EXAMINATION OF HNF6 AND DOWNSTREAM EFFECTORS IN POSTNATAL ENDOCRINE FUNCTION

By

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CHAPTER I

GENERAL INTRODUCTION

Significance

The pancreas is an endodermally-derived organ that is composed of two distinct tissue types (Figure 1). The exocrine portion constitutes approximately 98 percent of the adult pancreas and is comprised of acinar cells that secrete digestive enzymes into the ductal network (Figure 1B). The remainder of the pancreas consists of clusters of endocrine cells that are organized into islets of Lagerhans and scattered throughout the parenchyma (Figure 1C). These clusters contain 5 different hormone-producing cell types, including β (insulin), α (glucagon), δ (somatostatin), PP (pancreatic polypeptide), and ε (ghrelin) cells. Insulin is the predominant hormone produced by the pancreas, and it functions to regulate glucose homeostasis by communicating with peripheral tissues to modulate blood glucose levels. Diabetes mellitus results from inadequate levels of insulin, either due to an immune-mediated destruction of β cells (Type 1, T1D), or relative decreases in insulin production and secretion compounded by reduced insulin sensitivity in peripheral tissues (Type 2, T2D). The primary treatment for T1D is exogenous insulin therapy to maintain euglycemia; there has been limited success with transplants of cadaveric islets in which recipients achieve temporary insulin independence, but eventually the transplanted islets fail or succumb to autoimmunity. T2D can be treated with a variety of pharmaceuticals as well as exogenous insulin therapy. Drug therapies designed to enhance insulin sensitivity in peripheral tissues or decrease glucose output from the liver are often prescribed to treat T2D. Medicines targeted to pancreatic β cells that are designed to enhance insulin secretion, such as

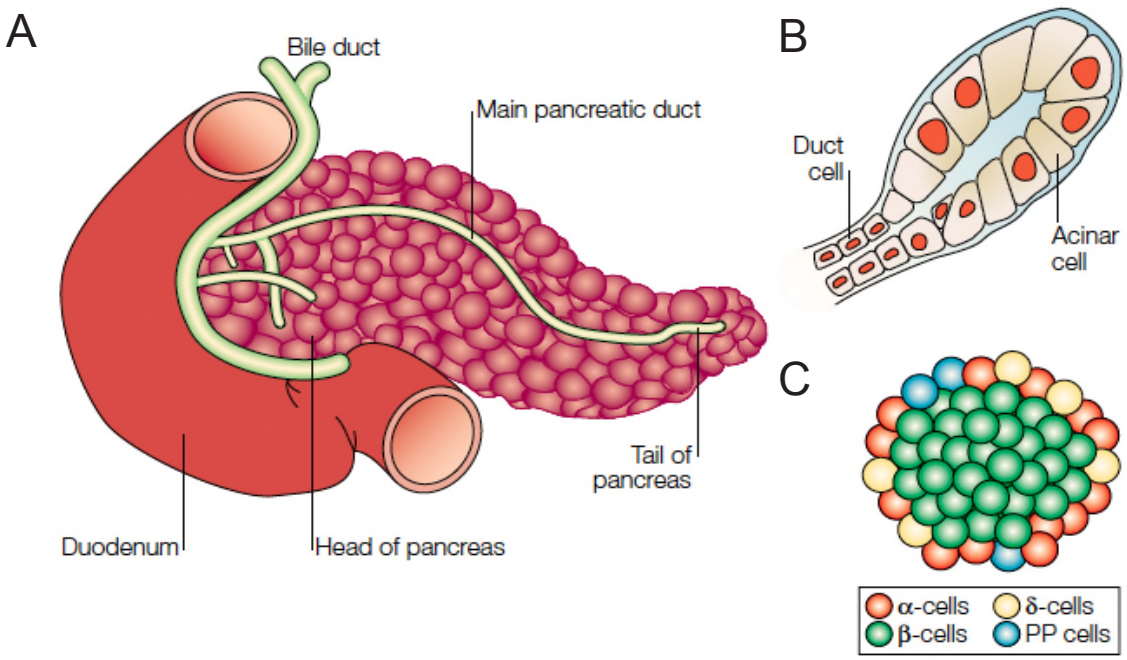


Figure 1. Vertebrate pancreas composition. (A) The vertebrate pancreas is an endodermally-derived organ that resides behind the stomach and in connection with the duodenum. (B) The exocrine portion of the pancreas is composed of digestive enzyme-secreting acinar cells and duct cells, which carry the digestive enzymes to the intestine. (C) The endocrine component of the pancreas consists of clusters of cells known as islets of Lagerhans. Islets contain five different hormone-producing cell types, including β (insulin), α (glucagon), σ (somatostatin), ε (ghrelin, not pictured)and PP (pancreatic polypeptide) and a characteristic morphology with a dense core of β cells. Figure modified and reprinted with permission from Edlund, 2002.

sulfonylureas or secretagogues, are often initially successful, but lose efficacy over time as β cells become exhausted (Hanefeld 2007). Thus, an ideal treatment for T2D would improve insulin secretion without subsequent fatigue and failure of the β cell.

In addition to the development of new pharmaceuticals that augment β cell function, much work within the diabetes community has focused on the derivation of insulin-secreting β cells from embryonic stem (ES) cells or induced pluripotent stem (iPS) cells. To this end, the developmental events that take place during pancreatogenesis, including the specification of endocrine and β cells, as well as the complexities of the *in vivo* microenvironment that contribute to the successful generation of endocrine cell types have been explored extensively. Of particular interest are signaling molecules and transcription factors that regulate endocrine cell fates or facilitate the differentiation or function of β cells.

Pancreas development

Specification of pre-pancreatic endoderm

In vertebrates, the pancreas originates from the posterior foregut endoderm after receiving both instructive and permissive signals from the notochord, developing blood vessels, and mesenchyme. Following gastrulation and formation of the gut tube (Figure 2), the prepancreatic dorsal portion of the endoderm contacts the notochord, and subsequently the fused dorsal aortae, while the developing ventral pancreas is in contact with the splanchnic lateral plate mesoderm (LPM, Figure 3A) (Kim, Hebrok, and Melton 1997a, b, Kumar et al. 2003). Secreted growth factor signals from these tissues provide

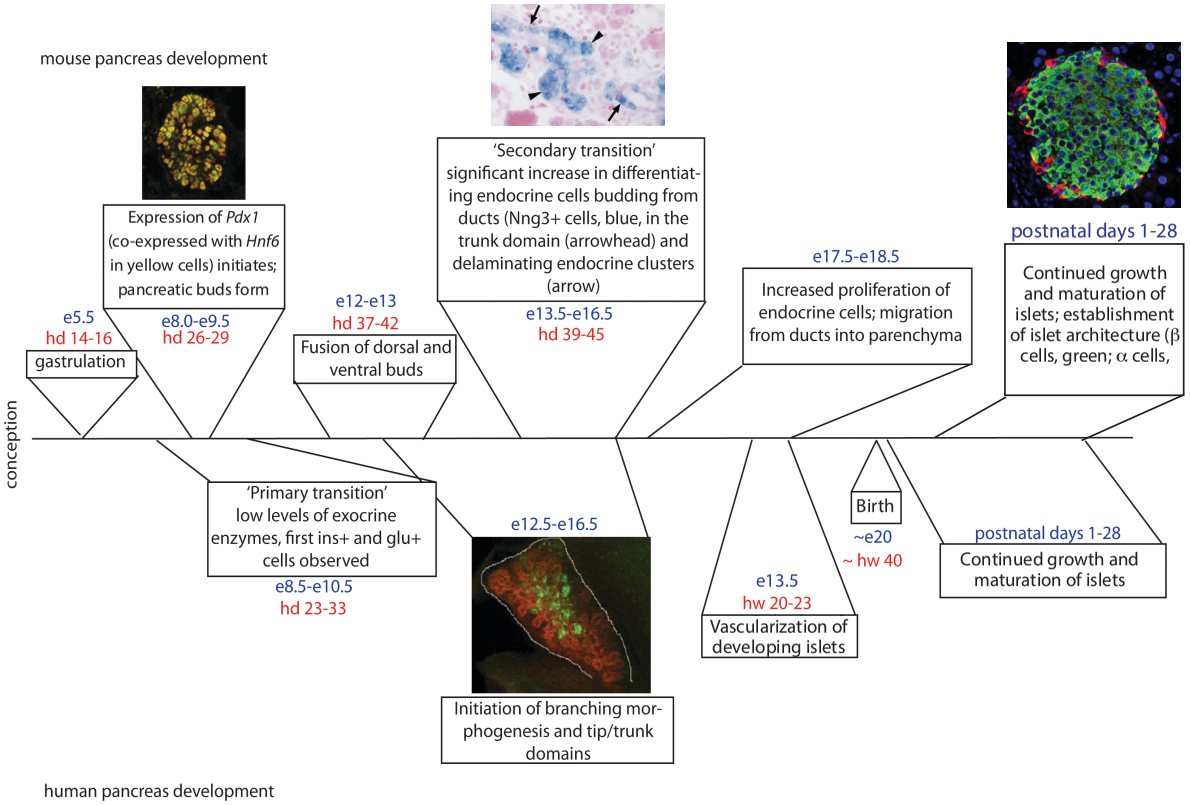


Figure 2. Important events in murine and human pancreas development. Murine time pints are listed in blue and human time points are listed in red. e, mouse embryonic day; hd, human embryonic day; hw, human embryonic week. Modified and reprinted with permission from Henley and Gannon, 2013.

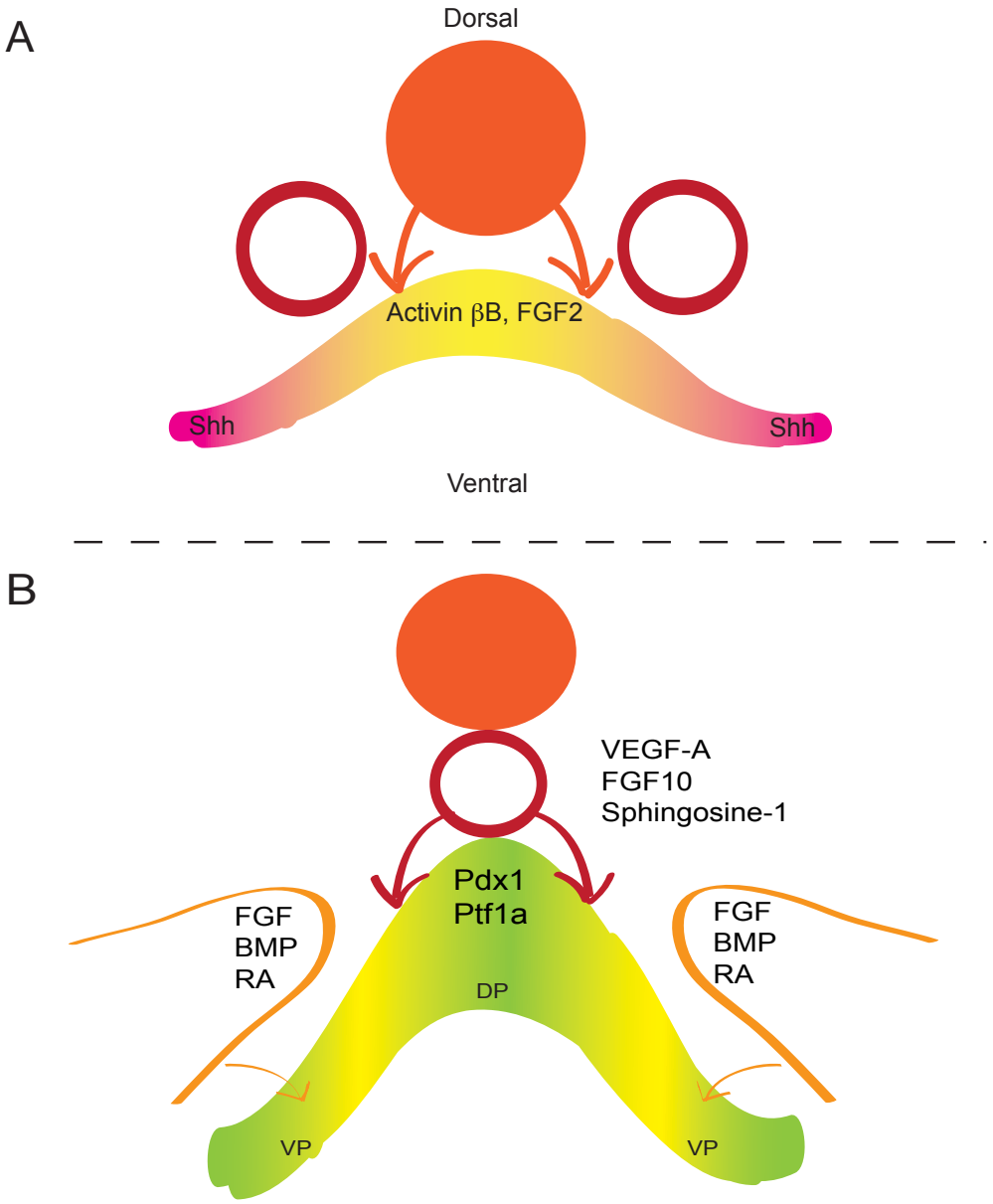


Figure 3. Signals from the notochord and dorsal aorta activate key pancreas-related transcription factors in the dorsal foregut endoderm. (A) Prior to e8.0 in the mouse, signals from the notochord (orange), including Activin b-B and FGF2, inhibit Shh expression (hot pink) in the dorsal endoderm (yellow) and permit pancreatogenesis to initiate. (B) After e8.0, fusion of the dorsal aortae (red) displace the notochord from the dorsal endoderm. Direct contact between the dorsal aorta and endoderm initiates Pdx1 and Ptf1a expression in the pre-pancreatic region (green). The dorsal aorta also secretes factors including VEGF-A, FGF10, and Sphingosine-1, which participate in proliferation of panreatic progenitors, pancraetic branching and establishment of endocrine mass. Signals from the LPM (light orange), such as FGF, BMP, and RA contribute to patterning of the gut tube, particularly induction of Pdx1 and Ptf1a in the developing ventral pancreas (VP). Figure modified and reprinted with permission from Henley and Gannon, 2013.

instructive cues allowing this region of the foregut endoderm to acquire the pancreatic fate, and stimulate the outgrowth and branching of the epithelium.

Contact between the notochord and dorsal pre-pancreatic endoderm is critical for branching of the pancreatic epithelium as well as expression of key hormones involved in endocrine differentiation and adult endocrine function (Slack 1995, Kim, Hebrok, and Melton 1997a, Hebrok, Kim, and Melton 1998). These events are mediated by the notochord-driven repression of *sonic hedgehog* (*Shh)* in the region of the endoderm from which the pancreas will derive (Figure 3A, yellow). Shh is a secreted morphogen involved in growth, differentiation, and function of many developing and adult organs; it promotes intestinal fates in the developing endoderm. Its expression is maintained rostrally and caudally to the prepancreatic endoderm, suggesting a direct link between the proximity of the notochord and repression of *Shh* (Hebrok, Kim, and Melton 1998, Apelqvist, Ahlgren, and Edlund 1997). Activin-βB and fibroblast growth factor (FGF) 2, both of which are secreted from the notochord, inhibit *Shh* expression in the dorsal pre-pancreatic region (Figure 3A) (Hebrok, Kim, and Melton 1998), thus allowing for pancreas development to initiate.

Following the fusion of the two dorsal aortae at the midline in the mouse at e8.0 (Figure 3B. red), the dorsal region of the developing pancreas is no longer in contact with the notochord. Signals derived from the dorsal aorta and other blood vessels are important to specification of the dorsal bud and expression of endocrine hormones, specifically insulin. *In vitro* culture experiments indicate that close proximity of the dorsal aorta to endoderm activates expression of *pancreatic and duodenal homeobox 1* (Pdx1, or *Insulin promoting factor 1, IPF1*) (Figure 3B) a transcription factor required for pancreas development and mature β cell function (Offield et al. 1996, Lammert, Cleaver,

and Melton 2001, Jonsson et al. 1994). Endothelial cells are also responsible for inducing expression of *pancreatic transcription factor 1a* (*Ptf1a*) in the dorsal bud of the developing pancreas (Figure 3B) (Yoshitomi and Zaret 2004). Like Pdx1, Ptf1a is crucial to pancreas development and differentiation of all pancreatic cell types, but unlike Pdx1, Ptf1a is required for acinar rather than endocrine function in the adult (Krapp et al. 1998);(Kawaguchi et al. 2002). Both *Pdx1* and *Ptf1a* are required for proper pancreatic bud outgrowth in mouse and human (Jonsson et al. 1994, Offield et al. 1996, Stoffers et al. 1997, Kawaguchi et al. 2002, Sellick et al. 2004). Signals secreted from the blood vessels, including vascular endothelial growth factor *(*VEGF)-A, sphingosine-1-P (S1P), and fibroblast growth factor (FGF) 10, play a role in proliferation of progenitors, pancreatic branching, and establishment of endocrine mass in mice (Figure 3B) (Lammert, Cleaver, and Melton 2001, Cai et al. 2012, Sand et al. 2011, Bhushan et al. 2001). The data regarding the effect of over-expression of VEGF-A on the development and function of the adult pancreas have been conflicting (Lammert, Cleaver, and Melton 2001, Cai et al. 2012), but nonetheless reflect the importance of blood vessel-derived molecules in pancreas specification and differentiation.

Regionalization of the foregut endoderm

After its specification, the foregut endoderm is designated into discrete regions that give rise to specific organs. Much like the patterning of the pre-pancreatic endoderm, neighboring structures and tissues secrete signals that enable regionalization of the endodermal gut tube along the anterior/posterior axis. Factors including FGFs, bone morphogenetic proteins (BMPs), activin, and retinoic acid (see below) are secreted from the adjacent LPM to regionalize the gut tube (Kumar and Melton 2003, Dessimoz et

al. 2005). The most anterior region of the foregut is demarcated by *Sox2* expression and gives rise to the lungs, esophagus, trachea, and forestomach (Figure 4). Posterior to the *Sox2*-positive foregut is a region of the gut tube which is marked by expression of *Pdx1, Ptf1a, motor neuron and pancreas homeobox 1 (Mnx1)* and *caudal type homeobox 2 (Cdx2).* This segment, called the posterior foregut, gives rise to the antral stomach, pancreas, and liver. BMP4, BMP7, and Activin-A, all of which belong to the transforming growth factor β (TGFβ) superfamily of signaling molecules (see BMP Signaling, below), are capable of activating *Pdx1* expression in the presumptive pancreatic region of endoderm (Kumar and Melton 2003). *Pdx1* expression is initially observed in the mouse at e8.5 in this region (Figure 3B), and later expands to the antral stomach, rostral duodenum, and common bile duct by e11.5 (Offield et al. 1996, Guz et al. 1995, Jonsson et al. 1994, Wessels 1967). At e9.5 in mice, a subset of cells within the *Pdx1* positive domain also express *Ptf1a*; cells double positive for both transcription factors give rise to the pancreas, whereas cells positive for only *Pdx1* contribute to the stomach and duodenum (Figure 4).

Posterior to the pancreatic domain is the midgut, which is marked by expression of *Pdx1* (in the most anterior portion) and *Cdx2* (Figure 4). This region consists of endoderm that will give rise to the duodenum (*Pdx1 and Cdx2),* and small intestine (*Cdx2)*. In the absence of *Ptf1a,* cells that are destined to become *Ptf1a*-positive pancreatic cells are instead incorporated into the spleen and duodenum, indicating the important role of *Ptf1a* in appropriately regionalizing and populating the developing pancreas (Krapp et al. 1998, Kawaguchi et al. 2002). The most posterior region of the digestive tract is the hindgut, which is marked by expression of *Cdx1* and *2* and gives rise to the colon (Figure 4).

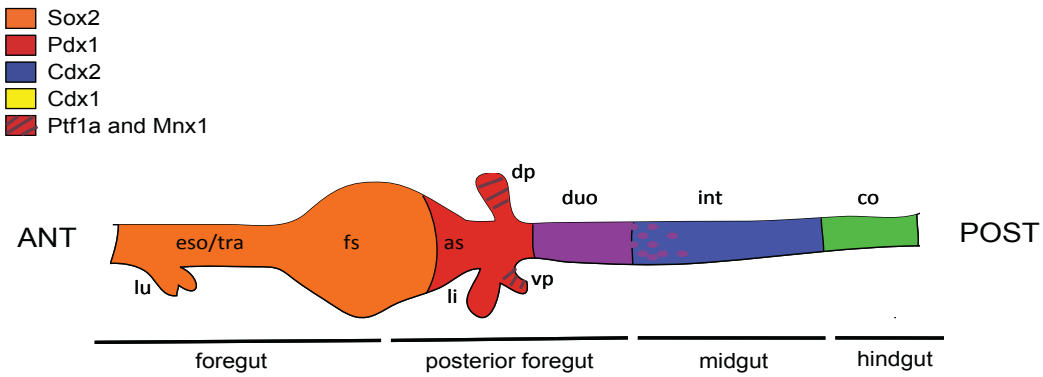


Figure 4. Regionalization of the gut tube during murine embryogenesis. The gut tube is organized in an anterior-to-posterior manner and is compartmentalized into discrete regions that give rise to specific organs. The most anterior portion of the gut tube is the foregut, which gives rise to the esophagus, trachea, lungs, and forestomach, and is marked by expression of *Sox2*. The posterior foregut is demarcated by expression of *Pdx1* and gives rise to the antral stomach and liver. Portions of the posterior foregut that also express *Ptf1a/Mnx1* and *Cdx2* develop into the dorsal and ventral pancreatic buds and duodenum, respectively. *Cdx2* is expressed throughout the midgut, from which the intestine develops, and throughout the hindgut, where it is co-expressed with *Cdx1* and gives rise to the colon. Figure reprinted with permission from Guney and Gannon, 2009.

Formation of the pancreatic buds

Development of the pancreas proper initiates around e9.5 in mice and gestational day 25 in humans (Pictet et al. 1972) (Figure 2, 5). Pancreatic bud formation is marked by the condensation of mesenchyme around the prospective pancreatic anlagen. Growth of the buds and subsequent branching morphogenesis are dependent on permissive signals secreted from the mesenchyme (Golosow and Grobstein 1962). The transcription factor *Bapx1* (Nkx3.2) is present in the mesenchyme at the time of budding and is responsible for the separation of spleen and pancreas in the developing mouse (Asayesh et al. 2006). FGFs are also highly expressed in the mesenchyme at this time, and signaling through FGF receptors in the developing pancreatic epithelium promotes proliferation and branching morphogenesis. The absence of *Fgf10* in mice results in blunted pancreatic branching, and loss of expression of the FGF receptor 2B (*Fgfr2b*) also reduces branching morphogenesis (Bhushan et al. 2001, Pulkkinen et al. 2003). Cells that co-express *Pdx1* and *Ptf1a* in the posterior foregut endoderm evaginate from the dorsal and ventral epithelium into the surrounding mesenchyme to form the dorsal and ventral pancreatic buds, respectively. In the mouse, the ventral bud emerges 12 hours after the dorsal bud, and 6 days after the dorsal bud in humans. The dorsal bud originates from the midline endoderm between the third and ninth somite pair, and the human ventral pancreas emerges from two lateral buds that originate from the same level and eventually fuse and then join the dorsal bud later in gestation (Nielsen et al. 2009, Kumar et al. 2003). The mouse also develops two ventral domains during early pancreatogenesis, but only one domain survives to fuse with the dorsal bud.

Despite their eventual fusion, the dorsal and ventral pancreatic buds in both mouse and human are two distinct entities that have differential signaling and transcription factor requirements and responses. For example, the ventral bud develops

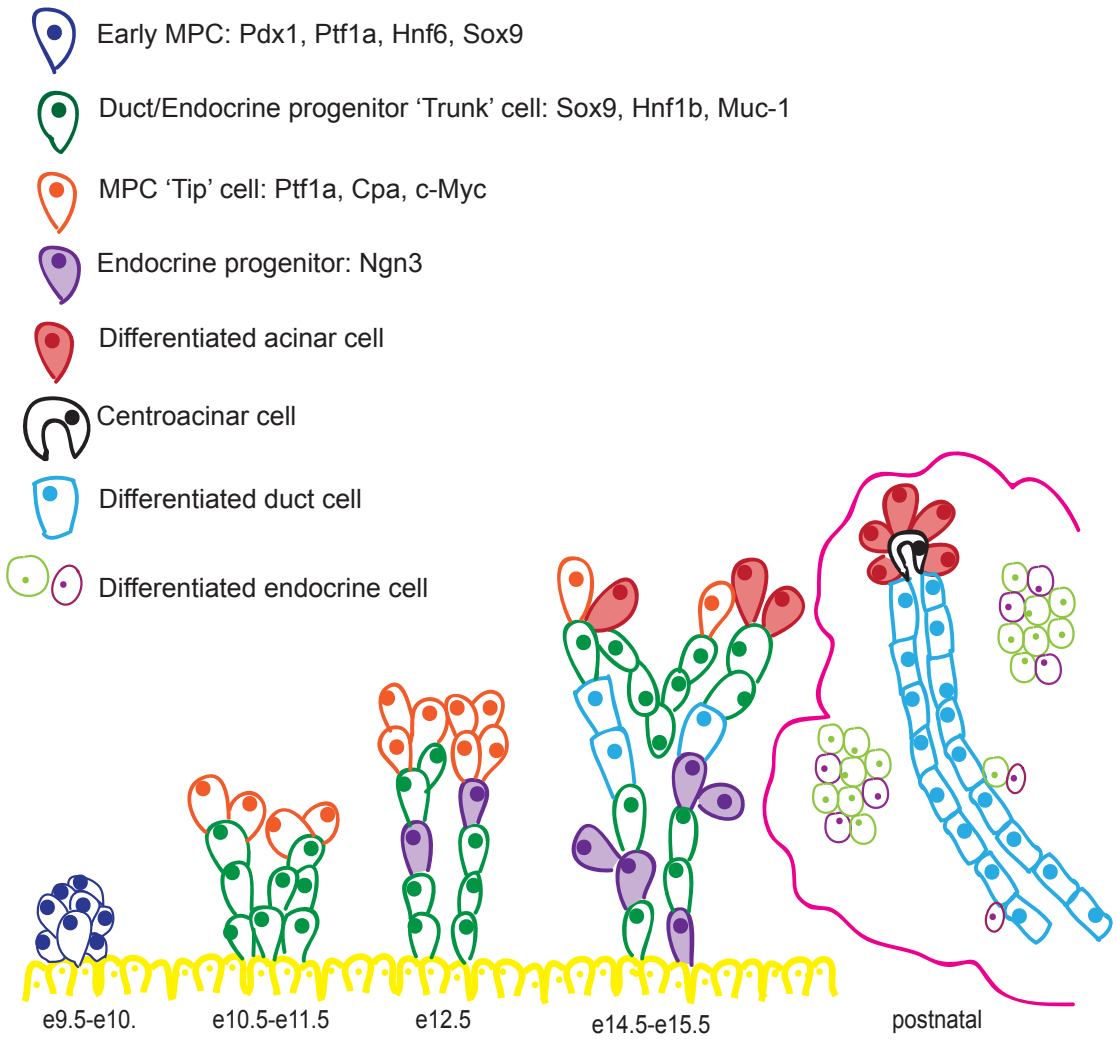


Figure 5. Developmental events during murine pancreatogenesis. See text for details. Figure modified and reprinted with permission from Henley and Gannon, 2013.

without associating with or receiving direct signals from the notochord, while the dorsal bud relies on notochord signaling for proper induction (Figure 3A). As mentioned above, *Mnx1* is a transcription factor that marks both dorsal and ventral pancreatic buds in the developing posterior foregut endoderm (Figure 4). Loss of *Mnx1* results in failure of the dorsal bud to develop, while surrounding endoderm and mesenchyme are mostly unaffected, although there are slight perturbations in β cell differentiation and islet organization in the adult organ (Harrison et al. 1999, Li et al. 1999). The dorsal bud is also differentially sensitive to expression of *islet-1* (*Isl1)*, a transcription factor expressed in the developing pancreatic epithelium, mesenchyme, as well as adult endocrine cells. Mice lacking *Isl1* fail to develop dorsal mesenchyme and thus specifically lack dorsal bud outgrowth and also exhibit a paucity of endocrine cells in the ventral bud (Ahlgren et al. 1997). Indeed, mutations in *ISL1* have also been associated with Type 2 Diabetes in the Japanese population (Deering, Chinn, et al. 2009). Retinoic acid (RA) signaling also plays a differential role in dorsal and ventral bud formation. Retinaldehyde dehydrogenase (Raldh*)* 2 is required for RA synthesis and, when deleted in the mouse, results in dorsal pancreatic agenesis (Martin et al. 2005, Molotkov, Molotkova, and Duester 2005). The ventral bud is affected when a dominant negative RA receptor is expressed in the pancreatic epithelium, indicating that the ventral bud is sensitive to RA signaling in general, but not expression of a particular RA synthesizing enzymes (Ostrom et al. 2008).

