuhan Hao and Guoqiang Gu, unpublished data). It is possible that these $Myt1^{+}Ngn3^{+}$ double positive cells are destined to become insulin expressing β cells. Moreover, Myt1 may be competent to drive $Ngn3^{+}$ endocrine progenitors towards the β cell fate. Ectopic expression of *Myt1* in $Ngn3^{+}$ cells may help to address this question.

Furthermore, Ngn3 alone or in combination with other transcription factors, is capable of reprogramming differentiated/determined cells into insulin-expressing endocrine cells (Yechoor et al., 2009; Zhou et al., 2008). Pancreatic β cell neogenesis after PDL is dependent on *Ngn3* (Xu et al., 2008). However, it remains unknown whether *Ngn3* controls the endocrine neogenesis in the adult pancreas in a similar manner as during embryogenesis. It is possible that $Ngn3^{high}$ cells induced in the adult pancreas by virus mediated over-expression or injuries have similar properties and follow the same multi-step differentiation process as embryonic endocrine progenitors, as suggested by Xu et al. (Xu et al., 2008). Alternatively, induced $Ngn3^{high}$ cells in the adult pancreas may possess different properties and go through different differentiation process. To distinguish between these two hypotheses, it is important to identify the origin of the induced $Ngn3^{high}$ cells, compare their differentiation potentials with embryonic

endocrine progenitors, and examine the downstream transcriptional networks activated in these Ngn3^{high} cells.

Concluding Remarks

This work demonstrated that two transcription factors, Myt1 and Ngn3, form a feed-forward expression loop to promote endocrine differentiation in the pancreas (Figure 6.4). Centering on this conclusion, we further revealed that *Myt1* is important for the proper endocrine differentiation and islet function in the pancreas by loss-of-function analyses. Moreover, we provided direct evidence to show that proper levels of Ngn3 within endocrine progenitor cells are essential for endocrine differentiation. Reduced Ngn3 levels significantly impair endocrine differentiation and endocrine/exocrine allocation. Additionally, we revealed that sustained *Ngn3* expression in hormone-expressing islet cells is required for endocrine maturation and function. These studies not only provide important information regarding the regulation of endocrine differentiation/function, but also open up new directions to improve islet function under diabetic conditions.

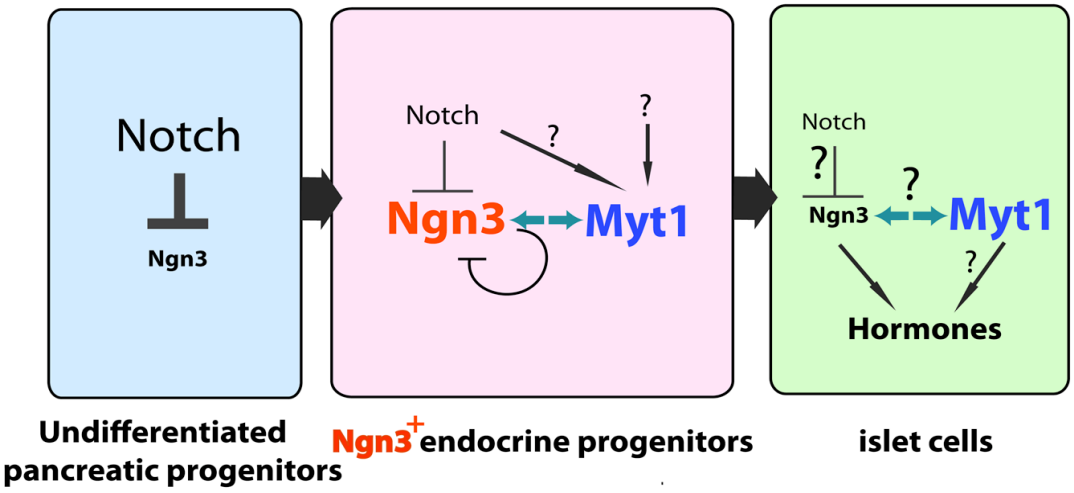

Figure 6.4 Myt1 and Ngn3 form a feed forward expression loop to drive endocrine differentiation in the mouse pancreas

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