The period of time following bud evagination from the endoderm is termed the 'primary transition' and takes place in the mouse from e8.5 to e10.5 and from approximately the 23rd to 33rd days of gestation in the human (Pictet et al. 1972) (Figure 2, 5). Little cellular ultrastructural differentiation is seen at this time, but low levels of

exocrine-specific enzymes can be detected and the first glucagon- and insulin- positive cells are observed. These hormone-positive cells do not express markers of mature endocrine cells and are not thought to contribute to the mature endocrine cell population of the pancreas (Herrera et al. 1994, Pang, Mukonoweshuro, and Wong 1994, Lee et al. 1999, Herrera 2000, Wilson, Kalamaras, and German 2002). Essentially, the role and fate of these hormone positive cells that arise during the primary transition is unclear.

Fusion of pancreatic buds

The evagination of the dorsal and ventral pancreatic buds into the surrounding mesenchyme is followed by growth and elongation of the buds and, at e12-13 in mice and gestational day 37 to 42 in humans, fusion to form a single organ (Figure 2). Fusion occurs as a result of organ elongation and rotation of the gut tube (Jorgensen et al. 2007). The process of bud fusion is poorly understood, but it has been determined that molecules belonging to the Shh family are involved. Absence of *Indian hedgehog (Ihh),* a secreted molecule within the Sonic hedgehog family of proteins, results in ectopic branching of the ventral pancreas and formation of an annulus encircling the duodenum in mice, which closely resembles annular pancreas seen in humans (Hebrok et al. 2000). Fusion of the buds also results in the connecting of the ventral bud duct with the distal portion of the dorsal bud duct, establishing the main pancreatic duct (duct of Wirsung) that runs the entire length of the organ and connects with the common bile duct.

Initiation of branching morphogenesis

The budding pancreas is initially a pair of small, rounded, and grossly morphologically identical fin-shaped buds on the dorsal and ventral sides of the midline foregut endoderm. After fusion of the buds, the pancreas undergoes organizational and morphological changes within the epithelium and ductal plexus, begnning around e12.5 in the mouse, to establish a branched organ with an intricate network of ducts (Figure 2, 5) (Villasenor et al. 2010). It was initially proposed that pancreatic branches arise from random buckling of endodermal epithelium (Slack 1995). It has since been determined that individual branches emerge from the proliferation of multipotent progenitor cell (MPC)-containing tip domains (Figure 5) (Zhou et al. 2008) from the epithelium and longitudinal growth, rather than perpendicular elongation of the tip domains from the epithelium (Villasenor 2010).

By e12.5 in the mouse, the buds have fused and form a tear-drop shaped structure with little to no external evidence of branching. At this time, however, a multi-lumenal ductal plexus has been established, and remodels to form a singular lumen as branching morphogenesis progresses (Villasenor et al. 2010). The pancreas is divided into two lobes; with time, the splenic lobe of the pancreas extends across the stomach from the duodenum to the spleen, and the gastric lobe lengthens along the posterior stomach. The majority of the dorsal pancreas is composed of the splenic lobe and is characterized by its anvil-shaped tail, named as such due to its likeness to the distal region of the human pancreas. Development of the tail begins at e12.5 in the mouse (and continues throughout development), followed at e13.5 by development of left lateral branches and smaller right lateral branches from the proximal portion of the splenic lobe.

At approximately e12-e12.5 in the mouse, the pancreas undergoes morphological and gene expression pattern changes that establish the 'tip' and 'trunk' domains of the branching organ (Figure 2, 5). Prior to this change, the pancreas is populated by MPCs that have the ability to differentiate into acinar, duct, or endocrine cells, and express a signature of transcription factors (*Pdx1, Ptf1a, Sox9 Nkx6.1,* hepatocyte nuclear factor 6 (*Hnf6)* and *Hnf1β* (Gu, Dubauskaite, and Melton 2002, Solar et al. 2009)). The MPC pool maintains itself through *Sox9*-mediated proliferation; loss of *Sox9* in mice results in near complete pancreatic agenesis due to failed MPC pool expansion (Seymour et al. 2007). Around approximately e12.5 in mice, the pancreas segregates into a 'tip' and 'trunk' domain that, in part, facilitates branching morphogenesis (Figure 5). Cells in the 'tip' regions are MPCs distinct from those found in the emerging bud; the tip pool of MPCs expresses *Ptf1a, c-Myc,* and carboxypeptidase A (*Cpa1),* and in absence of other acinar markers, has the potential to give rise to all three pancreatic cell types. This pool of progenitors also expresses *Hnf6* and *Pdx1*, but to a lesser extent than the MPCs found in the developing bud. The 'trunk' domain extends down from the MPC-containing tips to the main duct, and is populated by cells that can give rise to duct or endocrine cells. Marked by expression of *Hnf1β, Sox9, Mucin 1 (Muc1)*, *Nkx6.1, Hnf6* and *Pdx1,* the differentiation of bipotent trunk cells into endocrine progenitors is mediated by expression of the basic helix-loop-helix transcription factor *neurogenin 3* (*Ngn3).* Mice lacking *Ngn3* fail to develop intestinal and pancreatic endocrine cells, and humans with *NGN3* mutations suffer from malabsorptive diarrhea and neonatal diabetes (Wang et al. 2006, Pinney et al. 2011). The process of establishing the tip and trunk domains overlaps with the 'secondary transition' of pancreas development, which is described in detail below.

Regulation of branching morphogenesis

Branching morphogenesis is regulated by a diverse group of transcription factors and signaling molecules, several of which have been well characterized and are described here. The one cut domain-containing transcription factor *Hnf6* is expressed broadly in the developing endoderm and plays a crucial role in liver and pancreas development (Jacquemin et al. 2000, Jacquemin, Lemaigre, and Rousseau 2003, Zhang et al. 2009). Deletion of *Hnf6* within the *Pdx1*-expressing domain results in, among other profound changes, decreased pancreatic branching at e14.5 in the mouse and a subsequent hypoplastic pancreas (Zhang et al. 2009). Expression of the pro-endocrine factor *Ngn3* is also important to branching morphology, as mice null for *Ngn3* show significant reductions in branching as well as dilated ductal structures and stunted growth (Magenheim et al. 2011, Beucher, Martin, et al. 2012). As mentioned above, branching morphogenesis is also dependent on the expression of FGF ligands and receptors. Furthermore, animals globally lacking the ephrin receptors B2 and B3 (*EphB2* and *EphB3*, respectively), which are normally expressed in the pancreatic epithelium, show reduced pancreas size and decreased number and size of lateral pancreatic branches, suggesting that ephrin signaling is important to overall pancreatic growth as well as establishment of appropriate numbers of branches (van Eyll et al. 2006, Villasenor et al. 2010). Overall, branching morphogenesis is important not only to the growth of the pancreas as an organ, but also to generating the proper number of endocrine, duct, and acinar progenitors that will eventually populate the pancreas as mature cells. For example, mice lacking *Hnf6* in a global or tissue-specific fashion show reduced branching in addition to decreased number of *Ngn3*+ cells and reduced α and β cells at late gestation (Jacquemin et al. 2000, Zhang et al. 2009).

Establishment of ductal network

As an endocrine organ, the pancreas maintains glucose homeostasis in the body by releasing hormones directly into the blood stream, while the exocrine portion produces digestive enzymes in the acinar tissue and secretes them into the ductal network. Digestive enzymes are delivered to the gut by the main pancreatic duct, which is connected to smaller, intercalated ducts that are closely associated with enzyme-secreting acinar cells by inter- and intra-lobular ducts. The primordial ductal system is pre-formed prior to the initiation of branching morphogensis, and is reorganized throughout development to yield a mature ductal system. Initially, the ductal network is a multi-lumenal structure, as shown by whole mount immunolabeling using the ductal marker *Mucin 1* (*Muc1)* at e11.5 to 12.5 in mice (Figure 6) (Kesavan et al. 2009). Remodeling of the ductal plexi results in the formation of ducts with a single lumen by e15.0 in the mouse (Figure 6), and the remodeling of ducts is concurrent with the development of MPC tips into protruding branches (Villasenor et al. 2010).

Remodeling and branching of ducts is partially regulated by *Pdx1*. *In-vivo* culture experiments using murine-derived pancreatic ductal cells revealed a surge of *Pdx1* expression in cells undergoing branching (Wescott et al. 2009). Additionally, in the developing mouse pancreas as early as e12.5, cells negative for insulin but positive for *Pdx1* and the duct-marker *cytokeratin 19* (*K19*) exhibit branching morphogenesis, indicating that although *Pdx1* expression is not maintained in adult ducts, it is important for duct development. Branching events in the pancreas also rely on the Rho-GTPase Cdc42, which is ubiquitiously expressed in the developing pancreatic epithelium (Kesavan et al. 2009). Absence of *Cdc42* does not affect the establishment of polarity within an individual cell, but does prevent the formation of microlumens at e11.5, and subsequent fusion of lumens at e12.5 in mice (Kesavan et al. 2009).

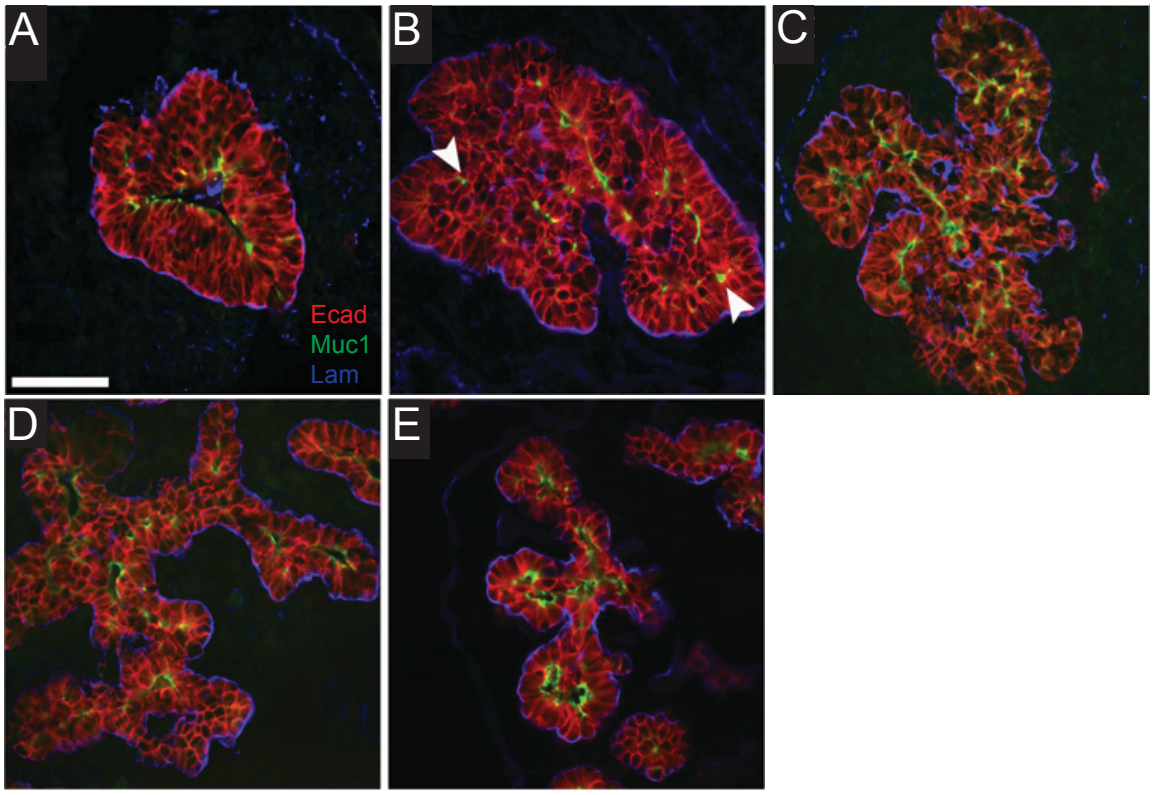


Figure 6. Duct modeling during pancreatogenesis. (A) Wild type (WT) multilayered pancreatic epithelium (E-cadherin, red) at e10.5 exhibiting a central lumen (Muc 1, green). (B) At e11.5, microlumens are formed (arrowheads) within the multilayered pancreatic epithelium. The number of microlumens increases and individual microlumens fuse to form larger lumens at e12.5 (C) and e13.5 (D). (E) By e15.5, Muc1-positive cells (green) have formed a ductal network with a single lumen within the developing pancreas. Figures modified and used with permission from Kesavan et al., 2009.

Secondary Transition

The secondary transition is the developmental time period characterized by a monumental increase in differentiation of both exocrine and endocrine cell types from the MPC population. This period was initially characterized by Pictet and Rutter with the observation of the great increase in exocrine enzymes at e14.5 in rats (Pictet et al. 1972, Rutter et al. 1968). The secondary transition marks the progression from a state of 'protodifferentiation' to that of enzyme and hormone production and establishment of discrete components of the pancreas via the tip and trunk model of branching morphogenesis (see above), and is also marked by vascularization of the developing endocrine clusters (Brissova et al. 2006) (Figure 2, 5). These events initiate at approximately e13.5 in the mouse and gestational day 39 in the human, and proceed through murine e16.5 and human gestational day 45 (Figure 2).

Exocrine development during the secondary transition

Prior to the secondary transition, there are low levels of enzymes and select hormones can be detected in the developing pancreas, and although the ultimate fate of the cells generating these products is not known, they are not presumed to contribute to adult tissues. The rapid increase in differentiating cells at the secondary transition translates into a large induction of enzyme gene expression from the emergent exocrine portion of the pancreas. Not surprisingly, this time period is also marked by development of the rough endoplasmic reticulum (ER) and zymogen granules in the forming acinar cells (Pictet et al. 1972). Indeed, the 'tip' region of cells within the tip/trunk model of branching morphogenesis is designated, in part, by expression of the acinar-associated enzyme *Cpa1* and transcription factor *Ptf1a. Cpa1* expression is initially detected at

e10.5 within MPCs, but is compartmentalized to and increased in the developing tip acinar clusters at e12.5 (Figure 5) (Chiang and Melton 2003, Gittes and Rutter 1992, Jorgensen et al. 2007). *Elastase* (*Ela*) expression also increases during the secondary transition, followed later in development by expression of *amylase (Amy*). Acinar differentiation is preceded by apical polarization, demonstrated by localization of *Muc1* at the apical surface of epithelial cells in the murine bud at e11.5 (Kesavan et al. 2009)

Plasticity of ductal cells during the secondary transition

In the tip/trunk model of branching morphogenesis, cells residing within the trunk domain that are destined to become endocrine or ductal cells express *Sox9, Hnf1β, Muc1, Hnf6,* and *Pdx1* (Seymour et al. 2007, Solar et al. 2009, Kopinke and Murtaugh 2010). A subset of these trunk cells lose their bipotentiality after induction of the pro-endocrine gene *Ngn3* (Figure 5); indeed, lineage labeling in the mouse suggests that these bipotent trunk cells have the ability to give rise to endocrine cells throughout development, but contention lies with the specific time at which they lose this ability to give rise to endocrine cells. Experiments that studied lineage labeled murine *Hnf1β*+ cells from the trunk domain throughout development revealed that this cell population cells loses the potentiality to give rise to both duct and endocrine cells at e15.5 (Solar et al. 2009). A different study showed that cells expressing *Sox9* have the ability to give rise to acinar, ductal, and endocrine cell types until postnatal day (P) 1 in mice, but after P7, the *Sox9*+ population only gives rise to acinar and ductal cells (Furuyama et al. 2011). A second study investigating the fates of *Sox9* lineage-labeled cells (which were marked in a pulse-chase fashion) determined that *Sox9*+ cells give rise to all three pancreatic cell types from e8.5 until e18.5, but only give rise to duct cells and non-β

endocrine cells after P5 in mice (Kopp, Dubois, Schaffer, et al. 2011). There is controversy surrounding these studies in terms of the efficiency of the lineage-tracing models used to label and track individual cells. What remains clear is that cells residing within the trunk domain experience a period of plasticity during which they can adopt an endocrine or ductal fate; the specific mechanisms behind the timing of these cell fate decisions has yet to be elucidated.

Centroacinar cells/terminal duct cells as a source of progenitors in the ductal epithelium

Centroacinar and terminal duct cells are two distinct, poorly characterized cell types that reside at the interface between acini and small ducts (Figure 5). The precise function of these cells is still being examined, and it remains unclear whether they are equivalent in their capacities. The specific role for centroacinar/terminal duct cells in pancreas development has not been assessed, and although some data suggest they do not contribute to the regenerating adult endocrine pancreas, these cells contribute to the adult acinar population and incorporate into developing endocrine tissue *in vitro*. *Sox9*+ centroacinar cells are a source of progenitor cells within the ductal/acinar boundary and contribute to maintenance of the adult acinar cell population, but they are not a source of newly formed endocrine cells after pancreatic injury in the adult mouse (Kopp, Dubois, Schaffer, et al. 2011). However, *in vitro* and *ex vivo* experiments revealed that centroacinar/terminal duct cells isolated from the adult mouse pancreas express progenitor cell markers, including *c-Met*, *Sox9,* and *Nestin*, and cluster into hormone-secreting, glucose-responsive 'pancreatospheres' in culture. This adult cell population is also capable of contributing to developing endocrine and exocrine tissue when injected into e12.5 mouse dorsal bud cultures (Rovira et al. 2010).

Both human and murine adult acinar cells exhibit plasticity in culture and *in vivo*. Human acinar cells readily differentiate into ductal cells in culture, exhibiting CK19, Hnf1β, and Sox9 protein expression, in a mitogen-activated protein kinase (MAPK)-dependent manner (Houbracken et al. 2011). Additionally, mouse acinar cells can undergo acinar-to-ductal metaplasia in response to elevated EGFR signaling, as TGFα and hepatocyte growth factor (HGF) signaling through EGFR results in transdifferentiation into ductal cells through a *nestin*-positive intermediate (Means et al. 2005). Furthermore, combined over-expression of *Ngn3, Pdx1,* and musculoaponeurotic fibrosarcoma oncogene homolog A (*MafA*) in adult murine acinar cells results in transdifferentiation of acinar cells into glucose-responsive, insulin-secreting β cells that partially restore serum insulin and blood glucose levels in adult mice lacking endogenous β cells (Zhou et al. 2008). This suggests that adult human and murine acinar tissue are relatively plastic in culture and *in vivo* and provides promise for the generation of other pancreas cell types from human acinar tissue.

Delamination of endocrine progenitors from the trunk domain

Delamination is a process in the epithelial-to-mesenchymal transition (EMT) during which the endocrine progenitors emerge from the epithelial trunk region and migrate into the parenchyma (Figure 5). *Ngn3*+ trunk cells lose contact with the trunk lumen and, after activation of *Snail2*, down-regulate expression of E-cadherin to facilitate delamination (Gouzi et al. 2011). The ability to delaminate is lost in *Ngn3* mutant animals, as cells fated to be endocrine progenitors remain closely associated with ducts (Magenheim et al. 2011, Beucher, Martin, et al. 2012). E-cadherin down-regulation is, in part, mediated by expression of the co-repressor *groucho-related gene (Grg) 3*; *ex vivo*

cultures of developing pancreas from mice lacking *Grg3* have endocrine progenitors that do not delaminate from the ductal epithelium and show decreased numbers of differentiated endocrine cells (Metzger et al. 2012). Delamination of differentiating cells is also associated with down-regulation of atypical protein kinase C (aPKC), an apical surface marker (Georgiou et al. 2008). Conversely, increased expression of *Eph3B* designates delaminating and differentiating endocrine progenitors during the secondary transition and is required for delamination (Villasenor et al. 2012, Villasenor et al. 2010).

Commitment to endocrine fate: Expression and action of Ngn3

Within the trunk domain of the developing pancreas, cells destined to become endocrine progenitors transiently express *Ngn3* (Figure 5). *Ngn3*+ cells can give rise to all five endocrine types (Gu, Dubauskaite, and Melton 2002). High expression of *Ngn3* is required for commitment to the endocrine lineage, as studies in mice have indicated that low levels of *Ngn3* expression due to *Ngn3* heterozygosity or hypomorphism result in the diversion of cells to the acinar or ductal fate; furthermore, complete loss of *Ngn3* results in the adoption of acinar or ductal fates by failed endocrine progenitors (Wang et al. 2010, Beucher, Martin, et al. 2012, Zhang et al. 2009). Upon expression of *Ngn3*, endocrine progenitors are temporarily post-mitotic, but following delamination and differentiation into specific endocrine cell types, the ability to proliferate is regained. Clonal analysis of *Ngn3*+ cells in the developing mouse pancreas indicates that endocrine progenitors expressing *Ngn3* are unipotent, each giving rise to a single endocrine cell (Desgraz and Herrera 2009). After commitment to the endocrine lineage, expression of *paired homeobox* (*Pax*) *4* or *aristaless related homeobox* (*Arx*) contribute

to adoption of β/δ or α cell fate, respectively (Sosa-Pineda et al. 1997, Collombat et al. 2005, Seymour and Sander 2011).

The ability of endocrine progenitors to differentiate into specific endocrine cell types is an property that changes over the course of pancreas development. *Ngn3+* endocrine progenitors exhibit windows of competence to differentiate into specific cell types (Johansson et al. 2007). For example, cells that differentiate early (prior to e10.5 in the mouse) become only α cells. Between e 10.5 and e12.5, differentiating endocrine cells become α or β cells, and after e12.5, progenitors differentiate into PP, β, and δ cells (Johansson et al. 2007) In accordance with this temporal differentiation pattern, over-expression of *Ngn3* early within the *Pdx1*-positive domain in mice results in the precocious differentiation of nearly all endocrine progenitors into α cells (Grapin-Botton, Majithia, and Melton 2001). While the permissive, instructive, or inhibitory signals that govern these windows of competence have not yet been thoroughly characterized, some key activators of specific endocrine programs have been identified and are described below.

Hnf6: Establishing endocrine mass and function

Hnf6 belongs to the cut-domain family of transcription factors and was initially characterized as a liver-enriched factor required for *Hnf3β* expression in rat hepatocytes (Samadani and Costa 1996). It is expressed in the developing and adult murine central nervous system (CNS), liver, and pancreas (Landry et al. 1997). In the CNS, *Hnf6* is required for maintenance of neuronal and projection identity and participates in the formation of hindlimb neuromuscular junctions (Chakrabarty et al. 2012, Espana and Clotman 2012a, b, Hodge et al. 2007, Roy et al. 2012, Stam et al. 2012, Audouard et al. 2012). Deletion of *Hnf6* in a global fashion results in early postnatal lethality of seventy

five percent of animals as a result of cholestasis-related liver necrosis (Jacquemin et al. 2000, Clotman et al. 2002). The surviving animals are initially hypoglycemic due to impaired liver function, and exhibit glucose intolerance at 10 weeks of age that results from impaired endocrine function (Jacquemin et al. 2000). *Hnf6* null animals exhibit reduced β, α, δ, and PP cells at e15.5, all of which are scattered within and close to the pancreatic ducts; islet morphology is not established until 2-3 weeks of age in these mutants, at which point the endocrine clusters still lack an α cell mantle and remain closely apposed to the ducts (Figure 7) (Jacquemin et al. 2000). Defects in endocrine differentiation observed in mice lacking *Hnf6* in a global or pancreas-specific fashion can be linked to decreased specification of endocrine progenitors, as expression of *Ngn3* and number of Ngn3-positive cells is reduced in both of these models (Figure 7, 8) (Jacquemin et al. 2000, Zhang et al. 2009). Consistent with this idea, over-expression of *Hnf6* in endocrine cells results in an increased number of Ngn3-positive cells during development (Figure 8) (Wilding Crawford et al. 2008). Indeed, *Hnf6* can stimulate *Ngn3* reporter activity *in vitro*, suggesting potential direct regulation of the endocrine program by *Hnf6* itself (in addition to other factors) (Oliver-Krasinski et al. 2009).

In the developing murine liver, *Hnf6* is required for biliary epithelial cell and gallbladder cell differentiation and works in concert with the Notch pathway to regulate development of the intrahepatic biliary system (Clotman et al. 2002, Vanderpool et al. 2012). In the adult liver, *Hnf6* regulates expression of *Gck* and glucose-6-phosphatase (*G6pase),* two genes required for regulation of glucose homeostasis (Lannoy et al. 2002, Streeper et al. 2001, Clotman et al. 2002). Hnf6 also functions upstream of *Foxa2* in the liver, and elevated *Foxa2* activity in mice is associated with defective glycogen storage (Rausa et al. 2000). Additionally, *Hnf6* antagonizes the blood glucose-elevating effects

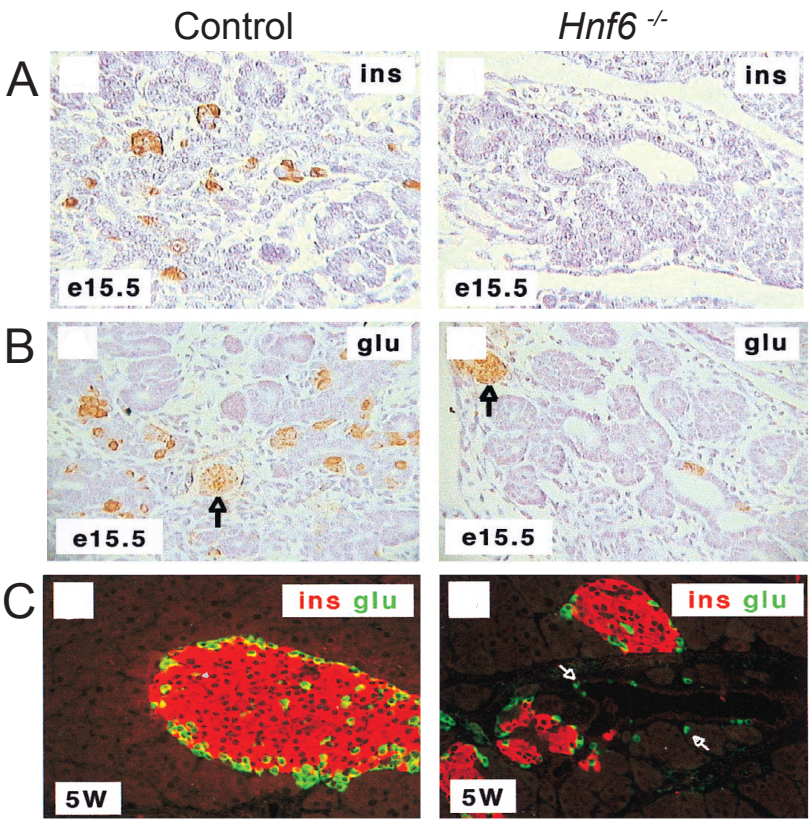


Figure 7. *Hnf6* is required for endocrine cell number and islet morphology. (A) *Hnf6* null animals, right, display fewer insulin-positive cells (brown) at e15.5 than control animals, left. (B) *Hnf6* null animals, right, display fewer glucagon-positive cells (brown), which remain closely apposed to ducts, compared to control animals, left. (C) Adult control animals, left, display appropriate islet morphology, including a β cell (red) core and an α cell (green) mantle, while *Hnf6* null animals exhibit smaller, disorganized islets that lack an α cell mantle and contain glucagon-positive cells (green) scattered throughout the acinar tissue. Figures modified and reprinted with permission from Jacquemin et al., 2000.

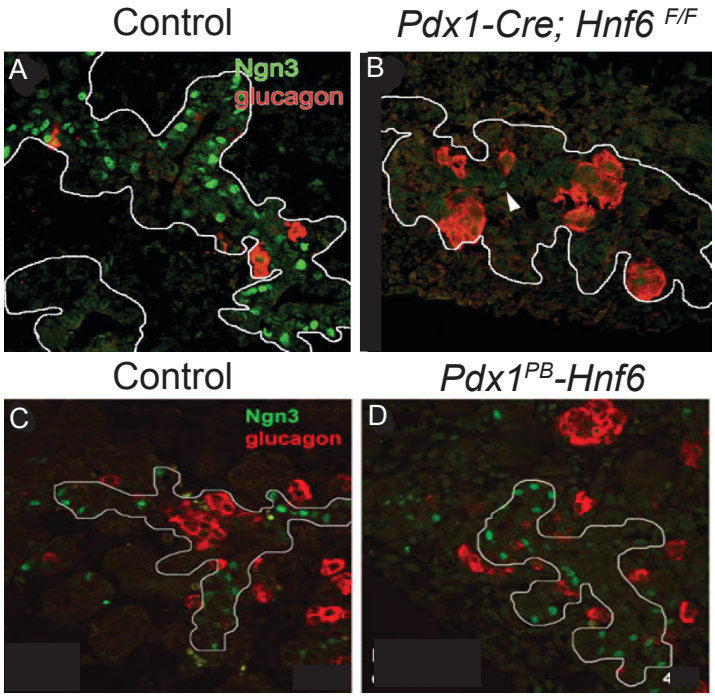


FIgure 8. *Hnf6* expression regulates endocrine progenitor number. (A) Control embryos possess Ngn3-positive endocrine progenitors (green) within the trunk epithelium at e13.5. (B) Embryos lacking *Hnf6* in the pancreatic epithelium (*Pdx1-Cre; Hnf6* $^{F/F}$) show a reduced number of Ngn3-positive endocrine progenitors (green), while glucagon-positive cells (red) are increased at e13.5. (C) Control embryonic pancreas showing Ngn3-positive cells (green) and glucagon-positive cells (red). (D) Over-expression of *Hnf6* under control of the islet-specific enhancer fragment of the *Pdx1* promoter increases the number of Ngn3-positive cells (green) at e13.5. Figures modified and reprinted with permission from Zhang et al., 2009 and Wilding-Crawford et al., 2008.

of glucocorticoids in the liver (Pierreux et al. 1999).

As described above, *Hnf6* is expressed in the primary MPCs of the pancreasand to a lesser extent in the secondary MPCs and the bipotent trunk domain of the pancreatic epithelium (Figure 2, 5). Based on the ability of *Hnf6* to activate *Ngn3* expression, it is thought that *Hnf6* expression in the trunk region is partially responsible for activating the endocrine program. Hnf6 protein is reduced in differentiated, hormone-positive endocrine cells (Zhang et al. 2009), but its expression persists in the Pdx1-positive population at low levels for a few days after birth in the mouse (Dr. Diana Stanescu and Dr. Doris Stoffers, unpublished data). Islets with sustained *Hnf6* expression exhibit increased α, δ, and PP cells at the expense of β cells, and endocrine clusters fail to migrate away from the ducts (Gannon et al. 2000). Furthermore, over-expression of *Hnf6* in islets prevents maturation of β cells, as marked by dramatically reduced expression of Glut2 and MafA. As a consequence, maintained expression of *Hnf6* in the postnatal islet causes diabetes (Gannon et al. 2000, Tweedie et al. 2006). These data are consistent with recently published work that suggests that *Hnf6* negatively regulates *MafA* expression in a β cell line by preventing the binding of *Foxa2* to the *Mafa* promoter (Yamamoto et al. 2013).

Pdx1: β cell lineage commitment and maintenance

Pdx1 expression is initiated in the pre-pancreatic endoderm and persists in the MPC population of the developing pancreatic buds (Figure 3, 5). As described above, it has multiple, distinct roles during pancreas induction, regionalization, organization, differentiation, and morphogenesis. Pdx1 is thought to play a role in the activation of the endocrine program, as *in vitro* studies indicated that *Pdx1* and *Hnf6* activate the *Ngn3* locus in a cooperative fashion (Oliver-Krasinski et al. 2009). Indeed, it is also necessary for establishing appropriate numbers of cells committed to the β cell lineage and

maintenance of the mature β cell phenotype (Jonsson et al. 1994, Offield et al. 1996, Ahlgren et al. 1998, Dutta et al. 1998, Brissova et al. 2002, Gannon et al. 2008). Expression of *Pdx1* is significantly reduced in *Ngn3*+ endocrine progenitors, and is restored in β cells at late gestation through adulthood; it is also expressed at lower levels in δ cells and in the acinar tissue. Global deletion of *Pdx1* results in pancreatic agenesis, although mutant animals develop a rudimentary dorsal bud and possess first-wave glucagon-positive cells (Jonsson et al. 1994, Offield et al. 1996). Reduced expression of *Pdx1* in animals homozygous for a hypomorphic *Pdx1* allele have fewer β cells, increased α and PP cells, disrupted islet architecture, and impaired glucose tolerance (Fujitani et al. 2006). Loss of *Pdx1* in β cells during late gestation (*RIP-Cre;Pdx1*$^{F/F}$) results in diabetes with age (Ahlgren et al. 1998). *Pdx1* heterozygosity causes reduced insulin secretion and glucose intolerance in adult mice due to decreased expression of genes regulated by *Pdx1* that are involved in β cell function and survival, including insulin, the glucose transporter 2 (*Glut2*), islet amyloid polypeptide (Amylin, *IAPP*), glucokinase (*Gck) MafA*, and *Pdx1* itself (Dutta et al. 1998, Brissova et al. 2002, Leibowitz et al. 2001, Waeber et al. 1996, Ohneda et al. 2000, Watada, Kajimoto, Miyagawa, et al. 1996, Watada, Kajimoto, Kaneto, et al. 1996, Raum et al. 2006, Marshak et al. 2000, Gerrish, Cissell, and Stein 2001).

Pdx1 is also involved in establishing the proper number of β cells. The burst of β cell proliferation just prior to birth (Figure 2) is mediated by *Pdx1* in mice, as deletion of *Pdx1* in β cells in mid-gestation results in decreased β cell proliferation and a concomitant increase in α cell replication (Gannon et al. 2008). Activation of *Pdx1* expression in *Ngn3*-positive cells biases some endocrine progenitors to the β cell fate, resulting in minor increases in β cells at the expense of α cells during the embryonic period. This may occur through through recruitment of the islet-enriched histone methyltransferase Set7/9 (Yang et al. 2011, Deering, Ogihara, et al. 2009). Postnatally,

this maintenance of *Pdx1* over-expression within differentiated endocrine cells promotes the conversion of α cells to β cells (Yang et al. 2011). Pdx1 is also thought to directly inhibit the α cell fate by repressing the *glucagon* promoter (Ritz-Laser et al. 2002).

Pax4 and Arx: β/δ vs. α lineage committment

As described above, *Ngn3*+ endocrine progenitors can give rise to all endocrine cell types and, in the presence of permissive factors, such as *Pdx1*, make specific endocrine cell fate decisions. *Pax4* is present in the pancreatic epithelium at e9.5 in the mouse, and is later restricted to β and δ cells (Sosa-Pineda et al. 1997). Ablation of *Pax4* in mice results in an increased number of ghrelin+ cells at the expense of β cells, and forced expression of *Pax4* in the developing pancreas and in adult α cells promotes the β cell fate (Collombat et al. 2009). These experiments indicate that *Pax4* is necessary and sufficient for commitment to the β cell lineage. Conversely, *Arx* is initially expressed in the mouse pancreatic anlagen at e9.5 and is later co-expressed with glucagon at e14.5 and also found in PP cells (Collombat et al. 2003, Collombat et al. 2007). *Arx* null mice show reduced numbers of α cells with a concomitant increase in β and δ cells, and ectopic expression of *Arx* throughout development or in adult β cells results in enhanced α cell differentiation and reduced β and δ cell number (Collombat et al. 2003, Collombat et al. 2007). Pax4 and Arx reciprocally inhibit the other's promoter (Collombat et al. 2005). The effects of *Pax4* and *Arx* modulation on the population of β/δ and α cells and their mutual antagonism suggests a governing role for these two transcription factors in endocrine cell type specification downstream of Ngn3. Interestingly, Pax4 and Arx exert similar control over the fates of enteroendocrine cells in the developing intestine (Beucher, Gjernes, et al. 2012).

Pax6: endocrine cell differentiation and adult α and β cell function

Pax6 is another paired homeodomain transcription factor expressed at e9.5 in the pancreatic epithelium and later in committed endocrine cells (Turque et al. 1994, St-Onge et al. 1997). *Pax6* is detected in both insulin- and glucagon-positive cells, and mice lacking *Pax6* show reduced numbers of β, δ, and PP cells, with a marked decrease in α cells and similar reduction in glucagon expression (Sander et al. 1997, St-Onge et al. 1997) . Similar to loss of *Pax4*, loss of *Pax6* results in an expansion of the ghrelin-expressing cell population, suggesting that it functions to divert endocrine cells away from this lineage (Heller et al. 2005). *Pax6* transactivates both *insulin* and *glucagon* promoters, and regulates expression of genes involved in adult β cell function (including *Pdx1, musculoaponeurotic fibrosarcoma (Maf) A,* and *Glut2,* among others) (Gosmain et al. 2012). In total, Pax6 is a key factor in endocrine cell differentiation as well as adult α and β cell function.

Nkx2.2: β cell lineage commitment

NK homeodomain factors are involved in specification of endocrine progenitors upstream of *Ngn3* expression and commitment to the endocrine cell lineage. *Nkx2.2* is expressed in the pancreatic bud and *Ngn3*+ endocrine progenitors, where it functions as a repressor of non-endocrine fates, and is later expressed in all endocrine cell types except δ cells (Sussel et al. 1998, Schwitzgebel et al. 2000, Doyle, Loomis, and Sussel 2007). Loss of *Nkx2.2* in mice results in the complete absence of β cells, reduced numbers of α and PP cells, and increased ghrelin+ ε cells, suggesting a strong role for Nkx2.2 in specification of the β cell lineage and partial control of other endocrine cell type lineage specification. Indeed, experiments in mice show that *Nkx2.2* and *Arx* work in concert to carefully regulate the population of δ and ε cells in the developing pancreas (Mastracci et al. 2011).

Exocrine development in late gestation

After the secondary transition, the acinar component of the exocrine pancreas expands through proliferation of pre-existing cells and maintains expression of digestive enzymes. Expansion of the acinar cell population throughout the secondary transition and late gestation is mediated by Wnt signaling (Murtaugh 2008). The ductal portion of the exocrine pancreas undergoes changes in gene expression and organelle formation at this time. Ducts reach maturity in the mouse between e17 and e18 (Wescott et al. 2009). Research performed in murine models suggests that *Hnf6* regulates expression of *prospero homeobox1 (Prox1)*, which is down-regulated in animals lacking *Hnf6* (Zhang et al. 2009). *Prox1* expression is required for pancreas development, where it is expressed in pancreas progenitors and maintained in ducts, islets, and centroacinar cells in the mouse (Wang et al. 2005). Absence of either *Hnf6* or *Prox1* in the pancreas results in increased ductal proliferation and dilated ducts, indicating the roles for both in ductal homeostasis and maintenance of differentiation (Zhang et al. 2009, Westmoreland et al. 2009). Late gestation is also characterized by the formation of primary cilia in ducts. These cilia are immotile and hypothesized to function as chemo- or mechano-sensory organelles in the pancreas (Davenport and Yoder 2005). Formation of primary cilia is mediated by expression of transcription factors including *Hnf6* and *Sox9*, and dysfunctional or reduced primary cilia result in duct dilation, pancreatitis, and cystic fibrosis (Zhang et al. 2009, Shih et al. 2012, Cano, Sekine, and Hebrok 2006). *Sox9* expression is upheld in differentiated ducts and also maintains the differentiated state, possibly through expression of polycystin *2* (*Pkd2)*, a component of primary cilia (Shih et al. 2012).

During late gestation, acinar cells also acquire their adult morphology and orientation. Zymogen granules form during the secondary transition (Pictet et al. 1972) and upon differentiation, acinar cells form a rosette structure at the ends of intercalatated

ducts (Figure 5) (Reichert and Rustgi 2011). Rosettes are organized such that the nuclei of each individual acinar cell is oriented at the basal membrane of the cell and zymogen granules containing digestive enzymes are positioned at the apical surface, where they are secreted into the ducts (Kesavan et al. 2009).

Endocrine development in late gestation

The period of time following the secondary transition and prior to birth in the mouse is characterized by increased proliferation of endocrine cells and their migration away from the ducts to form clusters within the exocrine tissue (Figure 2, 5). Islet morphogenesis is initiated at this time, although not fully established until postnatal stages. Additionally, β cells initiate changes in gene expression that are associated with maturity and glucose responsiveness (Figure 2).

Once endocrine progenitors acquire a specific endocrine fate and *Ngn3* expression is down-regulated, the cells regain their capacity to proliferate. The late gestational period of pancreas development marks the height of endocrine proliferation in the mouse, with the peak of proliferation just prior to birth at e18.5 (Gannon et al. 2008, Rhodes 2005). During early pancreatogenesis, endocrine cells are formed from *Ngn3*+ progenitors by neogenesis, but later in gestation and in the perinatal stages, endocrine mass is increased by proliferation of existing cells (Gu, Brown, and Melton 2003). The source of proliferative stiumuli at this time in murine gestation has not yet been completely characterized, but specific genes required for appropriate proliferation have been identified. Loss of *Pdx1* in insulin-producing cells of the developing mouse pancreas results in reduced proliferation of β cells and a subsequent increase in α cell proliferation and δ cell number, indicating the requirement for *Pdx1* to establish appropriate numbers of specific endocrine cell types (Gannon et al. 2008). Connective tissues growth factor (CTGF) also regulates β cell proliferation at late gestation, and loss

or over-expression of *CTGF* in a global or tissue specific manner results in altered ratios of endocrine cells in the developing pancreas (Crawford et al. 2009, Guney et al. 2011) . Additionally, loss of expression of PERK, an eIF2α kinase, is associated with permanent neonatal diabetes that is observed in human Wolcott-Rallison syndrome, and contributes to decreased β cell proliferation in fetal mice (Delepine et al. 2000, Zhang et al. 2006, Feng et al. 2009).

Concurrent with endocrine cell proliferation is the active migration of cells away from ducts (Figure 5) (Puri and Hebrok 2007). *Hnf6* and the Bmp, Wnt, and TGFβ modulator *CTGF* are both important to this process. *Hnf6* over-expression or loss of *Ctgf* results in endocrine cells that fail to migrate and remain apposed to the ducts (Gannon et al. 2000, Crawford et al. 2009). The extracellular matrix is normally remodeled as endocrine cells migrate into the acinar parenchyma, partially through TGF-β-mediated expression of matrix metalloproteases (Miralles et al. 1998, Perez et al. 2005). Integrin signaling is also involved in the emergence of cells from the ductal epithelium and their migration into the surrounding extracellular matrix (Cirulli et al. 2000). Additionally, the Wnt and epidermal growth factor (EGF) pathways contribute to endocrine cell migration, as does expression of E-cadherin. Inactivation of the Wnt ligand *Wnt5* in zebrafish and mouse impairs migration of endocrine cells (Kim et al. 2005). Global deletion of EGFR causes decreased β cell proliferation and impaired pancreatic branching in the mouse, partially through decreased expression of matrix metalloproteinases (Miettinen et al. 2000). Furthermore, over-expression of a dominant-negative Rho GTPase, *Rac1,* disturbs E-cadherin-mediated cell-cell contact and islet cell migration in the mouse (Greiner et al. 2009).

Establishment of islet morphology also occurs as endocrine cells migrate from the ductal epithelium (Figures 2, 5). Interaction between β cells is required for islet formation and function and is mediated by cell adhesion molecules, including cadherins

and neuronal cell adhesion molecule (N-CAM) (Dahl, Sjodin, and Semb 1996, Esni et al. 1999) . As endocrine cells migrate, they organize into clusters in which β cell contact each other directly. The arrangement of β cells in this manner and communication between cells via gap junctions and connexins allows for pulsatile insulin secretion in response to a glucose stimulus (Bergsten and Hellman 1993, Bosco, Haefliger, and Meda 2011, Head et al. 2012).

Maturation of β cells

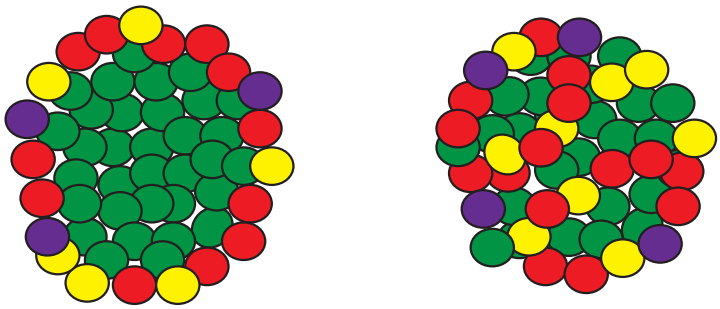
The hallmark of a mature β cell is regulated glucose responsiveness. Although β cells are still maturing at late gestation, key genes involved in adult function are first expressed at this time. The ability to respond to changes in blood glucose is dependent on expression of *Glut2* and prohormone convertase PC1/3, which cleaves proinsulin to active insulin (Guillam et al. 1997, Guillam, Dupraz, and Thorens 2000, Goodge and Hutton 2000) . Loss of either of these components affects insulin secretion in the postnatal animal. β cell maturation is also characterized by the transition of *MafB* to *MafA* expression (Nishimura et al. 2006, Artner et al. 2010). *MafB* is expressed in both insulin- and glucagon-positive cells at the primary and secondary transition, but its expression is restricted to α cells after birth (Artner et al. 2006). *MafA* expression is observed beginning at e13.5 at the secondary transtion in insulin-positive cells along with *MafB.* Activation of genes involved in β cell differentiation, including *Pdx1, Nkx6.1, Glut2,* and *MafA*, is promoted by *MafB* expression, and absence of *MafB* reduces the number of insulin-and glucagon-positive cells (Artner et al. 2007). Although MafA is not required for β cell development, it is a β cell-specific activator of insulin transcription and is required for glucose tolerance and insulin secretion in the adult animal (Zhang et al. 2005). The transition of *MafB* to *MafA* expression is coincident with the re-expression of *Pdx1* in β cells, which is consistent with the regulation of *MafA* expression by *Pdx1*

(Raum et al. 2006). Although *MafA* expression is seen in the mouse at e13.5, complete maturation of β cells does not occur until weaning (3 weeks of age) in rodents (Hang and Stein 2011) (Aguayo-Mazzucato et al. 2011).

β cell maturation is also associated with innervation of the pancreas, which initiates in the mouse at e10.5 with the migration of neural-crest derived cells into the pancreatic buds (Plank, Mundell, et al. 2011, Burris and Hebrok 2007) . Deletion of the transcription factor *Foxd3* from the neural crest population results in an expansion of endocrine mass. However, the expanded population of β cells fails to mature, as evidenced by decreased expression of *MafA* and *Glut2* (Plank, Mundell, et al. 2011). Thus, endocrine cell maturation is dependent on innervation of the pancreas, which initiates during mid-pancreatogenesis and completes postnatally around 3 weeks of age (Burris and Hebrok 2007).

Differences in mammalian islet morphology

After the burst of proliferation in the late gestational and early postnatal periods, pancreatic islets assume their characteristic morphology (Figure 2). The murine islet consists of a β cell core with α, δ, PP, and ε cells composing the peripheral mantle of the islet (Figure 9). This morphology is also conserved in porcine islets, although islets are



Rodent postnatal islet Human postnatal islet

Beta (β) cells: produce and secrete insulin to lower blood glucose levels; compose the core of the islet (rodents) or are in closer contact with other hormone-producing cells in the core (human)

Alpha (α) cells: produce and secrete glucagon to raise blood glucose levels; distributed around the perifery of the islet (rodents) or contacting other cells within the core of the islet (human)

Delta (δ) cells: produce and secrete somatostatin and work in concert with insulin to suppress glucagon production; distributed around the periphery of the islet (rodents) or more interspersed throughout the islet with other hormone-producing cells (human)

PP cells: produce and secrete the gut hormone pancreatic polypeptide, which regulates food intake and endocrine and exocrine pancreatic secretions; distributed around the periphery of the islet (rodents) or more interspersed throughout the islet with other hormone-producing cells (human)

Figure 9. Postnatal murine and human islet morphology. Figure modified and reprinted with permission from Henley and Gannon, 2013.

smaller and grouped into multi-islet clusters (Cabrera et al. 2006a). Interestingly, human and non-human primate islets differ in the organization of specific endocrine cell types compared to mice (Brissova, Fowler, et al. 2005). Humans and non-human primates have more of a 'mixed islet phenotype', with α, δ, and β cells found within the core of the islet, rather than compartmentalized to the periphery and core (Figure 9). Human islets are also heterogeneous and display different proportions of specific endocrine cell types compared to mice. In particular, human islets are composed of a greater percentage of α and δ cells, with a subsequent reduction in percentage of β cells, compared to murine islets (Brissova, Fowler, et al. 2005). It is currently unclear what accounts for the discrepancies between murine and primate islet morphology, but the reduced number of β cells in human or non-human primate islets may be related to their threshold of glucose sensitivity. Indeed, human and non-human primate islets have a lower set point for sensing glucose and respond lower levels of glucose than mouse islets (Henquin et al. 2006, Ling et al. 1996, Cabrera et al. 2006b, Cai et al. 2012). Perhaps primate islets are composed of fewer β cells because they are more sensitive to low levels of glucose, and therefore fewer β cells are required to maintain normal glucose levels within the organism. Currently, the field is assessing the species differences in islet development, composition, and glucose sensitivity in effort to better understand β cell function in the human context.

Murine islets are considered functionally mature upon glucose responsiveness and expression of *Glut2* and *MafA* and loss of *MafB*. Although human islets express *Glut2*, the predominant glucose transporter present in human β cells is the higher-affinity glucose transporter *Glut1* (De Vos et al. 1995, Ferrer, Benito, and Gomis 1995). Expression of this transporter may account for the increased sensitivity of human islets to low glucose levels. Also present in human β cells is *MafB*, which is normally restricted

to α cells in mice (Dai et al. 2012, Riedel et al. 2012). Cells that co-express insulin and glucagon are commonly observed in immature endocrine cells in the developing mouse and human pancreas. Surprisingly, adult human islets contain cells that continue to express both insulin and glucagon; while they lack *Pdx1, MafA,* and *Nkx6.1*, *MafB* and *Arx* can be detected in this population of cells (Riedel et al. 2012). These cells are thought to be mature α cells that transiently express *insulin* through an unknown mechanism, and this phenomenon is not observed in mouse islets (Riedel et al. 2012). It is not clear why adult human islets exhibit differential gene expression compared to murine islets, but these distinguishing features may partially explain why human β cells respond differently to glucose than murine β cells.

BMP and Wnt Signaling

BMP Signaling

BMP ligands and their receptors belong to the TGFβ superfamily of paracrine signaling molecules. This large group of secreted factors is divided into two subgroups based on sequence similarity and pathway activation. TGFβ, Activin, and Nodal comprise one subgroup, and BMP, Growth and differentiation factor (GDF), and Muellerian inhibiting substance (MIS) form the second subgroup. TGFβ signaling pathways are evolutionarily conserved and play a role in growth, differentiation, migration, adhesion, and apoptosis. BMP signaling is important for developmental processes including embryogenesis, neural development, somite formation, limb bud expansion, and bone formation (Gazzerro and Canalis 2006). Postnatally, BMPs are required for growth, differentiation, apoptosis, and proliferation, and have been

implicated in the pathophysiology of osteoporosis, kidney disease, cerebrovascular diseases, and cancer (Shi and Massague 2003).

TGFβ ligands are characterized by their cysteine knot structure and are secreted as functional disulfide-linked dimers. All ligands within the TGFβ superfamily bind to Type I and Type II trans-membrane serine threonine kinase receptors to initiate pathway activity; BMP ligands have a high affinity for Type I receptors, while TGFβ and Activin preferentially bind Type II receptors (Figure 10). BMP ligands (BMP 2, 4-7, 8a, 8b, 9, and 10) form hetero- or homodimers and bind to BMP receptor type I (BMPRI) or type II (BMPRII) to stimulate receptor heterodimerization and activation. BMP ligands bind weakly to Type II receptors alone, and with a much higher affinity when already bound to Type I receptors. Type II BMP receptors are constitutively active and phosphorylate Type I receptors after heterodimerization. Once activated, Type I receptors phosphorylate members of the Sma- and Mad-related (Smad) family of proteins, specifically receptor Smads (R-Smads) 1, 5, and 8, which then dimerize with the ubiquitously expressed co-Smad4 and translocate to the nucleus to facilitate transcription of target genes (Figure 10).

Regulation of BMP signaling

BMP signaling activity is regulated extracellularly and intracellularly at the ligand and co-factor level, respectively. BMP signaling can be antagonized intracellularly by inhibitory Smads 6 (BMP-specific) and 7 (a general TGFβ-family inhibitor), which compete with Smad4 for interaction with the activated Type I receptor (ten Dijke et al. 2003). Intracellular regulation is also maintained by the HECT-domain ubiquitin ligases Smurf1 and Smurf 2, which complex with Smad7 and target activated receptors for

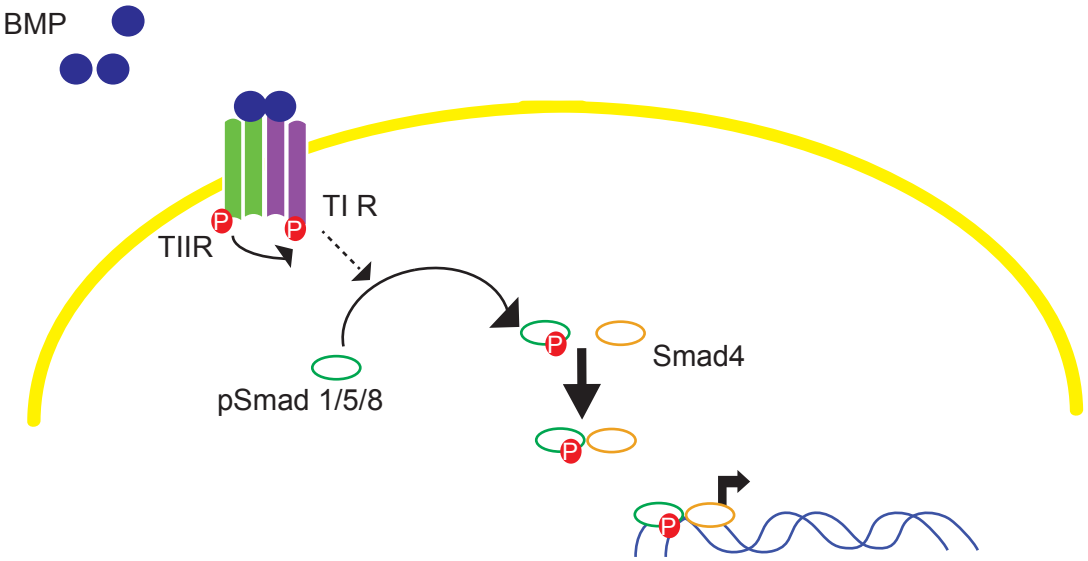


Figure 10. BMP signaling. Extracellular BMP ligands (blue circles) bind to Type I (TIR) and Type II (TIIR) serine threonine kinase receptors to facilitate hetero-oligomerization. The constitutively active TIIR phosphorylates and activates TIR after ligand binding. After activation, TIR phosphorylates BMP-specific Smads 1/5/8, which then dimerize with the ubiquitously expressed co-Smad4. This Smad complex translocates to the nucleus to activate transcription of target genes.

degradation (Ebisawa et al. 2001, Kavsak et al. 2000). TGFβ family antagonists bind to and sequester ligands to prevent their interaction with receptors. Extracellular antagonists of the BMP pathway are widely expressed throughout development and in adult tissues to modulate BMP signaling. Many BMP antagonists, including those belonging to the DAN/Cerberus/CAN family of inhibitors, have a cysteine knot structure similar to that of BMP ligands (Avsian-Kretchmer and Hsueh 2004). *Cerberus,* a member of the DAN family, induces ectopic head structures in *Xenopus* and plays a role in anterior neural induction and somite formation in mice; it binds to Nodal, Wnt, and BMP ligands, allowing modulation of the TGFβ and Wnt pathways (Pearce, Penny, and Rossant 1999, Avsian-Kretchmer and Hsueh 2004). *Gremlin,* also in the DAN family, was first isolated from *Xenopus* neural crest cells and is required for limb development (Khokha et al. 2003). In the kidney, *Gremlin* expression is induced by high glucose and is associated with several models of diabetic nephropathy (Lappin et al. 2002, Murphy et al. 2002). Other BMP antagonists, including DAN and SOST, are involved in axon outgrowth/guidance and negative regulation of bone mineralization, indicating the breadth of BMP antagonist location and function (Dionne, Skarnes, and Harland 2001, Kusu et al. 2003).

Differential effects of BMP signaling on pancreas development

As described above, both Activins and BMPs are important to the induction of pre-pancreatic and pancreatic endoderm. Interestingly, members of this superfamily have differential effects on pancreas development and function. While BMP4 and 7 promote *Pdx1* expression in the early endoderm in the chick, BMP signaling from the septum transversum promotes liver fate over pancreas fate in the mouse (Kim, Hebrok,

and Melton 1997a, Rossi et al. 2001). Later during development, over-expression of TGFβ1 under control of the rat insulin promoter (*RIP-Tgfβ1*) results in small,disorganized endocrine clusters in the mouse, suggesting that increased TGFβ signaling negatively affects endocrine development and organization (Sanvito et al. 1995). Consistent with this idea, over-expression of *Bmp6* in the pancreatic epithelium (*Pdx1-Bmp6*) results in pancreatic agenesis and reduced stomach and spleen size in the mouse (Dichmann et al. 2003). Interestingly, over-expression of *Bmp4* within the same domain has no effect on pancreas development, indicating specific roles for particular BMP ligands during pancreatogenesis (Goulley et al. 2007). In contrast to these studies, zebrafish carrying the truncated form of the BMP antagonist Chordin (*chordino)* exhibit an enlarged pancreatic primordia and increased expression of pancreatic markers (Tiso et al. 2002). Furthermore, the *swirl* zebrafish mutant, that possesses a mutation in the *Bmp2b* gene, exhibits decreased expression of the endocrine markers *NeuroD1* and *Islet1 (Isl1)* (Tiso et al. 2002*).* Removal of *CTGF*, a dual BMP/Wnt antagonist that shares functional similarity to *Sostdc1* (see below) in a conditional fashion causes reduced β cell proliferation and vascular density; when *CTGF* is over-expressed in an inducible fashion during embryogenesis, β cell mass is expanded by increased proliferation (Crawford et al. 2009, Guney et al. 2011). Interestingly, loss of the dual BMP and Wnt antagonist *Sostdc1* does not affect pancreas development (Henley et al. 2012). Clearly, there is a differential requirement for BMP signaling throughout pancreas development and across different species that requires further study to completely understand.

Wnt signaling

The Wnt pathway is a highly conserved signaling pathway that is involved in multiple developmental processes and includes over 20 mammalian ligands. Activation of the Wnt signaling pathway induces axis patterning, cell proliferation, differentiation, gene transcription, and cell adhesion (Nelson and Nusse 2004). Canonical Wnt signaling facilitates the stabilization of β-catenin and its transcriptional activation of target genes through disruption of the glycogen synthase kinase (GSK) 3/APC/Axin1 destruction complex (Figure 11). Wnt ligands bind to the 7-pass transmembrane domain Frizzled Fzd) receptor and LDL-related protein receptor (LRP) 5/6 to activate the intracellular phosphoprotein Dishevelled (Dsh). Dsh disrupts the formation destruction complex, which normally phosphorylates β-catenin to target it for degradation. The stabilization of β-catenin allows for its accumulation in the nucleus, where it functions to displace the transcriptional repressor Groucho from the TCF/LEF1 complex of transcription factors. In the absence of Wnt ligands, Dsh remains inactive and the destruction complex is able to phosphorylate β-catenin and target it for degradation (Figure 11).

Regulation of Wnt signaling

Activity of the Wnt pathway is regulated by extracellular antagonists that bind Wnt ligands or receptors. In particular, the *Dickkoph* (*Dkk)* family of extracellular Wnt

antagonists regulate pathway activity by binding LRP5/6 receptors and preventing interaction with Wnt ligand/Fzd receptor complexes (Zorn 2001). Wnt ligands are bound and sequestered from receptors by soluble Frizzled Related Proteins (sFRPs*)* or Wnt Inhibiting Factors (WIFs) (Kawano and Kypta 2003). sFRPS can also bind to Fzd receptors themselves, and may also stabilized Wnt ligands by binding them.

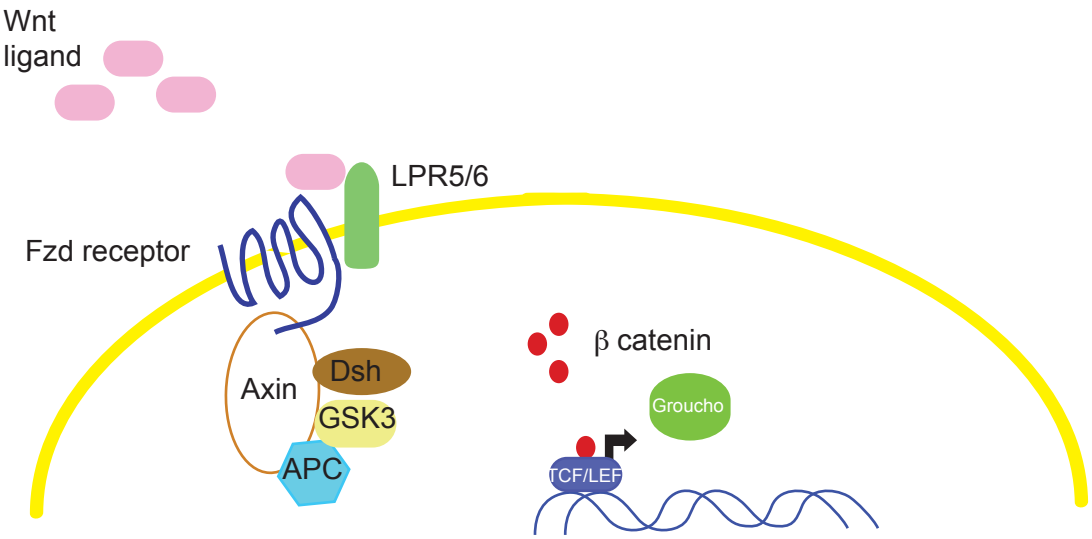


Figure 11. Canonical Wnt signaling. In the presence of Wnt ligand, Fzd and LRP5/6 receptors interact and activate Dsh, which prevents the Axin/GSK3/APC destruction complex from phosphorylating the coactivator β catenin and targeting it for degradation. When stabilized, β catenin can translocate to the nucleus, displace the co-repressor Groucho, and complex with TCF/LEF transcription factors to activate target genes.

Wnt signaling in the pancreas

Multiple Wnt ligands and receptors are expressed in the developing and adult pancreas (Heller et al. 2002, Heller et al. 2003). Studies in *Xenopus* have established the requirement for inhibition of Wnt signaling for proper formation of foregut endoderm and development of pancreas and liver (McLin, Rankin, and Zorn 2007). However, low levels of Wnt signaling are actually required to maintain foregut endoderm progenitors (Zhang, Rankin, and Zorn 2013). Later in development, Wnt signaling is absolutely required for expansion of the acinar cell population (Murtaugh 2008). As evidence, reduced Wnt signaling or ablation of β-catenin prior to or during the secondary transition results in a striking, sometimes complete, loss of exocrine tissue, with varying reductions in Pdx1-expressing progenitor cells and reduction in size and number of endocrine cells (Gittes 2009). Conversely, stabilization of β-catenin using the late (e11.5) *Pdx1-Cre* conditional system causes a 4.6-fold increase in pancreas size, indicating that Wnt signaling may play a role in late embryonic/post-natal growth (Heiser et al. 2006). Furthermore, conditional expression of constituitively active β-catenin under control of the *Pdx1* promoter increased β-cell size, enhanced insulin secretion, and improved glucose tolerance in mice (Rulifson et al. 2007). To contrast, islets from mice lacking *LRP5* exhibited decreased levels of intracellular Ca^{2+} and ATP after glucose stimulation and failed to increase insulin secretion after incubation with Wnt3a, which stimulates secretion in WT islets (Fujino et al. 2003). These data are especially interesting in light of the recent identification of the Wnt pathway transcription factor *Tcf7l2* as a T2D susceptibility gene. Of the *Tcf2l7* variants that have been associated with diabetes, the most likely candidate is a single nucleotide polymorphism (SNP), rs7903146, which lies within an intron (Gloyn, Braun, and Rorsman 2009). Although the exact role that *Tcf7l2* plays in the pathogenesis of diabetes has yet to be elucidated, it appears that

disregulated levels of *Tcf7l2* affect distribution of voltage gated Ca^{2+} channels in the membrane and ultimately alter insulin secretion (Shu et al. 2008, da Silva Xavier et al. 2009). In accordance with these data, carriers of the *Tcf7l2* risk allele exhibit decreased GSIS (Murtaugh 2008). Recent studies have revealed that deletion of *Tcf7l2* in the glucagon-expressing cells in the gut and brain, but not the pancreas, resulted in impaired glucose tolerance and blunted insulin secretion, suggesting that Wnt activity in non-pancreatic tissues can affect glucose homeostasis (Shao et al. 2013).

Sostdc1: BMP/Wnt antagonist regulated by Hnf6

While multiple studies have explored the effect of altered BMP signaling on pancreas development, fewer have examined the implications of irregular BMP pathway activity on adult pancreas function. Over-expression of *Bmp4* within the pancreatic epithelium (*Pdx1-Bmp4)* has no effect on pancreas development, but improves glucose homeostasis in adult animals and increases expression of genes involved glucose sensing and metabolism and in insulin production and secretion (Figure 12A, C) (Goulley et al. 2007). Furthermore, over-expression of a dominant negative *Bmpr1a* allele (*Pdx1-dnBmpr1a)* impairs glucose homeostasis, insulin secretion, and expression of genes involved in insulin release in mice (Figure 12B, D) (Goulley et al. 2007). These data indicate that positive or negative mediation of the BMP pathway through a specific ligand or receptor, respectively, affects islet function.

Similar to the *Pdx1-dnBmpr1a* model of impaired islet function, the *Hnf6* transgenic over-expression mouse model exhibits impaired glucose tolerance and reduced expression of *Glut2*, a gene associated with β cell maturity (Gannon et al. 2000, Tweedie et al. 2006). Analysis of changes in gene expression in pancreata from *Hnf6*

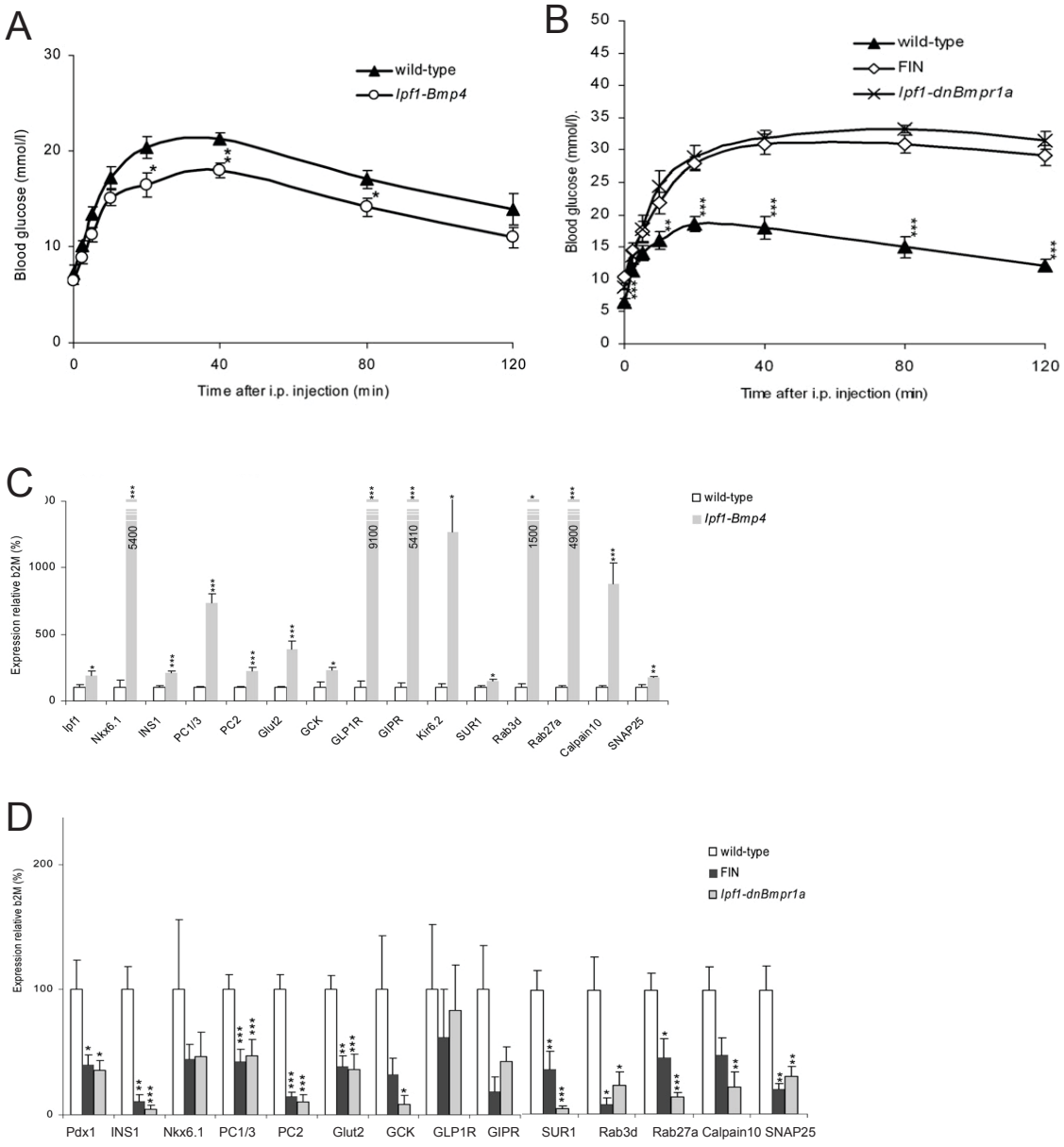


Figure 12. Characterization of *Pdx1-Bmp4* and *Pdx1-dnBmpr1a* animals. (A) Overexpression of *Bmp4* under control of the *Pdx1* promoter (*Ipf1-Bmp4*) improves glucose tolerance in adult mice. (B) Over-expression of a dominant negative BMP receptor *Bmpr1* under control of the *Pdx1* promoter (*Ipf1-dnBmpr1a*) impairs glucose tolerance. (C) and (D) Changes in gene expression in islets isolated from adult *Pdx1-Bmp4* and *Pdx1-dnBmpr1a* mice. Figures modified and reprinted with permission from from Goulley et al., 2007.

over-expressing mice at P1 revealed an up-regulation of the dual BMP/Wnt antagonist *Sclerostin domain-containing 1* (*Sostdc1; USAG-1, Ectodin, Wise;* Figure 13) (Wilding Crawford et al. 2008). Sostdc1 belongs to the 8-membered ring cysteine knot family of secreted BMP antagonists, including Cerberus, DAN, Gremlin, and Connective Tissue Growth Factor (CTGF) (Yanagita 2005). Sostdc1 shows overall 37% identity with mouse Sclerostin and 60% identity within the cysteine knot structure, and these two molecules form a distinct subgroup of DAN family BMP antagonists because they are secreted as monomers (Laurikkala et al. 2003). *Sostdc1* has been isolated from the uterus, testes, kidney, developing limb buds, teeth, bones, pancreas and regions within the developing and adult brain, and binds BMP2 and 4 with high affinity and BMP6 and 7 to a lesser extent (Laurikkala et al. 2003, Yanagita et al. 2004, Tanaka et al. 2008). Studies in tooth explants and hair placodes show that *Sostdc1* expression is induced by BMP ligands (Laurikkala et al. 2003, Narhi et al. 2008), consistent with the increased expression of BMP7 in the *Hnf6* transgenic model (Wilding Crawford et al. 2008). Sostdc1 regulates the size and placement of enamel knots through regulation of BMP signaling and inhibits BMP4- and 7-induced expression of muscle segment homebox *(*Msx) 2 in developing tooth ectoderm (Kassai et al. 2005, Laurikkala et al. 2003). Injection of *Sostdc1* mRNA in *Xenopus* embryos causes a hyper-dorsalized phenotype that is consistent with inhibition of BMP, a known ventralizing factor (Itasaki et al. 2003, Yanagita et al. 2004). In the kidney, Sostdc1 is the primary BMP antagonist and is localized with BMP7 during development and in adulthood, where the ligand functions to protect against renal fibrosis by inhibiting and reversing the epithelial-mesenchymal transition (EMT) (Yanagita et al. 2006). Mice lacking Sostdc1 are protected from kidney injury and exhibit supernumerary tooth formation and fused molars due to enhanced BMP signaling (Yanagita et al. 2004, Yanagita et al. 2006, Ahn et al. 2010) .

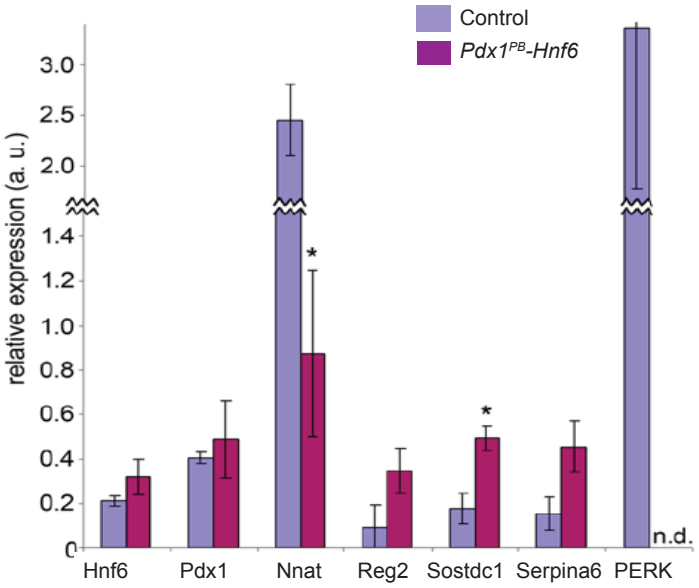


Figure 13. Gene expression changes in the islet-specific *Hnf6* over-expression model. RNA was collected from whole control and *Hnf6* over-expressing (*Pdx1*^{PB}*-Hnf6)* pancreata at P1 and changes in gene expression were detected by qRT-PCR. Genes of interest were normalized to *tubulin*. $P < 0.05$. Figure modified and reprinted with permission from Wilding-Crawford et al., 2008.

Overview and Aims of Dissertation

Previous studies in our lab have examined the function of *Hnf6* in endocrine differentiation and function using mouse models that exhibit reduced or augmented *Hnf6* expression in the developing and adult pancreas (Gannon et al. 2000, Tweedie et al. 2006, Wilding Crawford et al. 2008, Zhang et al. 2009). These pursuits have revealed that *Hnf6* participates in processes important to endocrine specification, differentiation, and postnatal function through regulation of genes required for these events. *Hnf6* is expressed in MPCs, bipotent progenitors, and in undifferentiated endocrine cells. Maintenance of *Hnf6* expression in endocrine cells causes islet dysmorphogenesis, increased α cells at the expense of β cells, hyperglycemia, impaired glucose tolerance, and the failure of β cells to mature. Removal of *Hnf6* reduces endocrine cell number during development and, in surviving animals, causes impaired insulin secretion and glucose tolerance (Jacquemin et al. 2000). These studies suggest that there is a temporal window during which *Hnf6* is required to establish endocrine mass and prime cells for appropriate function by activating specific genes.

A multi-pronged approach has been taken by the field to study, treat, and potentially cure both forms of diabetes. Efforts are being made to understand the immune insult that initiates T1D-mediated β cell destruction, while ongoing efforts to better comprehend β cell function, proliferation, and regeneration in the setting of T2D are also underway. Additionally, the specification and differentiation of endocrine cells is being assessed to improve methods for derivation of β cells *in vitro* or stimulation of their transidifferentiation from other cell types *in vivo.* The goal of the studies presented in this thesis are to further characterize roles for *Hnf6* and its downstream effectors in pancreatic endocrine function in the postnatal healthy and diseased states. We hypothesize that *Hnf6* regulates downstream effectors in a temporal-specific manner to

establish β cell maturity and function. This dissertation describes these efforts in four chapters. Chapter II describese methods by which the research presented in this thesis was performed. Chapter III presents experimental results testing the hypothesis that loss of *Sostdc1*, a dual BMP/Wnt inhibitor regulated by *Hnf6,* improves adult β cell function in the setting of metabolic demand. Chapter IV presents results regarding the cooperative role of *Hnf6* and *Pdx1* in regulation of genes involved in β cell maturity and function, and Chapter V details the proposed experiments involving the over-expression of *Hnf6* and *Pdx1* in a human ductal epithelial cell line to explore the potential of generating β cells from the ductal epithelium.

The following publications resulted from the research presented in this thesis:

'**Inactivation of the dual Bmp/Wnt inhibitor, Sostdc1, enhances pancreatic islet function**'. Henley, et al., American Journal of Physiology: Endocrinology and Metabolism. 2012 Sep 15;303(6).

'**Normal pancreatic development'.** Henley and Gannon, *Pathobiology of Human Disease: Dynamic Encyclopedia of Disease Mechanisms*. In press.

CHAPTER II

MATERIALS AND METHODS

Mice

Sostdc1 mutant allele

Sostdc1$^{LacZ/LacZ}$ animals were generated as described previously (Yanagita et al. 2006, Tanaka et al. 2008, Valenzuela et al. 2003). To summarize, a cytoplasmic *lacZ* allele replaced the open reading frame of *Sostdc1* to generate a knock-in allele (Figure 14). *Sostdc1*$^{LacZ/LacZ}$ animals were maintained on a mixed genetic background and genotyping of these animals was performed using primers designed to amplify the endogenous allele or the *LacZ* knock-in allele (Table 1).

BRE-LacZ transgenic mice

BRE-LacZ transgenic animals were generated as described elsewhere (Korchynskyi and ten Dijke 2002, Monteiro et al. 2004). Briefly, the coding sequence for β-galactosidase was inserted downstream of a fragment of the murine *Inhibitor of DNA binding 1* (*Id1)* promoter containing the Bmp response element (BRE) multimerized twice. Genotyping for the *BRE-LacZ* transgene was performed using primers specific for the transgene (Table 1).

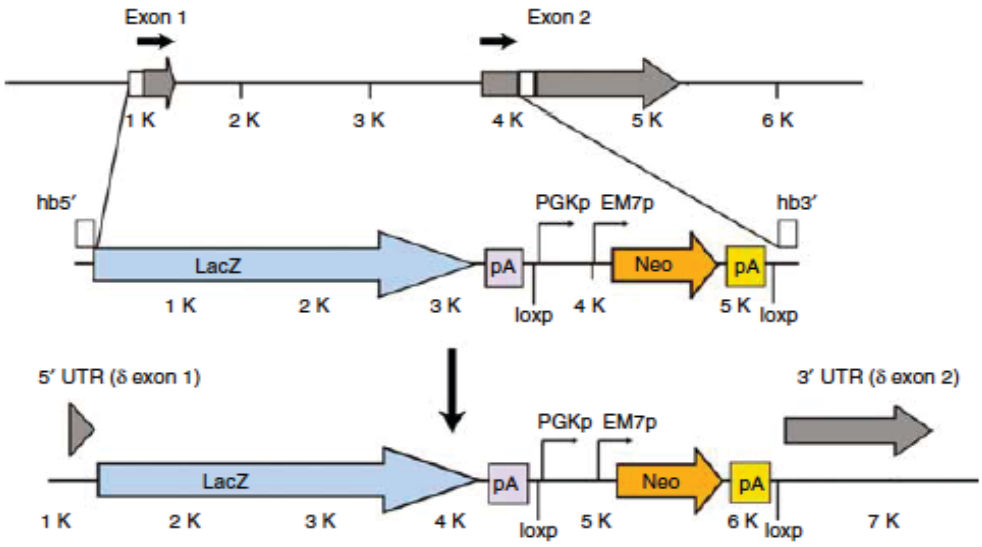


FIgure 14. *Sostdc1*^{LacZ} allele. The *Sostdc1*^{LacZ} allele was generated using homologous recombination. A cytoplasmic *LacZ* allele replaced the first exon of *Sostdc1* to generate a knock-in allele. The *LacZ* knock-in allele faithfully recapitulates Sostdc1 expression in the developing and adult kidney. Reprinted with permission from Tanaka et al., 2008.

Pdx1 mutant alleles

The *Pdx1*^{XSLacZ} (*Pdx1*^{LacZ}) allele was generated as previously described (Offield et al. 1996). In summary, a nuclear-targeted *LacZ* allele was fused in-frame with the 5' end of the second exon of *Pdx1*, replacing the DNA binding homeodomain (Figure 15). This allele generates a truncated protein that produces β *galactosidase* within the *Pdx1* domain. *Pdx1*^{LacZ} animals were maintained on a mixed genetic background. Genotyping for the *Pdx1*^{LacZ} allele was performed using primers specific for the *LacZ* knock in (Table 1). *Pdx1*^{KO} animals were generated by homologous recombination, in which intron and coding region sequences of the second exon were replaced with an MC1neo^{r} cassette (Figure 15) (Offield et al. 1996). Genotyping for animals carrying the *Pdx1*^{KO} allele were performed using primers specific for the MC1neo^{r} cassette (Table 1). All *Pdx1* mutant animals were maintained on a mixed genetic background.

Hnf6 floxed allele

The *Hnf6* floxed allele was generated as previously described (Zhang et al. 2009). In summary, *loxP* sites were introduced 5' and 3' of the cut domain within the first exon (Figure 16). *Cre*-mediated recombination of this locus generates a null allele. Primers designed to amplify the cut domain and the recombined allele were used for genotyping (Table 1). *Hnf6* floxed animals were maintained on a mixed genetic background.

Cre transgenes

The *Pdx1-Cre* and *Protamine-Cre (Prm-Cre)* transgenes were generated as described previously (Hingorani et al. 2003, O'Gorman et al. 1997). In summary, Cre recombinase expression is driven by the 4.3kb region of the murine *Pdx1* promoter (between *XhoI* and *SacI* restriction sites), and activates *Cre* expression as early as e9.5 in the antral

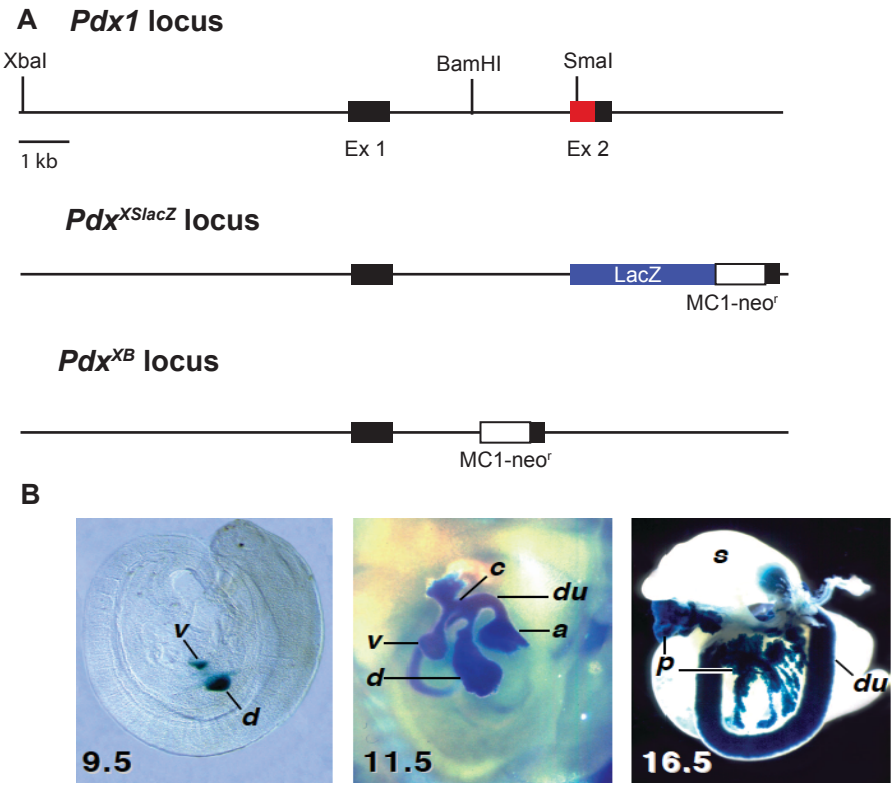
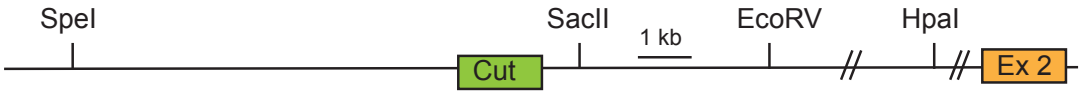


Figure 15. *Pdx1*^{XSLacZ} and *Pdx1*^{XB} alleles. (A) To generate the *Pdx1*^{XSLacz} (*Pdx1*^{LacZ}) allele, homologous recombination was used to replace the segment of *Pdx1* between *XbaI* and *SmaI* sites. A nuclear-targeted β-galactosidase allele that is fused in-frame with the 5' end of the second exon of *Pdx1* was inserted, replacing a portion of the homeodomain (middle locus; homoeodomain is shown in red). Homologous recombination was used to replace the segment of *Pdx1* between the *XbaI* and *SmaI* sites (including an intron and coding regions of the second exon) with an MC1-neo^r cassette (lower locus). Animals carrying two copies of the *Pdx1*^{XB} allele display pancreatic agenesis, consistent with other *Pdx1* null models (data not shown). (B) X-gal staining of embryos carrying the *LacZ* knock-in allele revealed evidence of β-galactosidase activity appropriately within the *Pdx1* domain. Numbers in (B) represent embryonic days of development. V, ventral bud; d, dorsal bud; a, antral stomach; du, duodenum; c, common bile duct; p, pancraes; s, stomach. Schematic in (A) was modified from Offield et al, 1996. Figures modified and reprinted with permission from Offield et al, 1996.

Wild type *Hnf6* allele



***Hnf6* targeting construct**

+ FLPe

***Hnf6* floxed allele**

Figure 16. *Hnf6* floxed allele. To generate the *Hnf6* floxed allele, a loxP-FRT *Hnf6* $^{flox\text{-}neo}$ construct with loxP sites flanking the cut domain (exon 1, green) was designed (middle schematic). The construct was electroporated into ES cells and positive clones were selected for neo^r and TK (gancyclovir) resistance. The neo^r cassette was removed by FLPe recombinase. The resultant floxed allele (bottom schematic) was microinjected into C57BL/6 blastocysts to generate chimeric mice. Figure adapted from Zhang et al., 2009.

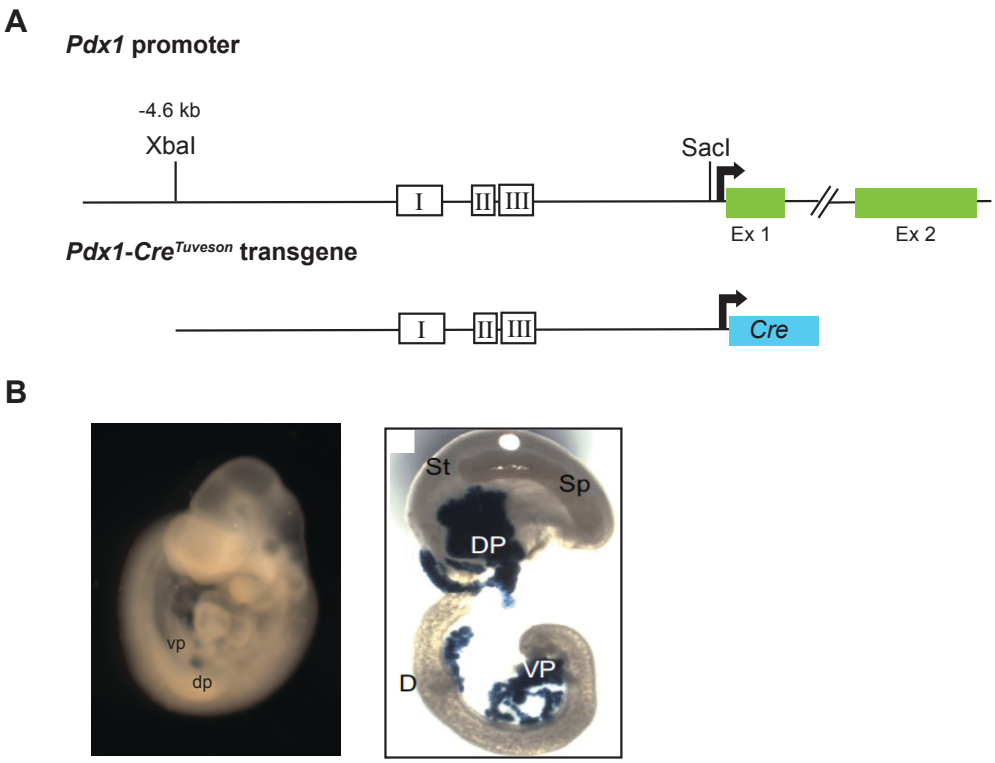


Figure 17. *Pdx1-Cre* transgene. (A) In this transgene, expression of *Cre* recombinase is under control of the 4.3 kb fragment between *XbaI* and *SacI* restriction sites of *Pdx1* promoter. This promoter contains areas I, II, and III, and is sufficient to drive *Cre* expression in the antral stomach, rostral duodenum, and throughout the entire pancreatic epithelium.(B) Recombination of the *R26R* reporter allele using this transgene is observed as early as e9.5 in the mouse (left) and throughout the pancreatic epithelium by e16.5 (right). St, stomach; sp, spleen; d, duodenum; dp, dorsal pancreas; vp, ventral pancreas. E9.5 whole mount image courtesy of Dr. Jennifer Plank and e16.5 image reprinted with permission from Wicksteed et al., 2010.

stomach, rostral duodenum, and pancreatic epithelium (Figure 17). *Prm-Cre* expression is driven by a 652-bp fragment of the murine *Protamine1* promoter and is expressed in the male germ line. *Prm-Cre* is active in haploid sperm, and recombines target alleles in the 2 to 4 cell stage. Primers designed to amplify the *Cre* transgene were used for genotyping (Table 1). All animals carrying the *Cre* transgene were maintained on a mixed genetic background.

***EYFP reporter* allele**

The enhanced yellow fluorescent protein (EYFP) reporter allele was generated as described previously (Srinivas et al. 2001). Briefly, homologous recombination was used to introduce an allele containing a transcriptional stop site flanked by *loxP* sites upstream of *EYFP* into the *ROSA26* (R26R) locus. In the presence of *Cre* recombinase, the sequence between *loxP* sites is recombined, removing the termination signal and allowing transcription of the *EYFP* allele. Primers designed to amplify the endogenous *R26R* locus and the *EYFP* insert were used for genotyping (Table 1).

All animals were maintained on a 12-hour light/dark cycle, provided food and water *ad libitum*, and all experiments involving animals were approved by the Institutional Animal Care and Use Committee of Vanderbilt University Medical Center.

Genotyping

Genotyping was performed using tail or ear punch DNA and the primer sets listed in Table 1.

Table 1. List of genotyping primers.

Primer name	**Sequence**
Sostdc1 WT 5’	CCTTCTCTGTGTTTTCACTCCG
Sostdc1 WT 3’	TGATTCAGGGTGCTGTTGC
LacZ Mut 3’	CCGTAATGGGATAGGTCACG
BRE-LacZ 5’	CCTTTCGCTATTACGCCAG
BRE-LacZ 3	TTAAGTTGGGTAACGCCAGG
LacZ 5’ (For *Pdx1*^{LacZ})	GCCGTCTGAATTTGACCTGA
LacZ 3’ (For *Pdx1*^{LacZ})	TCTGCTTCAATCAGCGTGCC
Pdx1^{XB} 5’	CAAGCCAGGGAGTTAAATC
Pdx1^{XB} 3’	AGGTGAGATGACAGGAGATC
Hnf6 Flox 5’	GTCGTCGACCTCTCTCTGTCTCCCTC AGTATCC
Hnf6 Flox 3’	ATAAGCGGCCGCCCTCCCTCTCTC TTT CCATC
Hnf6 recombined 3’	ATAAGCGGCCGCGCCTGCAGGGTTT GGAATCTGTG
Cre 5’	TGCCACGACCAAGTGACAGC;
Cre 3’	CCAGGTTACGGATATAGTTCATG
R26R 5’ (for eYFP)	AAAGTCGCTCTGAGTTGTTAT
R36R 3’ (for eYFP)	GGAGCGGGAGAAATGGATATG
eYFP Mutant 3’	AAGACCGCGAAGAGTTTGTC

Percent C57Bl/6 contribution.

Genetic background of *Sostdc1*$^{LacZ/LacZ}$ and wild-type littermates was determined by Jackson Laboratories (www.jax.org) using genome scanning for strain-specific single nucleotide polymorphisms (SNPs) (Petkov et al. 2004). Percent contribution of C57BL/6 to overall genetic background was calculated by adding the number of C57Bl/6-specific SNPs and one-half of the number of total SNPs heterozygous for C57Bl/6 and dividing by the total number of SNPs assessed.

Tissue dissection, preparation, and histology

For embryonic studies, the morning of the vaginal plug was considered to be e0.5. Digestive organs were dissected fixed for 1-4 hours in 4% paraformaldehyde (PFA) at 4°C, dehydrated in an ascending ethanol series, cleared in Citrisolv (Fisher) or xylenes, and embedded in paraffin. For frozen embedding, tissue was fixed as described above and placed in a 30% sucrose solution overnight. Following a 30 minute incubation in a 50/50 solution of FSC22 (Frozen Section Compound, Leica) and 30% sucrose, frozen tissues were embedded in 100% FSC22 and frozen on dry ice. Paraffin-embedded and FSC22 embedded tissues were cut at 5 μm and 7μm, respectively.

Paraffin-embedded tissues were deparaffinzed in Citrisolv or xylenes, rehydrated in a descending ethanol series to distilled water; frozen tissues were allowed to thaw for 30 minutes at room temperature and permeabilized in 0.1% Triton in 1X PBS for two 15 minute intervals. Indirect protein localization was obtained by incubation of tissue with primary antibodies (Table 2). Primary antibodies for Glut2, Hnf6, MafA, Sox9, and Synaptophysin required antigen retrieval (Table 2). Detection of primary antibodies was

Table 2. List of primary and antibodies used for immunohistochemistry.

Primary antibody	Source	Dilution	Antigen retrieval
Rab anti-glucagon	Millipore	1:1000	none
Rab anti-Glut2	DAKO	1:300	1X TEG buffer, pH 9.0, 1 minute fast boil, 7.5 minute slow boil
Gp anti-insulin	DAKO	1:1000	none
Goat anti-Hnf6	Santa Cruz	1:250	1X Sodium Citrate, pH 6.0, 2 hour pressure cooker
Rab anti-Hnf6	Santa Cruz	1:50	1X Sodium Citrate, pH 6.0, 2 hour pressure cooker
Rab anti-MafA	Bethyl	1:1000	1X TEG buffer, pH 9.0, 1 minute fast boil, 7.5 minute slow boil
Rab anti-Sox9	Millipore	1:5000	1X 10mM Citrate buffer, pH 6.0, 10 minute boil
Mouse α synaptophysin	Millipore	1:500	1X Sodium Citrate buffer, pH 6.0, 14 minute boil

achieved by labeling with species-specific secondary antibodies conjugated to either Cy2 or Cy3 fluophores and diluted 1:300.

For X-gal staining, tissues were permeabilized for 15–30 min in 2 mM MgCl2, 0.01% sodium deoxycholate, 0.02% Nonidet, P-40 in 1x PBS (permeabilization solution) at 4°C, and reacted with 5-bromo-4-chloro-3-indolyl-b-Dgalactopyranoside (X-Gal) substrate solution overnight at room temperature. Tissues were post-fixed in 4% PFA for 1 hour at 4°C.

Fluorescent and bright field images were captured using an Olympus BX41 research microscope or the Aperio ScanScope microscope and slide scanner (Vista, CA). Digital images were captured using MagnaFire software (Optronics Engineering, Goleta, CA) or the ImageScope software of the Aperio software suite (Vista, CA).

Analysis of α:β ratio and α/β cell area

Whole pancreata were serially sectioned at 5μm and sections were immunolabeled for insulin and glucagon. At least 1-2% of the entire pancreas was imaged (approximately 1 section every 250 μm throughout the pancreas). Slides were imaged as described above and using MetaMorph software (v7.7, Molecular Devices) or a macro built in Genie (Aperio System, Vista, CA), insulin- and glucagon- positive cells were counted or the area of insulin- and glucagon- positive cells was measured, respectively. The ratio of α to β cells was calculated by dividing the number of glucagon-positive cells by the number of insulin-positive cells. Insulin/glucagon-positive area was calculated by adding the insulin-positive and glucagon-positive area of each section and dividing it by the total pancreas area of each section.

Analysis of β cell mass

Sections ~250 μm apart (10 sections per animal) were immunolabeled for insulin followed by a peroxidase-conjugated secondary antibody, visualized using a DAB Peroxidase Substrate Kit (Vector Laboratories), and counterstained with eosin. Total pancreatic and insulin-positive areas of each section were measured using MetaMorph Software. β-cell mass was calculated by the ratio of insulin-positive area to total pancreas area of all sections for each animal multiplied by the tissue wet weight.

Analysis of β cell proliferation

Pancreatic sections 600–900 μm apart (3 sections per animal) were co-labeled for insulin and PH3 followed by species-specific Cy2- or Cy3-conjugated secondary antibodies. The PH3 primary antibody required antigen retrieval in 10 mmol/l sodium citrate, pH 6.5, followed by 0.2% Triton X-100 in PBS. Nuclei were labeled with 1.5 g/ml 4=,6=-diamidino-2-phenylindole (DAPI, Molecular Probes) in mounting medium. All insulin-positive cells on each section were imaged at 400X magnification on an Olympus BX41 microscope with a digital camera using Magnafire (Optronics). A minimum of 3,000 β-cells per animal were counted. Percent proliferating β-cells equaled the number of insulin/PH3 double-positive cells divided by the total number of insulin-positive cells.

Assessment of liver glycogen deposition by Periodic acid-Schiff (PAS) stain

5 μm sections of adult liver were randomly selected for assessment of glycogen deposition by PAS stain. Tissue sections were deparaffinzed and rehydrated in deionized water and stained according to manufacturer's instructions (Sigma).

Islet isolation and RNA acquisition

Isolation of islets from newborn or neonatal pancreas required dissection of the organ, collagenase digestion of whole pancreas at 37°C and hand-picking of islets from exocrine tissue. Isolation of islets from 4, 8, 12, and 16 week-old animals was performed by collagenase digestion from the main pancreatic duct and hand-picking from the exocrine tissue. Picked islets were placed immediately in 500µl Trizol reagent or RNALater (Ambion), lysed by vortexing or homogenized using a Tissuemiser (Fisher Scientific), and RNA was isolated using the RNAqueous (Ambion) or RNeasy Micro/Mini kits (Qiagen) according to manufacturer's instructions and eluted in 10-30 µl elution buffer. RNA concentration and integrity were assessed using a ND-1000 Spectrophotometer (NanoDrop) and the 2100 Electrophoresis Bioanalyzer (Agilent) at Vanderbilt Technologies for Advanced Genomics (VANTAGE) Core. cDNA generated from neonatal islets required amplification with the SMARTer Pico PCR cDNA synthesis kit according to manufacturer's instructions (Clontech).

Quantitative Real-Time PCR

cDNA was prepared from 50-350 ng islet or pancreas RNA using the Superscript III First-Strand synthesis system (Invitrogen). Real-time reactions were carried out in technical duplicate with iQ SYBR Green supermix (Bio-Rad) according to the manufacturers instructions on a CFX Real-Time PCR Detection system (Bio-Rad) in the Vanderbilt Molecular and Cellular Biology Resource Core. Primers used to assess gene expression are listed in Table 3.

Table 3. List of primers used for quantitative real-time PCR (qRTPCR).

	Forward Primer	**Reverse Primer**
HPRT *(*internal control)	AGT CAA CGG GGG ACA TTAA AA	TGC ATT GTT TTA CCA GTG TCA A
h18S (internal control)	CGG ACA TTG ACA GAT TGA TAG	TGC CAG AGT CTC GTT CGT TAT CG
Sostdc1	AAC AGC ACC CTG AAT CAA GC	CAG CCC ACT TGA ACT CGA C
Chordin	GCA GTG GTT CCC AGA GAA TCA	AAC AAT CGT CCC GCT CAC AGT
Ctgf	CTG GGG ACA ATG ACA TCT	GTT CGT GTC CCT TAC TTC CT
Id2	CCG CTG ACC ACC CTG AAC	ACA TAA GCT CAG AAG GGA ATT CAG AT
Nkx6.1	TCA GGT CAA GGT CTG GTT CCA	CGG TCT CCG AGT CCT GCT T
Smad1	TGT GAA CCA CGG GTT CGA	ATC CTG CCG GTG GTA TTC G
Glut2	GGG GTT GGT GCC ATC AAC	CAC AAG CAG CAC AGA GAC AGC
Bmp4	TGA TAC CTG AGA CCG GGA AG	AGC CGG TAA AGA TCC CTC AT
Bmp7	GGG CTT ACA GCT CTC TGT GG	TGG TCA CTG CTG CTG TTT TC
Bmpr1a	AAC GCT TGC GGC CAA TC	GAC ATT AGC TTC AAA ACT GCT CGA A
Wnt3a	ATG GCT CCT CTC GGA TAC CT	GGG CAT GAT CTC CAC GTA GT
Cyclin d1	GGC ACC TGG ATT GTT CTG TT	CAG CTT GCT AGG GAA CTT GG
Cyclin d2	GTT TGA GAG CAG GGC TTG AC	CTG TCT GCC TTT CGT CCT TC
Cdk4	TAG CCG AGC GTA AGG TGA GT	AT CGC ACT AGG CAC AAA GA
MMP7	GGT GAG GAC GCA GGA GTG AAC	GAA GAG TGA CTC AGA CCC AGA GAG T
c-Myc	CTC AGT GGT CTT TCC CTA CCC G	TGT CCA ACT TGG CCC TGT TGG C
PPAR-Delta	GGC CAT GGG TGA CGG AGC	GAT CTT GCA GAT CCG ACT GC
Chordin	GCA GTG GTT CCC AGA GAA TCA	AAC AAT CGT CCC GCT CAC AGT
Gremlin	AGA AAC AGC GCC TGA GCA AGA	AAA AGC GGC TGC CTA GGT CAT
Hnf6	AGG AGC CGG AGT TCC AGC	TGA AGA CCA GCC TGG GCT
Hnf6 OC domain	ATC CTC ATG CCC ACC TGA ATG	GTT TGA GCT CGG TGG TGA TAC
Pdx1	CGC GTG GAA AGG CCA GTG	CTG CCA GCT CCA CCC GGC GG
hHnf6	GGA GAC CTT CCG GAG GAT GTG	CCT ACC CTT CCT CCT TTG GTC
hPdx1	CTG CCT TTC CCA TGG ATG AAG	TCC GAC CCG GGA TAA TCC AAG

Analysis of gene expression by TaqMan Low Density array (TLDA)

To assess changes in islet gene expression, islet RNA was isolated as described above and 150-300ng cDNA was prepared using the SuperScript III First Strand Synthesis System Kit according to manufacturers instructions (Invitrogen). Genes were analyzed using TaqMan Universal PCR Mastermix (with UNG, Applied Biosystems) on

TLDAs were run on a 7900HT Fast Real-Time PCR PCR system and data were analyzed using SDS RQ Study software (Applied Biosystems, Life Technologies). All samples were run in triplicate.

Analysis of gene expression by RNA-Seq

RNA was extracted from whole pancreata at e15.5 (see above) and RNA libraries were prepared according to the University of Pennsylvania protocol (http://ngsc.med.upenn.edu, Lab protocol, Library preparation for RNA-Seq from total RNA). Libraries were single-end sequenced to 100bp on an Illumina hiSeq2000. Reads from ribosomal RNA and genomic repeats were identified by aligning the 5' 50bp of each read to ribosomal sequences and the human repeats in RepBase (version 14.10) using Bowtie (Langmead et al., 2010) and allowing for up to three mismatches. The remaining reads were processed with RUM (Grant et al. 2011) and aligned to the set of known transcripts included in RefSeq, UCSC known genes, and ENSEMBL transcripts, and the human genome (hg18, NCBI build 36.1). Transcript-, exon- and intron-level quantification was done using only the uniquely aligning reads (Langmead 2010).

Western blotting

Islets were isolated from 8-wk-old animals as described above. Following isolation, islets were immediately lysed in RIPA buffer and protein content was quantified using the Bio-Rad DC protein assay (Bio-Rad). Twenty micrograms of protein per sample were electrophoresed on 4–12% Bis-Tris gels under denaturing conditions and blotted onto PVDF membrane using the NuPAGE Western blotting system (Invitrogen). Blots were then blocked in 5% nonfat milk in TBS (pH 7.6) for 1 h at room temperature and probed with the following primary antibodies diluted in 3% nonfat milk in TBS and incubated overnight at 4°C: rabbit anti-phospho-Smad1 (Cell Signaling, 1:1,000) and mouse-anti-_-actin (Santa Cruz Biotechnology, 1:5,000). Blots were washed in 0.1% Tween 20 in TBS for 30 min at room temperature with three changes of buffer. HRP-conjugated species-specific secondary antibodies were diluted to 1:5,000 anti-rabbit and anti-mouse, Jackson ImmunoResearch) in 1% nonfat milk in 1X TBS and incubated for 1 h at room temperature. Following washes as previously described, protein detection was facilitated by an ECL detection system (Amersham) per the manufacturer's instructions using Kodak X-Omat Blue film. Protein levels were quantified using ImageJ software and presented graphically using GraphPad Prism software.

In vivo analysis of glucose homeostasis

Animals were fasted for 16 hours prior to *in vivo* intra-peritoneal glucose tolerance testing (IPGTT). Fasting blood glucose was measured from the tail vein using an Accuchek glucometer and glucose strips. Animals received an intraperitoneal glucose injection of filter-sterilized glucose (2mg dextrose/g body weight) and blood glucose was measured at the 15, 30, 60, 90, and 120 minute time points following injection.

Insulin tolerance testing

Animals were fasted for 6 h prior to an insulin tolerance test (ITT). Fasting blood glucose was measured as described above, and animals received an intraperitoneal injection of 0.075 U/ml insulin (recombinant human insulin, Sigma-Aldrich, no. I9278) in filter-sterilized 1X PBS at 0.1 ml/10 g body wt. Subsequent changes in blood glucose were measured at 15-, 30-, 60-, 90-, and 120-min intervals following injection.

Islet perifusion

Islet perifusions were performed as previously described (Brissova et al. 2002) Islets were isolated from the pancreata of mice on standard chow and high fat diet (HFD, 60% kcal from fat, BioServ) by collagenase digestion from the main pancreatic duct and hand-picked from exocrine tissue. Islets were perifused in a parallel, four-column apparatus controlled by peristalsis pumps and submerged in a circulating 37°C water bath. Islet effluent was plumbed into four fraction collectors at 3.0 min/fraction and saved for analysis of insulin secretion. Twenty to forty islets from each mouse were loaded per column and perifused with low (2.8 mmol/l) glucose perifusion medium (38.1 mmol/l sodium bicarbonate, 4.0 mmol/l L-glutamine, 1.0 mmol/l sodium pyruvate, 0.5% phenol red, 5.0 mmol/l HEPES, and 0.1% BSA in 1.0 l Dulbecco's modified Eagle's medium without glucose, pH 7.4) for a 30-min equilibration period (baseline) followed by high glucose (16.8 mM), high glucose plus 100μM 3-isobutyl-1-methylxanthine (IBMX), and high glucose plus 300 μM Tolbutamide. Insulin was measured by solid-phase radioimmunoassay (^{125}I-labeled insulin, Diagnostic Products) for mouse anti-insulin (MP Biomedical).

Generation of transgenes for over-expression of *Pdx1* and *Hnf6*

TetO-HA-Pdx1

To generate a tagged, inducible form of Pdx1, murine Pdx1 cDNA was cloned from the PZL1 plasmid, provided by Dr. Christopher Wright (Vanderbilt University). Primers were designed to add a 5' *EcoRV* site and an HA tag and an *EcoRV* site at the 3' end to *Pdx1* cDNA, generating an ~800 bp N-terminal HA-tagged version of Pdx1 flanked by *EcoRV* sites. PCR was used to generate HA-tagged Pdx1 containing 5' and 3' *EcoRV* sites from 100ng *Pdx1* cDNA and Platinum Taq (Invitrogen). The resultant product was digested with *EcoRV* and gel purified using a Gel Extraction Kit (Qiagen), according to manufacturer's instructions. Concentration and purity of the fragment was determined by ND-1000 Spectrophotometer (NanoDrop, (VANTAGE). EcoRV-HA-Pdx1-EcoRV was cloned into the pTeto$_7$CMV/BGH.polyA/pBSIISK(*AscI)* (pTetO) vector, provided by Dr. Timothy Blackwell (Vanderbilt University), following *EcoRV* digestion and dephosphorylation of the pTetO vector by Calf Intestinal Phosphatase (NEB) . pTetO and EcoRV-HA-Pdx1-EcoRV were incubated overnight at 15°C with T4 DNA Ligase according to manufacturer's instructions (NEB). Ligation products were transformed in DH5α by standard technique, allowed to recover for 1 hour in a shaking incubator at 37°, and 100µl were plated on Luria broth (LB) plates containing Ampicillin. Plates were incubated overnight at 37°C. Resultant colonies were picked and cultured overnight in 1ml LB containing 50ng/µl Ampicillin. Cultures were purified using a Miniprep Kit (Qiagen) according to manufacturer's instructions and eluted in 30 µl elution buffer. Presence of the HA-Pdx1 insert was verified by *EcoRV* digestion and sequencing (Figure 18).

Figure 18. Schematic of the pTetO-HA-Pdx1 plasmid. The HA-Pdx1 insert was cloned into the *EcoRV* site of the p(TetO)$_7$CMV/BGH.polyA/pBS II SK (*AscI*) plasmid to generate the TetO-HA-Pdx1 construct. The TetO-HA-Pdx1 fragment was linearized by digestion with *AscI*. pTetO$_7$CMV promoter ~ 500 bp; HA-Pdx1 fragment ~ 800 bp; BGH polyA tail ~ 300 bp. Plasmid not drawn to scale.

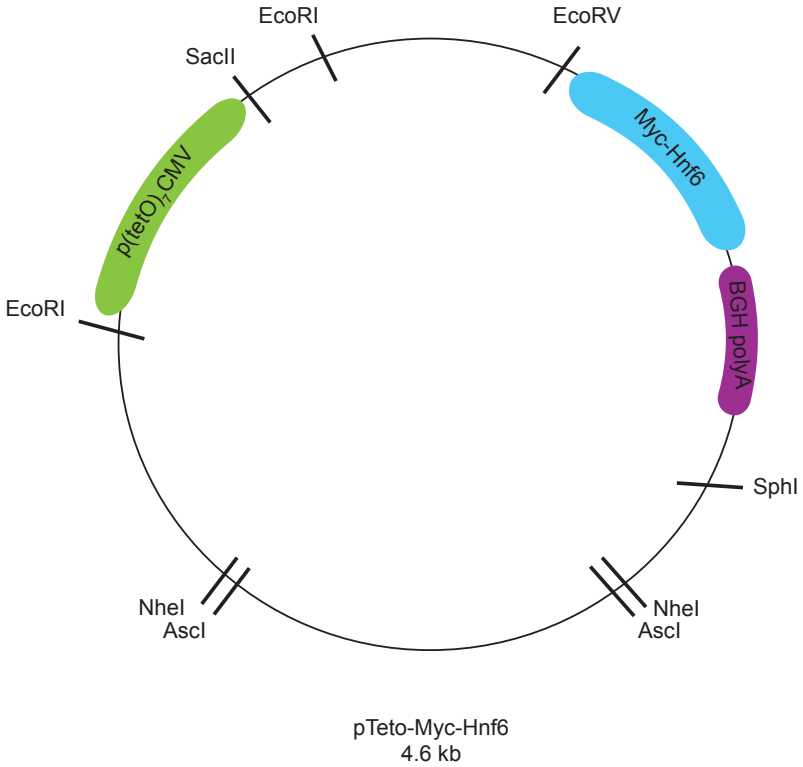


Figure 19. Schematic of the pTetO-Myc-Hnf6 plasmid. The Myc-Hnf6 insert was cloned into the *EcoRV* site of the p(TetO)$_7$CMV/BGH.polyA/pBS II SK (*AscI*) plasmid to generate the TetO-Myc-Hnf6 construct. The TetO-Myc-Hnf6 fragment was linearized by digestion with *NheI*. pTetO$_7$CMV promoter ~ 500 bp; Myc-Hnf6 fragment ~ 1.6 kb; BGH polyA tail ~ 300 bp. Plasmid not drawn to scale.

TetO-Myc-Hnf6

To generate a tagged, inducible form of Hnf6, murine *Hnf6* cDNA was cloned from the pGEM-1 parent plasmid, a gift from Dr. Robert Costa (University of Chicago). Primers were designed to add a 5' *EcoRV* site and a Myc tag and an *EcoRV* site at the 3' end to Hnf6 cDNA, generating an 1.6 kb N-terminal Myc-tagged version of Hnf6 flanked by *EcoRV* sites. PCR was used to generate Myc-tagged Pdx1 containing 5' and 3' *EcoRV* sites from 100ng *Hnf6* cDNA and Platinum Taq (Invitrogen). Cloning into the pTetO vector was performed as described above with one alteration; additional *Nhe1* restriction sites were added to the pTetO vector by PCR to facilitate the linearization of the TetO-Myc-Hnf6 fragment (Figure 19). Presence of the Myc-Hnf6 insert was verified by *EcoRV* digestion and sequencing.

Generation of *Pdx1* and *Hnf6* lentiviral vectors

pLVX-Pdx1-mCherry

Murine *Pdx1* cDNA was cloned from the PZL1 expression plasmid. PCR was used to add a 5' *XhoI* and 3' *SacII* restriction sites to *Pdx1* cDNA. Following introduction of these restriction sites, the XhoI-Pdx1-SacII fragment was digested with *XhoI* and *SacII* and gel purified. The pPRIG vector, provided by Dr. Patrick Martin (Université de Nice), was linearized by double *XhoI/SacII* digestion, dephosphorylated with Calf Intestinal Phosphatase (NEB) and gel purified. To add an internal ribosomal entry site (IRES) downstream of Pdx1, the XhoI-Pdx1-SacII insert and linearized pPRIG vector were ligated, transformed, cultured, and purified as described above. PCR was performed on pPRIG vectors containing the Pdx1 insert to add a *BamHI* restriction site 3' of the Pdx1-

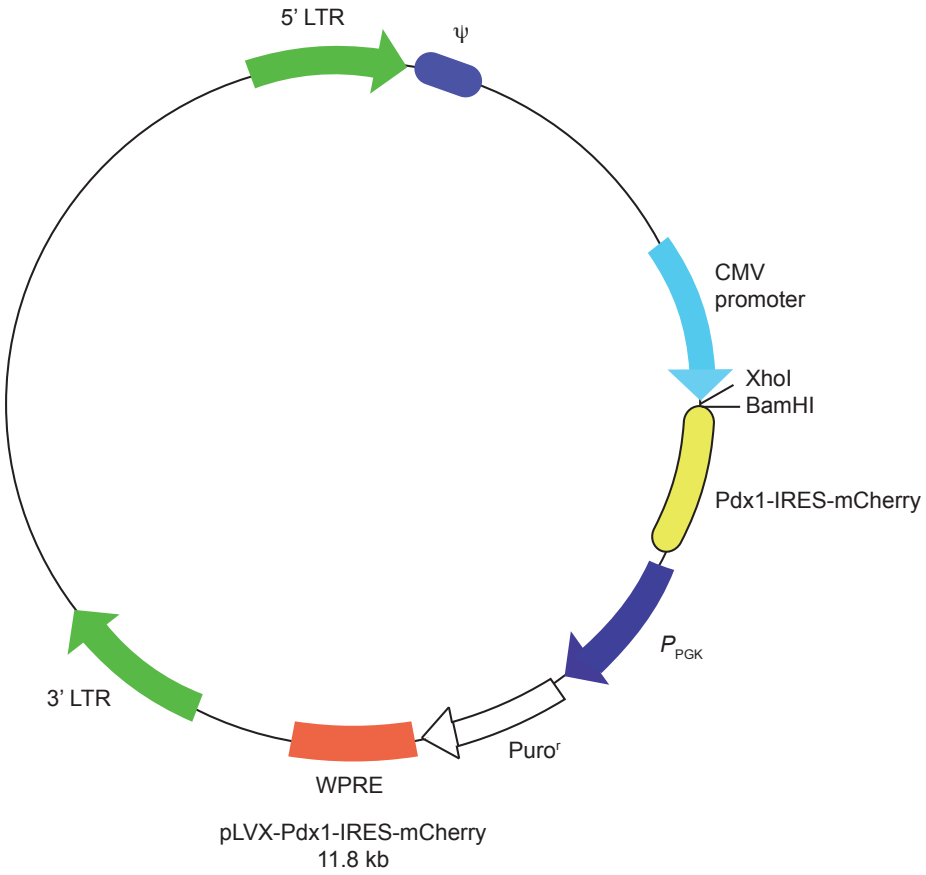


Figure 20. Schematic of pLVX-Pdx1-IRES-mCherry lentiviral plasmid. To generate a bicistronic vector for lentiviral over-expression of murine *Pdx1*, the 1.4 kb Pdx1-IRES insert was digested with *XhoI* and *BamHI* cloned into the pLVX-N3-GW-mCherry vector. Schmetaic includes components relevant to lentiviral production (ψ, packaging signal) and transcription (RRE, Rev-response element; P_{PGK}, posphoglycerate kinse promoter; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element). This plasmid map is modified from the pLVX-N3-mCherry vector map (Clontech, Dr. Matthew Tyska laboratory) and is not drawn to scale.

IRES fragment. Following gel purification, pPRIG vectors containing the Pdx1 insert were double digested with *XhoI* and *BamHI* to release the 1.4 kb Pdx1-IRES fragment. The pLVX-N3-GW-mCherry lentiviral vector, provided by Dr. Matthew Tyska (Vanderbilt University), was double digested with *XhoI* and *BamHI*, dephosphorylated as described above, and ligated with the XhoI-Pdx1-IRES-BamHI fragment as described above to generate a Pdx1-IRES-mCherry fragment (Figure 20). The resultant colonies were purified and sequenced to verify the presence of the *Pdx1-IRES* insert.

pLVX-TRE3G-Myc-Hnf6

To generate an inducible lentiviral vector that over-expresses murine *Hnf6*, the N-terminal Myc tagged *Hnf6* cDNA described above was cloned into the pLVX-TRE3G response plasmid (Clontech). The pLVX-TRE3G response plasmid is part of the Lenti-X Tet-On 3G Inducible Expression system, which includes a pLVX-Tet3G regulator plasmid containing a modified form of the Tet-On Advanced transactivator protein. In the presence of Doxycycline, the Tet-On 3G protein made by the pLVX-Tet3G vector binds the *tet* operator sequences located in the pLVX-TRE3G vector and activates gene expression. pLVX-TRE3G was digested with *SmaI* to yield a blunt end, linearized plasmid, and ligated with 1.6 kb Myc-Hnf6 as described above (Figure 21). After transformation, culturing, and purification, samples were digested and sequenced to confirm presence and orientation of the Myc-Hnf6 fragment.

Cell culture

Human ductal epithelial cells (HPDE6) were provided by Dr. Ming-Sound Tsao (Ontario Cancer Institute) (Furukawa et al. 1996). To generate this cell line, pancreatic tissues were obtained from human pancreatic specimens by Whipple's procedure, immortalized by transfection with human papillomavirus 16-derived E6 and E7 genes,

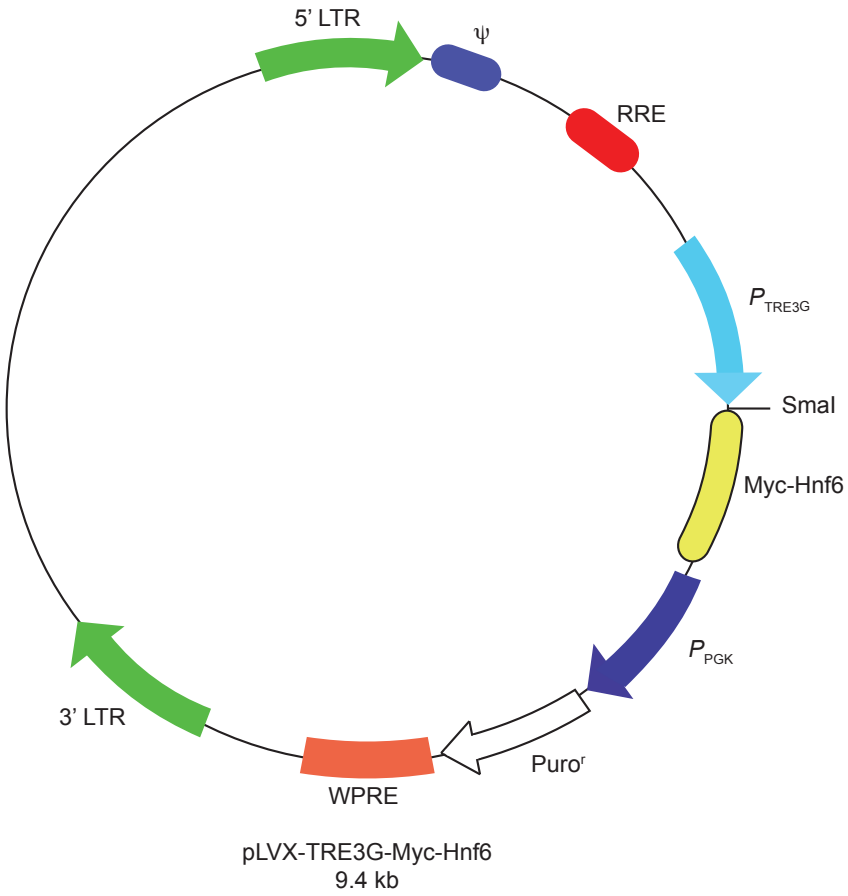


Figure 21. Schematic of pLVX-TRE3G-Myc-Hnf6 lentiviral plasmid. To generate an inducible model of murine *Hnf6* lentiviral over-expression, the 1.6 kb Myc-Hnf6 insert was digested with *SmaI* and cloned into the pLVX-TRE3G vector. Schmetaic includes components relevant to lentiviral production (ψ, packaging signal) and transcription (RRE, Rev-response element; P_{PGK}, posphoglycerate kinse promoter; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element). This plasmid map is modified from the pLVX-TRE3G vector map (Clontech) and is not drawn to scale.

and cultured on collagen gels. HPDE6 cells display a near normal phenotype, form a monolayer on collagen gels, express CK8/18 and CK19, are polarized with surface microvilli, and do not form tumors in mice or colonies in soft agar (Furukawa et al. 1996, Ouyang et al. 2000). HPDE6 cells were cultured in Keratinocyte Basal Medium, supplemented with bovine pituitary extract (20-30 µg/ml) and recombinant epidermal growth factor (rEGF, 0.1-0.2 ng/µl) (Lonza, Clonetics). Cells were grown in 25cm^2 cell culture flasks (Corning) at 37°C.

Statistics

Results are expressed as mean $\pm$ SEM. Statistical significance was calculated by Student's T Test, One-way, or Two-way ANOVA where applicable. $P \leq 0.05$ was considered significant.

CHAPTER III

GLOBAL LOSS OF THE DUAL BMP/WNT INHIBITOR SOSTDC1 ENHANCES PANCREATIC ISLET FUNCTION UNDER METABOLIC STRESS

Introduction

Our lab became interested studying the dual BMP/Wnt antagonist Sostdc1 in the context of pancreatic islet function when we observed a 1.8-fold increase in *Sostdc1* expression in pancreata of *Pdx1*^{PB}*-Hnf6* transgenic animals at P1 (Figure 13) (Wilding Crawford et al. 2008). Over-expression of *Hnf6* in endocrine population causes islet dysmorphogenesis, diabetes, and failure of β cells to mature in the postnatal animal, as evidenced by reduced expression of Glut2 and MafA (Gannon et al. 2000, Tweedie et al. 2006, Wilding Crawford et al. 2008). Increased expression of a BMP/Wnt antagonist in the setting of islet dysfunction was of interest to us as it had been documented that augmenting BMP signaling in the pancreas improves insulin secretion and glucose homeostasis by increasing expression of genes involved in insulin production, secretion, glucose sensing, and metabolism (Figure 12A, C) (Goulley et al. 2007). Conversely, expression of the dominant negative *Bmpr1a* receptor in the pancreatic epithelium has the opposite effect, impairing glucose homeostasis and reducing expression of key genes (Figure 12B, D) (Goulley et al. 2007); concordantly, global heterozygosity for the BMP receptor *Bmpr1a* diminishes insulin secretion and causes variability in expression of *Glut2, Pdx1*, *Gck*, and *Insulin* in islets (Scott et al. 2009).

The Wnt pathway is also implicated in islet function. Stabilization of the Wnt pathway effector β catenin in the *Pdx1* domain enhances islet size, insulin secretion, and glucose tolerance in mice (Rulifson et al. 2007). Glucose homeostasis is impaired and

insulin secretion is reduced in animals lacking the Wnt co-receptor *LRP5*, suggesting a direct role for Wnt signaling in β cell function (Fujino et al. 2003). Additionally, reduced expression of *Tcf7l2*, a Wnt-specific transcription factor, is associated with diabetes and disrupted insulin secretion in multiple populations and experimental models (Gloyn, Braun, and Rorsman 2009). Taken together, these data suggest a role for both BMP and Wnt signaling in postnatal islet function.

To examine the role of Sostdc1 in pancreas development and function, we utilized animals globally null for *Sostdc1*. *Sostdc1* mutants survive to adulthood and are fertile. Due to enhanced BMP signaling in developing and adult teeth, *Sostdc1* null animals exhibit supernumerary tooth formation and fused molars (Murashima-Suginami et al. 2007, Ahn et al. 2010). Augmented BMP signaling in the kidney also protects animals lacking *Sostdc1* from kidney injury (Tanaka et al. 2008).

We determined that *Sostdc1* null animals have no developmental phenotype and exhibit *ad lib* blood glucose values that are slightly reduced compared to control littermates, but have comparable glucose homeostasis profiles, when metabolically unchallenged. When placed on HFD, a subset of *Sostdc1* mutants are protected from diet-induced glucose intolerance and exhibit enhanced insulin secretion (Henley et al. 2012). These phenotypic changes are not related to the percent genetic contribution of C57BL/6 to the animals' genetic background. Prolonged exposure to HFD elicits a compensatory reduction in expression of *CTGF* and *Gremlin* in control islets, presumably to enhance BMP pathway activity. Loss of *Sostdc1* enhances the reduction of these genes in the setting of HFD exposure, thus preserving BMP signaling in the islet and improving function. This study was the first to examine the effects of augmented BMP/Wnt signaling in the context of pancreas function by capitalizing on the absence of a dual antagonist. The results of this study have been published in Henley, et al., 2012.

Results

Sostdc1 is not required for pancreas development or islet morphogenesis

Mice carrying a cytopmasmic *LacZ* allele knocked into the *Sostdc1* locus and replacing the coding region were used to study the role of *Sostdc1* in pancreas development and function (Valenzuela et al. 2003, Yanagita et al. 2004, Tanaka et al. 2008). *Sostdc1* null and heterozygous animals were born at expected ratios and possessed pancreata that were grossly morphologically similar to control pancreata (data not shown). To characterize the expression pattern of *Sostdc1* during pancreatogenesis, $Sostdc1^{LacZ/+}$ embryos were dissected at e14.5 and e18.5 and immunolabeled to detect β galactodisase activity. At both time points, X-gal staining was observed in the acinar component of the developing pancreas and absent from the developing insulin-positive clusters (Figure 22A). In the adult pancreas, X-gal reactivity was maintained in the acinar cells and excluded from the islets (Figure 22B), indicating that Sostdc1 is mainly expressed in the acinar tissue, and is not expressed at a level that can be detected by X-gal staining in the endocrine component of the pancreas at any time assessed.

To determine if loss of *Sostdc1* affects pancreas islet morphogenesis, sections of adult control and *Sostdc1* knock out (KO) pancreas were immunolabeled for insulin and glucagon. We observed normal islet morphology in *Sostdc1* mutants, with a dense β cell core and an α cell mantle, which were comparable to control littermates (Figure 22D vs. 22C) These data imply that islet morphogenesis in the mouse is not affected by loss of *Sostdc1.*

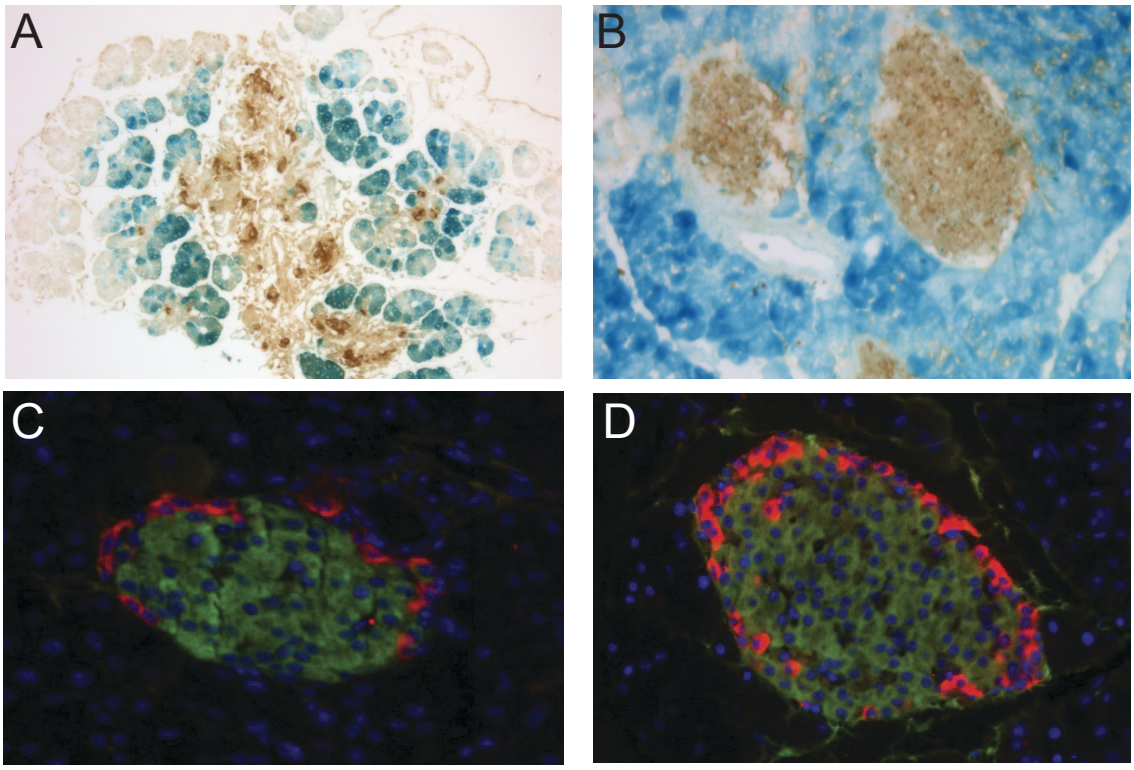


Figure 22. Sostdc1 localization in the developing and adult pancreas and influence on islet morphogenesis. (A) Sostdc1 (blue) is localized to the acinar clusters at e18.5 and is excluded from the insulin-positive (dark brown) endocrine tissue. (B) In the adult pancreas, Sostdc1 remains localized to the acinar tissue (blue) and is not detected by X-gal staining in the islets (brown). (C) Sections of pancreas from control animals exhibit proper islet morphology, with β cells composing the core (green) and α cells distributed around the periphery (red). (D) Sections of pancreas from *Sostdc1* null animals show islet morphology comparable to control littermates. Figures modified and reprinted with permission from Henley et al., 2012.

Loss of *Sostdc1* improves basal and stimulated islet function

To evaluate whether global loss of *Sostdc1* influences islet function, *ad lib* plasma insulin and blood glucose levels were measured in adult control and *Sostdc1* KO animals (data provided by Dr. Aris Economides, Regeneron Pharmaceuticals Figure 23A). Loss of *Sostdc1* causes elevated plasma insulin in a subset of animals and significantly reduced *ad lib* blood glucose in male and female adult mice. When challenged with an IPGTT, 8 week old males lacking *Sostdc1* are glucose tolerant and have glucose clearance profiles that are indistinguishable from control male littermates (Figure 23B). These data indicate that loss of *Sostdc1* reduces *ad lib* blood glucose levels, but does not alter glucose homeostasis without metabolic stress.

Control and *Sostdc1* KO littermates were placed on HFD for 4, 8, and 12 week intervals to determine if the absence of *Sostdc1* improves islet function under metabolic stress. Islets were isolated from control and mutant animals after exposure to HFD for 8 weeks and subjected to perifusion with high glucose and the secretagogues IBMX and Tolbutamide (Figure 24A). Stimulation with high glucose (16.7 mM) caused a non-significant increase in glucose secretion from *Sostdc1* null islets, but no differences were observed after IBMX and Tolbutamide stimulation (Figure 24A). It was noted that basal insulin secretion from control islets was consistent, while *Sostdc1* null islets segregated into clusters of low basal insulin secretion ($\leq$ 1.00 ng/100 IEQ/min) and and high insulin secretion ($\geq$ 2.00 ng/100 IEQ/min) (Figure 24B). When results from *Sostdc1* KO islets were separated based on basal insulin secretion and graphed accordingly, we observed a significant increase in total insulin secretion from islets with higher basal insulin secretion (Figure 24B, C). High glucose elicited an 11.3- and 8.3-fold increase in peak insulin secretion from control and *Sostdc1* KO islets with low basal insulin secretion, and a 4.86-fold increase from KO islets with high basal insulin secretion. Despite the reduced

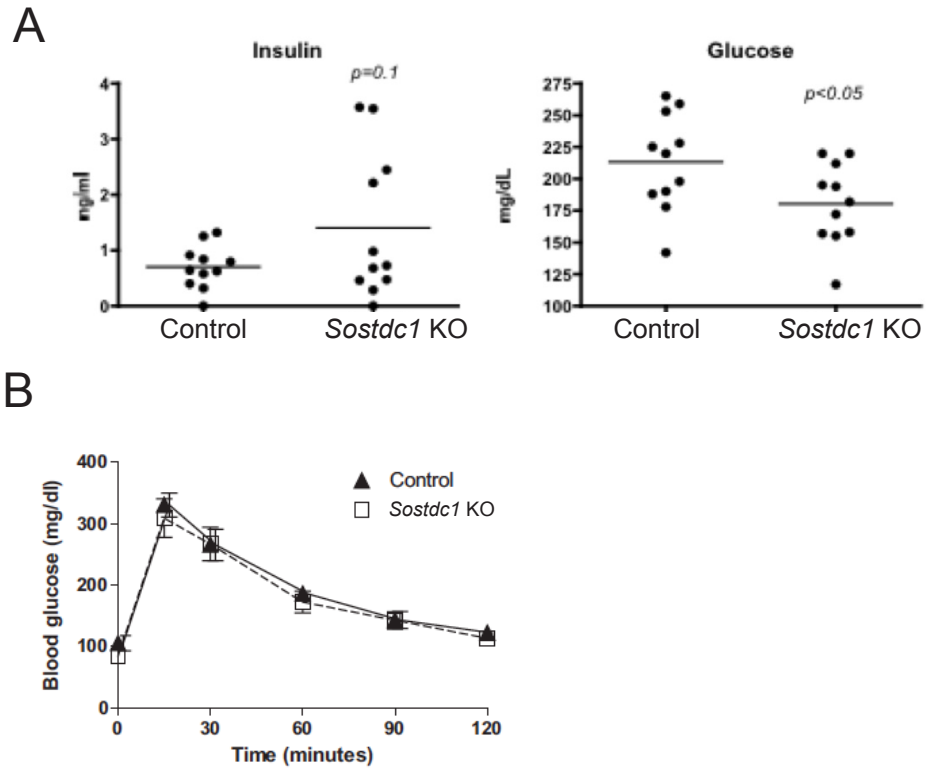


Figure 23. Loss of *Sostdc1* improves basal blood glucose levels. (A) A subset of adult male and female *Sostdc1* KO animals exhibit a trend toward increased *ad lib* serum insulin (left), and show significantly reduced *ad lib* blood glucose levels compared to controls. (B) At 8 weeks of age, control and *Sostdc1* KO male mice have indistinguishable glucose clearance profiles compared to controls. For (B), Control, n = 3; *Sostdc1* KO, n = 5. Error bars represent SEM. Figure (B) modified and reprinted with permission from Henley et al. 2012.

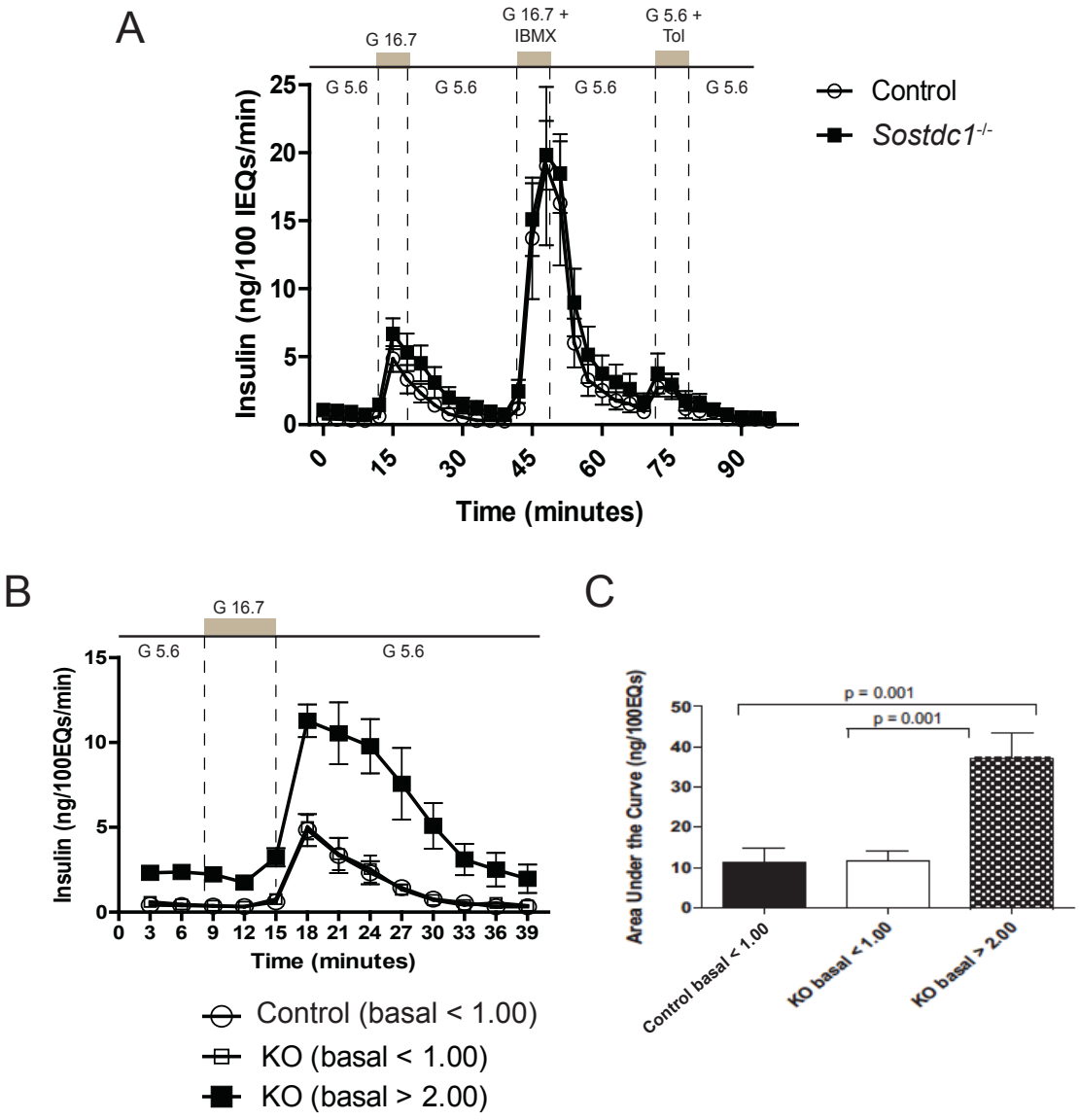


Figure 24. Loss of *Sostdc1* improves insulin secretion from isolated adult islets. (A) Loss of *Sostdc1* does not significantly increase insulin secretion from islets isolated from 8 week old mice after stimulation with IBMX or Tolbutamide, but elicits a non-significant increase after stimulation with high glucose. Control, n = 6; *Sostdc1* KO, n = 10. (B) Separation of *Sostdc1* null islets based on low (≤ 1.00 ng/100 IEQs/ min) and high (≥ 2.00 ng/100 IEQs/min) basal insulin secretion reveals that a subset of *Sostdc1* null islets exhibit increased insulin secretion in response to high glucose. Control islets (basal secretion < 1.00 ng/100 IEQs/min), n = 6; *Sostdc1* KO islets (basal secretion < 1.00 ng/100 IEQs/min), n = 6; *Sostdc1* KO islets (basal insulin secretion > 2.00 1.00 ng/100 IEQs/min), n = 5. (C). Quantification of data presented in (B). Error bars represent SEM. Figures modified and reprinted with permission from Henley et al., 2012.

fold change in insulin secretion in response to elevated glucose, the *Sostdc1* mutants with elevated basal insulin secretion exhibited a three-fold increase in insulin release overall in response to high glucose compared with the other two groups (Figure 24C). Collectively, these data provide evidence that loss of *Sostdc1* improves basal and glucose-stimulated insulin secretion after exposure to HFD in an *in vitro* setting.

We next examined *in vivo* glucose homeostasis by intraperitoneal glucose tolerance test (IPGTT) in control and *Sostdc1* male mice exposed to HFD for 8 and 12 weeks. At both time points, we observed that control and null animals segregated into two groups based on fasting blood glucose (FBG, Figure 25A). Animals exhibited low FBG (>125 mg/dl) or high FBG (< 125 mg/dl). During the glucose challenge, *Sostdc1* mutant animals with low FBG exhibited improved glucose clearance compared to both control and null animals with elevated FBG (Figure 25A). When subjected to an insulin tolerance test (ITT) after 8 and 12 weeks HFD, control and KO animals displayed comparable insulin tolerance (Figure 25B and data not shown). These results support the idea that improvements in glucose homeostasis can be attributed to augmented islet function, also supported by the perifusion data, and do not reflect changes in insulin resistance in peripheral tissues.

Analysis of endocrine mass and proliferation in *Sostdc1* null animals

The literature suggests that both BMP and Wnt signaling regulate proliferative processes in developing endocrine tissue and adult β cells; animal models display reduced or expanded endocrine compartments when BMP or Wnt pathways are inhibited or augmented, respectively (Papadopoulou and Edlund 2005, Dessimoz et al. 2005, Heiser et al. 2006, Hua et al. 2006, Crawford et al. 2009, Guney et al. 2011).

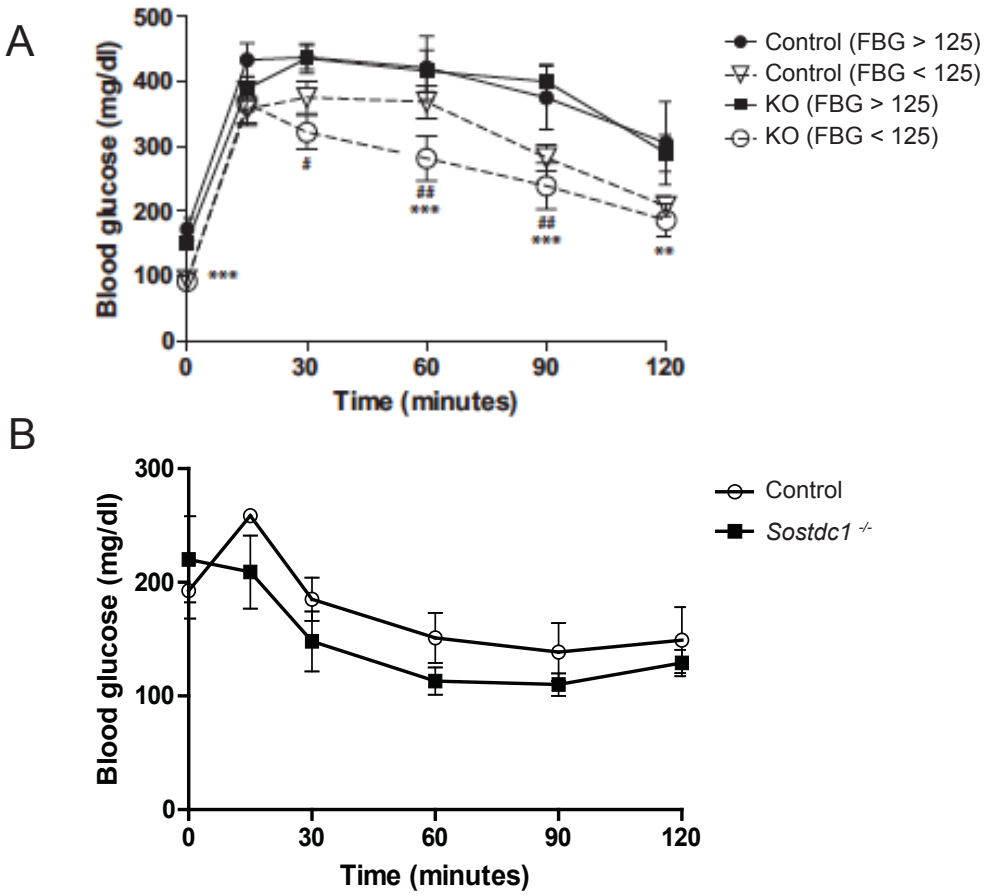


Figure 25. Loss of *Sostdc1* improves glucose homeostasis and does not affect insulin sensitivity. (A) A subset of *Sostdc1* null animals with FBG < 125 mg/dl exhibit improved glucose homeostasis compared to control and *Sostdc1* null animals with FBG > 125 mg/dl. Control (FGB > 125), n = 6; Control (FBG < 125), n = 11; KO (FBG > 125), n = 10; KO (FBG < 125), n = 8). KO (FBG < 125) vs KO (FBG > 125), * P = 0.1, ** P = 0.05. KO (FBG > 125) vs Control (FBG > 125), $^{\#}$ P = 0.01, $^{\#\#}$ P = 0.05. (B) Control and *Sostdc1* mutant male mice exhibit comparable glucose clearance when challenged with insulin. Error bars represent SEM. Figures modified and reprinted with permission from Henley et al., 2012.

Thus, it is possible that the absence of a dual BMP/Wnt regulator may enhance proliferation due to activation of either or both of these pathways. To determine if improvements in glucose homeostasis in the absence of *Sostdc1* could be attributed to increased endocrine mass, we measured β cell mass in control and null animals after exposure to chow and HFD for 12 weeks (Figure 26A, B). At the time points evaluated, there were no differences in β cell mass between genotypes; these data are consistent with the IPGTT data from chow-fed *Sostdc1* KO animals, which do not display improved glucose clearance compared to controls (Figure 26A). β cell proliferation was also assessed by immunolabeling for phosphohistone H3 (PH3) after 8 weeks of chow diet and 12 weeks HFD (Figure 26C, D). At the time points examined, there were no differences in proliferation between genotypes, indicating that loss of *Sostdc1* does not affect adult β cell proliferation in the basal or stimulated state.

Assessment of genetic background in *Sostdc1* null animals

Investigation of insulin secretion and glucose homeostasis revealed a divergent phenotype in *Sostdc1* null animals. In particular, we observed a deviation in basal insulin secretion from isolated islets after exposure to HFD for 8 wk (Figure 24B) and in FBG after exposure to HFD for 12 wk (Figure 25A). Together, these results indicate that in a subset of mutant animals, the absence of *Sostdc1* benefits β cell function. The *Sostdc1* null animals used in these experiments were of mixed genetic background, consisting of C57Bl/6, DBA, and 129Sv. To determine whether the divergent phenotype could be attributed to the varying contribution of these different genetic backgrounds, we utilized genome-scanning analysis to identify background-specific genetic markers that might be correlated with the improved phenotype (Petkov et al. 2004). To this end, tissue samples

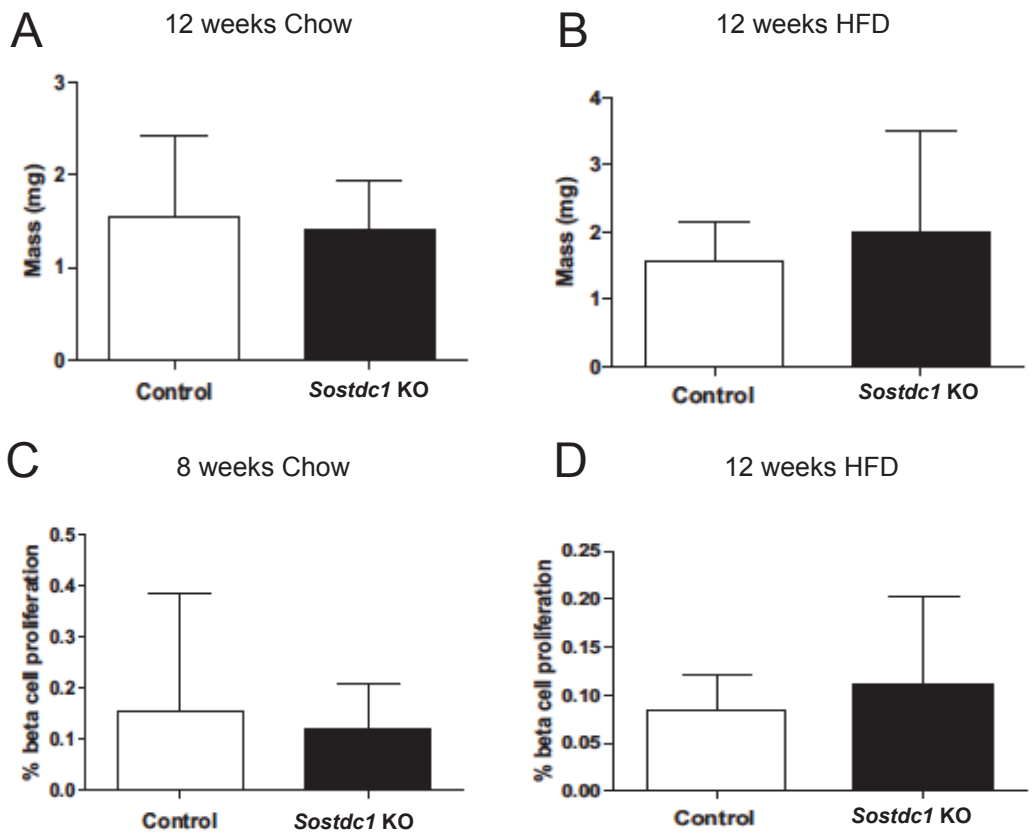


Figure 26. Analysis of β cell mass and proliferation in the absence of *Sostdc1*. Quantification of β cell mass in control and mutant male mice after 12 weeks chow (A) or HFD (B). Quantification of β cell proliferation by immunolabeling for phospho-histone H3 (PH3) in control and *Sostdc1* null male mice after 8 weeks chow (C) or 12 weeks HFD (D). For all figures: Control, n ≥ 2; *Sostdc1* KO, n ≥ 4. Error bars represent SEM. Figures modified and reprinted with permission from Henley et al., 2012.

were collected from control and *Sostdc1* KO animals and analyzed for SNP markers associated with C57Bl/6, DBA, and 129Sv genetic backgrounds (Appendix A). It has been well established that the C57Bl/6 strain is prone to impaired glucose tolerance and insulin secretion with age due to a mutation within the nicotinomide nucleotide transhydrogenase (*Nnt*) gene (Toye et al. 2005). Thus, we predicted that loss of *Sostdc1* would improve insulin secretion in animals predisposed to β cell dysfunction and anticipated that null animals showing the improved phenotype would also show higher C57Bl/6 genetic contribution. Interestingly, genome scanning analysis did not reveal any striking variations in composition of genetic background within *Sostdc1* null animals. Indeed, of the mutant animals assessed, contribution of C57Bl/6 to genetic background consistently ranged from 44.3 to 54.49%, indicating that broad differences in genetic background do not play a role in the divergent phenotype of *Sostdc1* KO animals (Appendix A). In addition, we assessed whether an increased number of *Nnt* mutant alleles in a given animal was associated with improved glucose homeostasis in the absence of *Sostdc1*. However, we found that the presence of this mutant allele could not account for the observed phenotype (data not shown).

Examination of changes in gene expression in the absence of *Sostdc1*

Following analysis of genetic background using strain-specific SNPs, we were unable to correlate percentage of specific genetic background with improved insulin secretion and glucose tolerance observed in a subset of *Sostdc1* null mice. To determine whether loss of *Sostdc1* affects changes in gene expression that may contribute to the observed phenotype, islets were isolated from 8 and 12 week old mice and differential gene expression was interrogated by quantitative RT-PCR (qRT-PCR). We chose to asses expression of genes that are regulated by BMP and involved in β cell function, including *Bmp4, Bmp7, Bmpr1a, Smad1, Nkx6.1, Id2,* and *Glut2* (Goulley et al.

2007). We also examined Wnt-responsive genes that have been linked to β cell function, including *PPAR-delta*, *c-Myc, matrix metalloprotienase 7 (MMP7), Wnt5a, Cdk, CyclinD1, and CyclinD2* (Liu and Habener 2008, Liu et al. 2008, Krutzfeldt and Stoffel 2010). After exposure to regular chow diet for 4 weeks (8 weeks of age), there were no significant changes in expression of genes assessed in male *Sostdc1* KO mice compared to controls (Figure 27A). After 4 weeks HFD, male *Sostdc1* null animals exhibit increased expression of the BMP-regulated glucose transporter *Glut2*, but all other genes assessed remain unchanged (Figure 27B). Interestingly, after extension of HFD exposure to 8 weeks, *Glut2* expression was normalized, while expression of the BMP-regulated transcription factor *Nkx6.1* and BMP pathway cofactor *Smad1* were significantly down-regulated in null islets (Figure 28A). Furthermore, expression of functionally similar BMP/Wnt modulators *Ctgf* and *Gremlin* were also significantly reduced in the absence of *Sostdc1* (Figure 28A).

We were interested in quantifying expression levels of *Ctgf* and *Gremlin* in chow-fed control animals after having observed the significant reduction in these genes after exposure to HFD. Control animals exhibited a trend toward decreased expression of *Sostdc1* and a significant reduction in expression of *Ctgf* after 8 weeks HFD, whereas *Gremlin* expression increased without significance (Figure 28B). As described above, we observed a significant reduction in *Sostdc1, Ctgf,* and *Gremlin* in islets from animals lacking *Sostdc1* after 8 weeks HFD (Figure 27B). The data presented here reveal a selective reduction in dual BMP and Wnt modulators after exposure to HFD that is greatly enhanced in the *Sostdc1* null background.

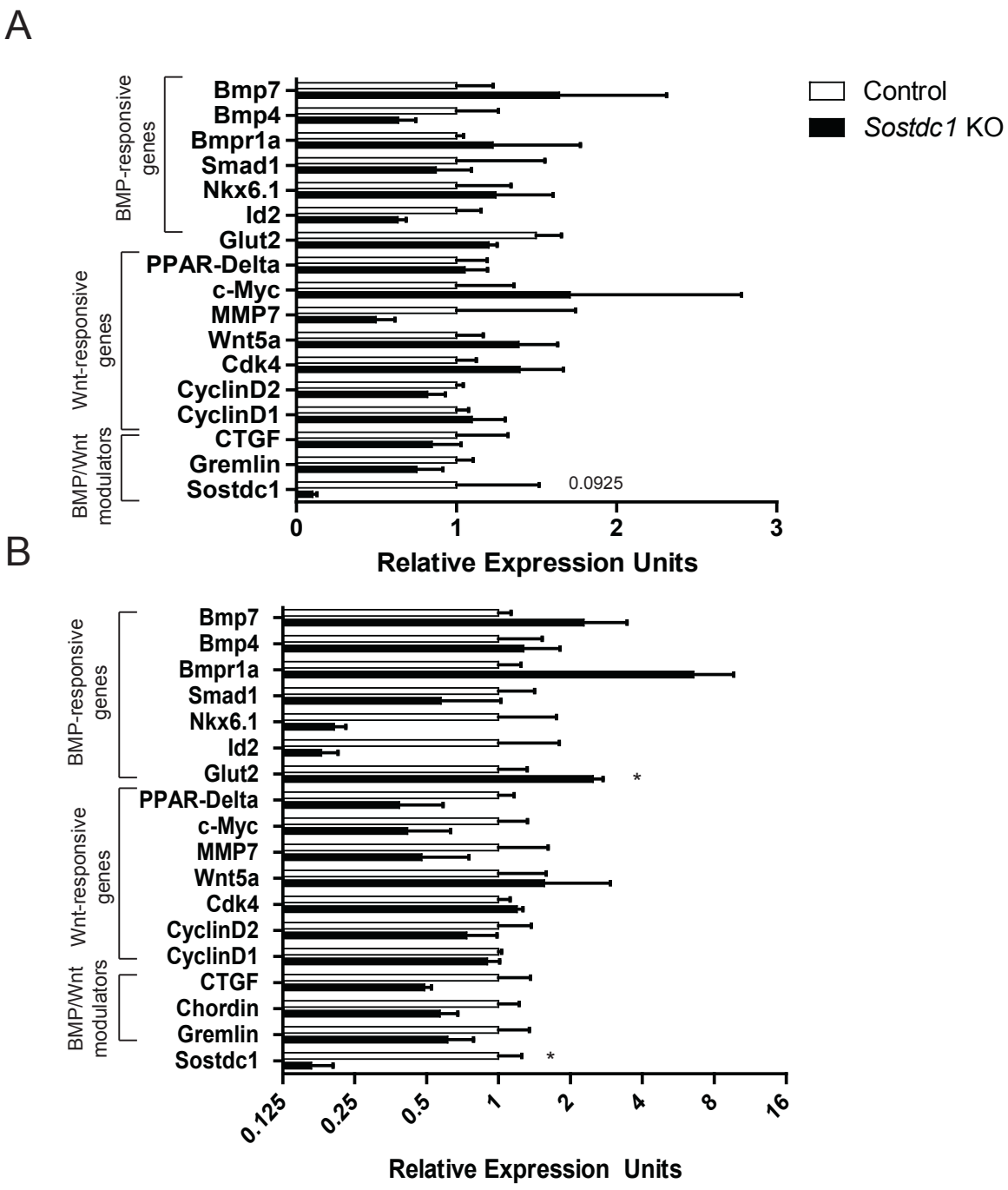


Figure 27. Gene expression in Sostdc1 null islets. (A) Analysis of selected BMP/Wnt-responsive gene expression in control and Sostdc1 null islets after 4 weeks chow. (B) Analysis of selected BMP/Wnt-responsive gene expression of control and Sostdc1 null islets after 4 weeks HFD. For each experiment, $n \geq 3$ for each genotype. Expression levels were normalized against ubiquitiously expressed Hprt. * P = 0.05. Error bars represent SEM. Figures modified and reprinted with permission from Henley et al., 2012.

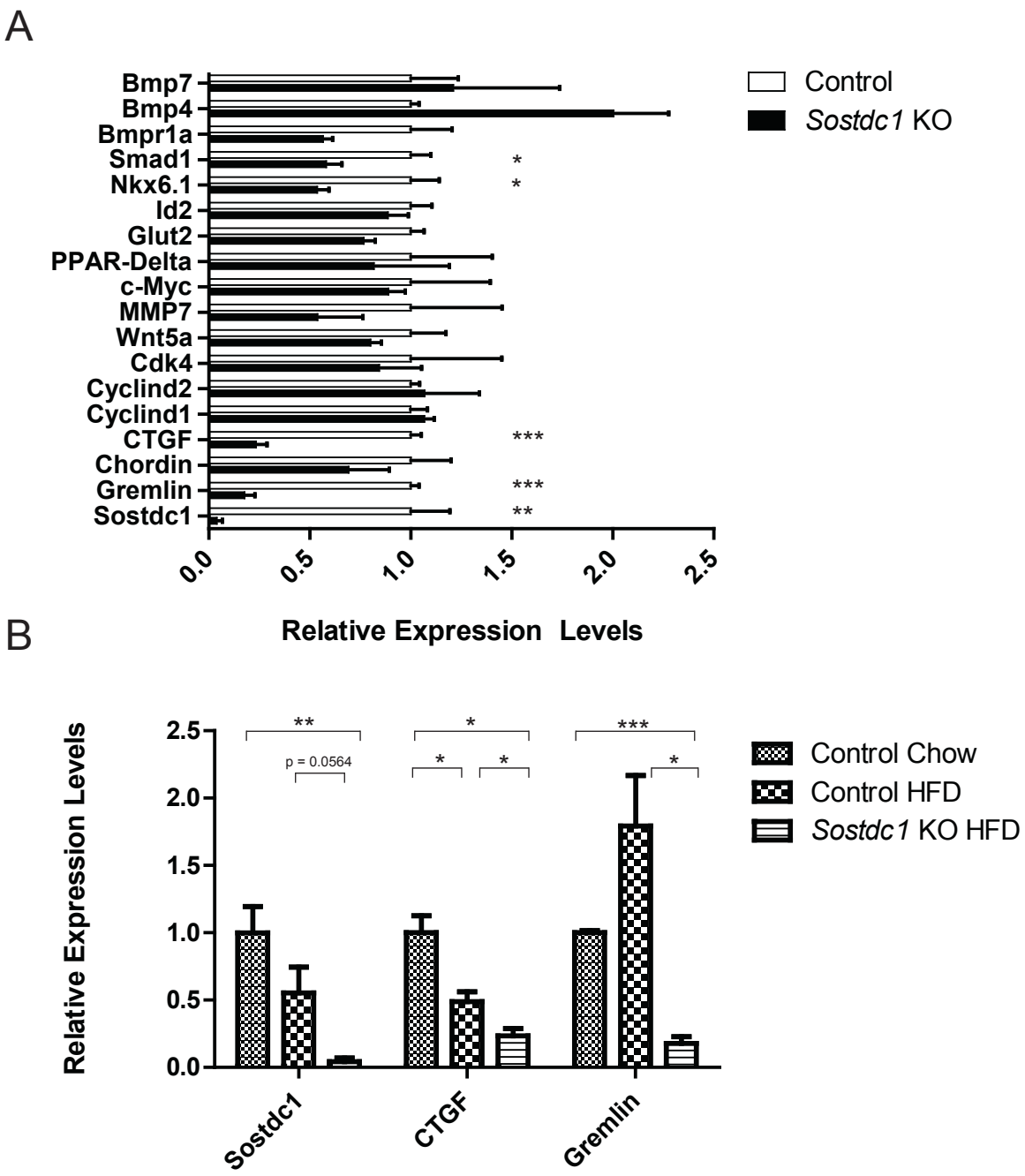


Figure 28. Gene expression in *Sostdc1* null islets. (A) Analysis of selected BMP/Wnt-responsive gene expression in control and *Sostdc1* null islets after 8 weeks HFD. (B) Quantification of changes in specific BMP/Wnt antagonists in control and *Sostdc1* null islets after exposure to chow or HFD for 8 weeks. For each experiment, n ≥ 3 for each genotype. Expression levels were normalized against ubiquitiously expressed *Hprt.* * P = 0.05; ** P = 0.01; *** P = 0.001. Error bars represent SEM. Figures modified and reprinted with permission from Henley et al., 2012.

Evaluation of BMP pathway activity in the absence of *Sostdc1*

Gene expression data revealed that, of the genes assessed, BMP/Wnt modulators and BMP-regulated genes are altered in the absence of *Sostdc1.* This suggests that the BMP pathway is preferentially targeted by Sostdc1 in islets. To evaluate whether BMP signaling is augmented in islets of *Sostdc1* null animals, we crossed *Sostdc1*$^{LacZ/LacZ}$ mice with those carrying the *BRE-LacZ* transgene, which expresses β galactosidase under control of the BMP response element-containing fragment of the *Id1* promoter (Korchynskyi and ten Dijke 2002, Monteiro et al. 2004). We evaluated activation of the BMP pathway by assessing β-galactosidase activity in islets of *Sostdc1*$^{LacZ/LacZ}$*;BRE-LacZ* mice compared to *BRE-LacZ* mice. We predicted that elevated BMP signaling in the absence of *Sostdc1* would result in activation of the *BRE-LacZ* transgene (and thus X-gal staining) in islets. However, *Sostdc1*$^{LacZ/LacZ}$*;BRE-LacZ* animals did not exhibit X-gal staining in islets (data not shown). These data were not surprising, given that un-stimulated *Sostdc1* KO animals did not display basal improvements in glucose homeostasis (Figure 23B). We predicted after exposure to HFD for 4 weeks, a situation in which *Sostdc1*$^{-/-}$ animals show improved β cell function, we would observe enhanced BMP signaling. *Sostdc1* null animals carrying the *BRE-LacZ* transgene and control animals (*BRE-LacZ* transgene or *Sostdc1* KO alone) did not exhibit β galactosidase reactivity in islets (data not shown). We did observe X-gal staining in the kidneys of animals carrying the *BRE-LacZ* transgene, as reported by others (Blank et al. 2008), indicating that the transgene is operative in BMP-responsive tissues (data not shown). Lack of *BRE-LacZ* activity in islets could be due to activation below the level of detection or may indicate that loss of *Sostdc1* does not increase BMP activity in islets.

As a secondary means of evaluating BMP pathway activity in *Sostdc1* null animals, levels of phosphorylated (activated) Smad1 were assessed by western blotting (Figure 29A). At 8 weeks of age, islets from *Sostdc1* mutant animals exposed to chow diet did not show significantly altered pSmad levels compared to control animals (Figure 29B). These data indicate that under basal conditions, BMP pathway activity is not substantially increased in islets from *Sostdc1* null mice.

Discussion

Our studies have explored the modulation of BMP and Wnt signaling in adult islet function, an area of study that still requires additional characterization. By utilizing animals globally lacking the dual BMP and Wnt inhibitor Sostdc1, we evaluated the role of this protein in insulin secretion and glucose homeostasis. Current literature indicates that both BMP and Wnt positively regulate β cell function and that loss of components of these pathways, either globally or in a tissue-specific manner, impairs β cell function, insulin secretion, and glucose homeostasis (Fujino et al. 2003, Dessimoz et al. 2005, Goulley et al. 2007, Liu and Habener 2010). The transgenic model of *Hnf6* over-expression generated by our group exhibits defects in insulin secretion that are reminiscent of those observed in *Bmpr1a* and *Lrp5* mutant animals (Fujino et al. 2003, Goulley et al. 2007, Scott et al. 2009). Concordantly, in *Hnf6* transgenic animals there is a 2-fold up-regulation of *Sostdc1*, a dual BMP and Wnt inhibitor that interacts with both BMP4 and the LRP6 receptor (Lintern et al. 2009). Taken together, these data suggest that irregular modulation of BMP and/or Wnt signaling may contribute to impaired insulin secretion observed in certain mouse models of diabetes.

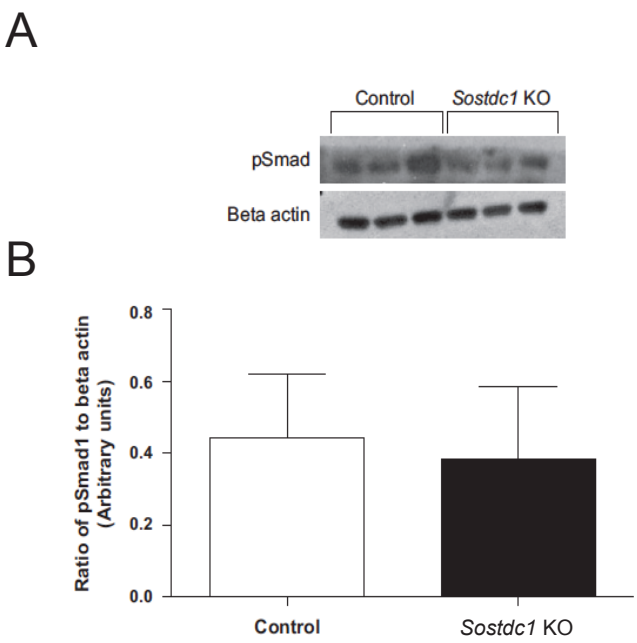


Figure 29. Quantification of pSmad in *Sostdc1* null islets. (A) Western blot representing pSmad1 levels in control and *Sostdc1* null islets isolated from 8 week old male mice on chow diet. (B) Quantification of and graphical representation of (A) using ImageJ. Error bars represent SEM. Figures modified and reprinted with permission from Henley et al., 2012.

The detection of *Sostdc1*-driven β-gal activity in the acinar tissue but not the islets of *Sostdc1*$^{LacZ/+}$ animals implies that Sostdc1 may be secreted from acinar tissue to affect islet function. To date, no studies have indicated a role for BMP or Wnt inhibitors in pancreatic acinar tissue. However, we were able to quantify a decrease in expression of endogenous *Sostdc1* in adult islets exposed to HFD compared to control chow (Figure 28B), suggesting that Sostdc1 is indeed expressed in islets, but at levels too low to detect by X-gal staining.

We saw no alterations to pancreas development in *Sostdc1* null animals in terms of endocrine cell number or islet morphology. This observation is consistent with the previously published model of BMP4 over-expression in the pancreatic epithelium (*Pdx1-Bmp4*, (Goulley et al. 2007), which did not exhibit a developmental phenotype. Although the role of Wnt signaling in endocrine pancreas development is somewhat controversial, it has been well established that Wnt signaling is required for exocrine pancreas development (Dessimoz et al. 2005, Murtaugh et al. 2005). Loss of Sostdc1 does not grossly affect exocrine development despite the essential need for tightly-regulated Wnt activity during this time period. Although we did not detect changes in expression of Wnt target genes in islets isolated postnatally (Figure 27, 28), it remains possible that the Wnt pathway is altered during development in the absence of *Sostdc1.* Overall, it appears that deletion of *Sostdc1* has a specific regulatory effect on the BMP pathway in pancreatic islets. Despite being a dual BMP and Wnt antagonist, the loss of *Sostdc1* did not result in significant modifications in expression of the Wnt responsive genes assayed in this study. This came as a surprise as, in HEK293 cells, Sostdc1 preferentially interacts with the LRP6 receptor over binding to BMP4 (Lintern et al. 2009).

We observed divergent insulin secretion and glucose homeostasis phenotypes in animals lacking *Sostdc1* and hypothesized that this discrepancy was related to genetic

variability. Exploring this idea is relevant not only to the present study, but also to the human population, which is composed of individuals of mixed genetic background. Indeed, murine phenotypes observed on mixed genetic backgrounds may better mimic complex genetic traits in humans. Because C57Bl/6 mice are predisposed to β cell dysfunction, and because an independently generated *Sostdc1* mutant mouse line displays a molar fusion phenotype with variable penetrance that correlates positively with a higher percent of C57Bl/6 alleles (Toye et al. 2005, Ahn et al. 2010), we predicted that animals with a greater contribution of C57Bl/6 to their genetic background would exhibit improved insulin secretion and glucose homeostasis in the absence of *Sostdc1*. However, we were unable to correlate low FBG (< 125 mg/dl) or enhanced insulin secretion with a higher percentage of C57Bl/6 contribution. In fact, the overall contribution of C57Bl/6 did not vary greatly among the animals used in this study. Although we observe significantly altered gene expression from *Sostdc1* null animals compared to control animals, these changes may only positively affect a specific subset of animals due to other as yet undefined modifying genetic factors. It remains possible that inheritance of a specific modifier gene(s) or genes that are controlled by *Sostdc1,* rather than overall strain contribution, is responsible for the observed phenotype. We did evaluate whether the *Nnt* mutation, previously shown to contribute to impaired insulin secretion and glucose homeostasis in the C57Bl/6 strain of mice, correlated with the *Sostdc1* mutant phenotype, but found no evidence suggesting a correlation. The analysis of gene expression presented here is limited to BMP and Wnt pathway-related genes, and thus may not be comprehensive enough to identify modifier genes that are responsible for the improved phenotype. In the future, global gene expression analyses comparing control and *Sostdc1* mutant islets would clarify which genes may play a role in the phenotype and which signaling pathways are most affected.

The preceding data reveal that loss of *Sostdc1* results in decreased expression of BMP-responsive genes and BMP and Wnt modulators in islets after HFD feeding for 8 weeks (Figure 28A). These results were surprising to us and contradicted the originial hypothesis, suggesting that *Sostdc1* may not antagonize BMP signaling in this particular situation. However, these data could also reflect a novel means of antagonist regulation in islets. The slight reduction of *Sostdc1* and significant decrease in *Ctgf* expression seen in control animals on HFD compared to chow for 8 weeks implies a mechanism in which expression of dual BMP/Wnt modulators is down-regulated in the setting of increased metabolic demand. Given that BMP and Wnt signals positively regulate islet function, decreased expression of antagonists of either of these pathways may improve or maintain glucose homeostasis in a situation of metabolic stress. It is unclear why expression of the BMP-selective inhibitor *Gremlin* trends toward increased expression in control animals after 8 weeks HFD feeding. *Gremlin* is involved in the differentiation of preadipocytes to mature adipocytes, and therefore may be systemically up-regulated during exposure to HFD (Rose et al. 2003). Gremlin has also been implicated in pancreatic cancer and the pathology of diabetic nephropathy (Namkoong et al. 2006), but has not been studied in normal pancreas function. Of the three BMP and Wnt modulators assessed, *Gremlin* is the most specific of antagonists, as it targets only the BMP pathway. This specificity may affect β cell function in control animals on HFD, but this idea needs to be tested directly. Nonetheless, we observed consistent reductions in both *Ctgf* and *Gremlin* expression in the *Sostdc1* mutant background after 8 weeks HFD feeding. As described above, we also observed reductions in *Smad1* and *Nkx6.1* expression in *Sostdc1* mutant islets and a trend toward reduced *Glut2* expression. As an integral part of the BMP-signaling pathway, Smad1 mediates expression of BMP-responsive genes and it is therefore possible that decreased expression of *Smad1* alone may contribute to the decreased levels of other BMP-related genes. Roles for *Nkx6.1* in

endocrine development and differentiation have been established, but its function in the adult pancreas is less clear. *In vitro* studies showed that adenoviral mediated over-expression of *Nkx6.1* in rat and human islets resulted in augmented insulin secretion and β cell proliferation (Schisler et al. 2008). Conversely, *in vivo* over-expression of *Nkx6.1* in adult murine β cells did not result in significant alterations in glucose homeostasis, insulin secretion, or β cell proliferation (Beucher, Martin, et al. 2012). However, *Nkx6.1* participates in maintaining β cell identity by repressing the α cell-specific transcription factor *Arx,* and loss of *Nkx6.1* in mature β cells results in conversion to δ cells (Schaffer et al. 2013). Because *Nkx6.1* is a BMP-responsive gene in islets, reduced *Nkx6.1* expression in *Sostdc1* mutant mice may reflect a reduction in BMP pathway activity with prolonged HFD exposure, and not impaired function or loss of β cell identity.

In an effort to analyze BMP pathway activity, we also examined whether loss of *Sostdc1* would increase activity of a *BRE-LacZ* reporter transgene in the setting of HFD exposure. We initially hypothesized that the absence of *Sostdc1* would enhance BMP signaling activity in islets when animals were exposed to HFD, and we expected to observe β-galactosidase activity in the islets of *Sostdc1* KO animals carrying the *BRE-LacZ* transgene. We did not find evidence that this reporter is active in islets of *Sostdc1* null animals. This may be because the animals were not exposed to HFD for a sufficient amount of time to activate the transgene; however, we observed a significant increase in expression of the BMP-responsive gene *Glut2* after 4 weeks of HFD, implying that some BMP target genes are responding at this time. It may also be that the *BRE-LacZ* transgene is not expressed in islets, although its expression was verified in the kidneys of the same animals.

Given the available published data, it is challenging to explain how decreased expression of BMP-responsive genes is associated with improved glucose homeostasis

and insulin secretion. Recent literature suggests, however, that the deletion of *Id1*, a BMP-responsive gene, protects animals from HFD-induced obesity and enhances insulin secretion (Akerfeldt and Laybutt 2011). This implies that modulation of specific components of the BMP pathway may have differing affects on the β cell. We propose that in the *Sostdc1* mutant background, there is a significant reduction in expression of BMP and Wnt modulators after exposure to HFD that is not observed in control animals (Figure 28B). We hypothesize that reduced expression of BMP antagonists enables maintenance of sufficient levels of BMP signaling activity to preserve glucose homeostasis in the setting of increased metabolic demand. Control animals fail to reduce expression of BMP and Wnt modulators, and perhaps BMP signaling activity is augmented in control animals to counteract the presence of these antagonists. Interestingly, impairments in insulin secretion and glucose homeostasis still occur. The changes in signaling activity are reflected in the expression levels of BMP target genes, as expression of genes such as *Smad1* and *Nkx6.1* are reduced in *Sostdc1* null animals compared to controls.

In conclusion, we provide evidence that loss of *Sostdc1* enhances insulin secretion and glucose homeostasis in animals under metabolic stress, in part by reducing expression of other BMP and Wnt modulators. Changes in BMP-responsive gene expression are present in animals lacking *Sostdc1*, which implies that Sostdc1 preferentially targets the BMP pathway in the pancreatic islet. To our knowledge, this is the first study to provide evidence for diet-induced regulation of BMP and Wnt modulators as well as coordinated regulation by the modulators themselves. These data further support a role for BMP signaling in islet function as shown by others (Goulley et al. 2007, Scott et al. 2009), and suggest that factors that limit BMP antagonist activity could potentiate the positive effects that BMP signaling has on islet function. Future

studies examining the loss of *Sostdc1*, *CTGF* and *Gremlin* (either alone or in combination) in adult islet function and regulation of BMP and Wnt signaling should elucidate whether these extracellular regulators may be targets for drug therapy to enhance islet function.

CHAPTER IV

COOPERATION BETWEEN HNF6 AND PDX1 IN THE REGULATION OF GENES INVOLVED IN POSTNATAL ISLET FUNCTION

Introduction

Development and function of the endocrine pancreas rely on precise spatiotemporal expression of genes that coordinate endoderm specification, endocrine differentiation, proliferation, glucose sensing, hormone production and secretion. A key factor involved in all of these processes is *Pdx1*, which is initially expressed in the foregut endoderm and persists, at varying levels, throughout pancreas development (Figures 2-5). *Hnf6* is expressed prior to *Pdx1* in the presumptive endoderm and is also maintained throughout pancreas development. These two transcription factors are initially co-expressed in primary MPCs, and to a lesser extent in bipotent trunk precursors (Figure 2, 5) (Zhang et al. 2009). *Pdx1* expression is temporarily down-regulated in *Ngn3*-positive endocrine precursors, but is re-expressed at high levels in differentiating β cells. Conversely, *Hnf6* expression persists in endocrine precursors until their differentiation and induction of hormone expression, at which point *Hnf6* expression is down-regulated. However, *Hnf6* expression is maintained at high levels in the pancreatic ducts and to a lesser extent in the acinar tissue, where it is required to maintain differentiation.

As described previously, both *Pdx1* and *Hnf6* regulate expression of genes that are involved in endocrine specification, differentiation, and mature endocrine function (Jonsson et al. 1994, Ahlgren et al. 1998, Watada, Kajimoto, Kaneto, et al. 1996, Watada, Kajimoto, Miyagawa, et al. 1996, Dutta et al. 1998, Ohneda et al. 2000,

Marshak et al. 2000, Leibowitz et al. 2001, Jacquemin et al. 2000, Gerrish, Cissell, and Stein 2001, Jacquemin, Lemaigre, and Rousseau 2003, Brissova, Blaha, et al. 2005, Raum et al. 2006, Wilding Crawford et al. 2008, Yamamoto et al. 2013). Their initial co-expression in primary MPCs and co-regulation of the pro-endocrine gene *Ngn3* suggest that these two transcription factors function, in part, to activate the endocrine program (Zhang et al. 2009, Oliver-Krasinski et al. 2009). Certain genes may require a threshold of *Pdx1* and *Hnf6* expression to be sufficiently activated; thus, decreased expression of *Pdx1* and *Hnf6* together may result in reduced or lack of expression of target genes. We wanted to explore whether haploinsufficiency for both factors would negatively affect the development of the endocrine compartment of the murine pancreas. Indeed, single *Pdx1* heterozygous animals develop impaired insulin secretion and glucose intolerance with age, but no developmental phenotype for either heterozygous animal has been documented (Dutta et al. 1998, Brissova, Blaha, et al. 2005). Thus, we hypothesized that double heterozygosity would result in abnormal endocrine pancreas development and function due to decreased expression of key genes.

The first approach utilized animals globally heterozygous for *Pdx1* and conditionally heterozygous for *Hnf6* in the pancreatic epithelium (*Pdx1*$^{LacZ/+}$*;Pdx1-Cre;Hnf6*$^{F/+}$, henceforth denoted as *Pdx1*$^{LacZ/+}$*; Hnf6*$^{\Delta panc/+}$). Using this model, changes in blood glucose or pancreas weight were not observed at postnatal day 1 (P1). In agreement, endocrine mass and α:β cell ratio were unchanged between single and double heterozygous animals at P1. Surprised by these observations, we evaluated the efficiency of the *Cre* recombinase system to confirm the timing of *Hnf6* recombination. Based on reported data, *Pdx1-Cre* recombination of the *R26R-LacZ* reporter allele takes place at e9.5, a time deemed appropriate for the experiments at hand because it precedes the overlap of *Pdx1* and *Hnf6* expression in primary MPCs at e10.5. However, examination of *Cre*-mediated *Hnf6* recombination revealed inefficient deletion by e10.5,

indicating that this specific model of *Hnf6* heterozygosity is not appropriate for the purposes of this study.

In order to ensure early *Hnf6* inactivation, we generated conditional global double heterozygous animals (*Pdx1*$^{LacZ/+}$*;Prm-Cre; Hnf6*$^{F/+}$) to circumvent the issue of *Cre*-mediated timing. Using this approach, it was determined that body weight, pancreas weight, and blood glucose levels in double heterozygous at birth are comparable to single heterozygous and control littermates. After one day, however, double heterozygous animals exhibited elevated blood glucose and reduced body and pancreas size compared to controls. Interestingly, these changes are resolved by weaning, suggesting compensatory mechanisms that restore islet function in double heterozygotes. Currently, we are assessing gene expression changes at birth and weaning to determine which genes are primarily affected.

Results

Conditional double heterozygosity does not affect endocrine development or early postnatal function

The initial approach to determine whether double heterozygosity for *Pdx1* and *Hnf6* affects pancreas development and function made use of mice that are globally heterozygous for *Pdx1* and heterozygous for *Hnf6* in the *Pdx1* expression domain which includes the pancreatic epithelium (*Pdx1*$^{LacZ/+}$*; Hnf6*$^{\Delta panc}$). Pancreas weight was measured at P1 in single and double heterozygous animals, and at this time, no changes were observed between genotypes (Figure 30A). Similarly, there were no appreciable differences in blood glucose between single and double heterozygous

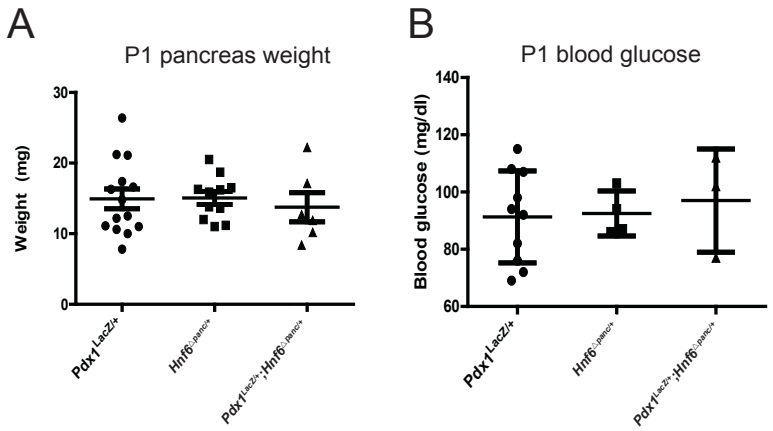


Figure 30. Analysis of pancreas weight and blood glucose at P1. (A) Pancreas weight in single and double heterozygotes was measured at P1. (B) Blood glucose in single and double heterozygotes was measured at P1. Error bars represent SEM.

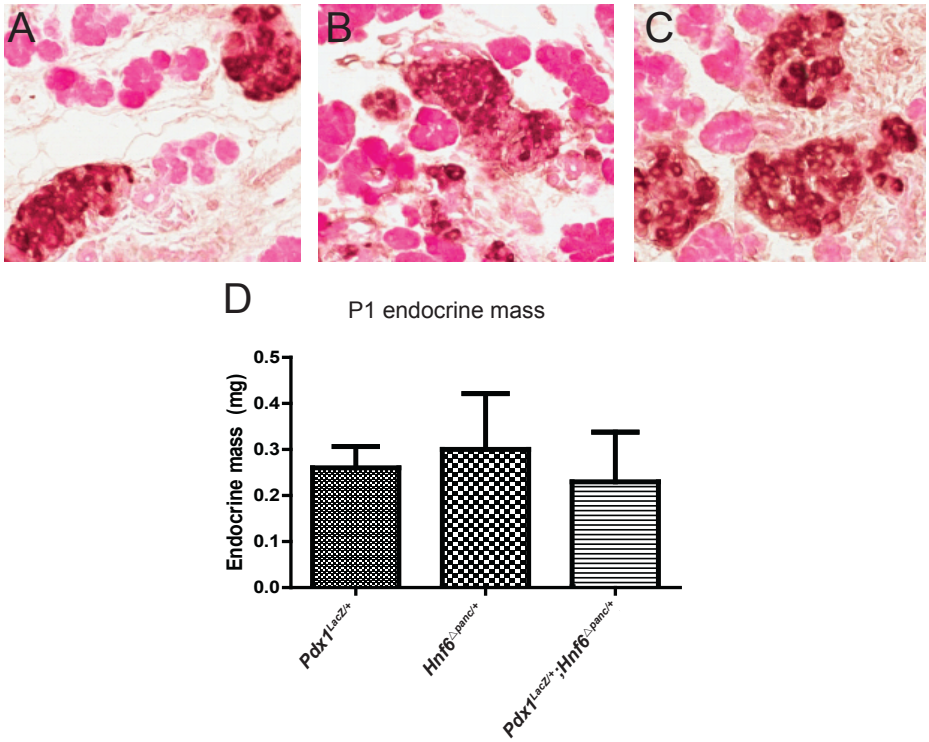


Figure 31. Assessment of endocrine mass at P1. Distribution of insulin-producing β cells (brown) within acinar tissue (pink) in *Pdx1*$^{LacZ/+}$ (A), *Hnf6*$^{\triangle panc/+}$ (B), and *Pdx1*$^{LacZ/+}$;*Hnf6*$^{\triangle panc/+}$ (C) at P1. (D) Quantification of endocrine mass by synaptophysin immunolabeling in single and double heterozygotes. Error bars represent SEM. For (D), *Pdx1*$^{LacZ/+}$ n = 4, *Hnf6*$^{\triangle panc/+}$, n = 2, *Pdx1*$^{LacZ/+}$;*Hnf6*$^{\triangle panc/+}$, n = 3.

animals at P1 (Figure 30B), indicating that endocrine function is not perturbed using this particular model of double heterozygosity.

Considering the role that *Pdx1* and *Hnf6* play in regulation of the pro-endocrine gene *Ngn3*, we were interested in evaluating whether endocrine mass was negatively affected in the setting of double heterozygosity. Sections of pancreata from single and double heterozygotes were immunolabeled with insulin antibodies to examine the distribution of β cells within endocrine islets (Figure 31A-C). In general, there were no gross differences between genotypes based on this evaluation. Endocrine mass was quantified by synaptophysin immunolabeling, and at P1, double heterozygous animals do not exhibit changes in endocrine mass compared to single heterozygotes (Figure 31D). Co-labeling with insulin and glucagon to visualize islet morphology revealed no obvious differences between single and double heterozygotes (Figure 32A-C). The ratio of α to β cells was also similar between genotypes (Figure 32D). Taken together, these data suggest that the $Pdx1^{LacZ/+};Hnf6^{\Delta panc/+}$ model of double heterozygosity does not elicit considerable changes in the endocrine population.

Evaluation of timing and efficiency of *Pdx1-Cre*-mediated recombination

In the aforementioned experiments, one copy of the *Hnf6* floxed allele was recombined using *Cre* recombinase driven by the 4.3kb fragment of the *Pdx1* promoter to achieve *Hnf6* heterozygosity in the pancreatic epithelium (Figure 17) (Zhang et al. 2009, Hingorani et al. 2003). This particular *Cre* transgene is operative as early as e9.5 and facilitates reporter allele recombination efficiently throughout the entire pancreatic epithelium (Figure 17) (Wicksteed et al. 2010). Because the $Pdx1^{LacZ/+};Hnf6^{\Delta panc/+}$ double heterozygous model did not exhibit endocrine defects at P1, we became concerned that the conditional *Hnf6* allele was being not recombined at the expected time during pancreatogenesis. Specifically, we wanted to determine the timing of recombination in

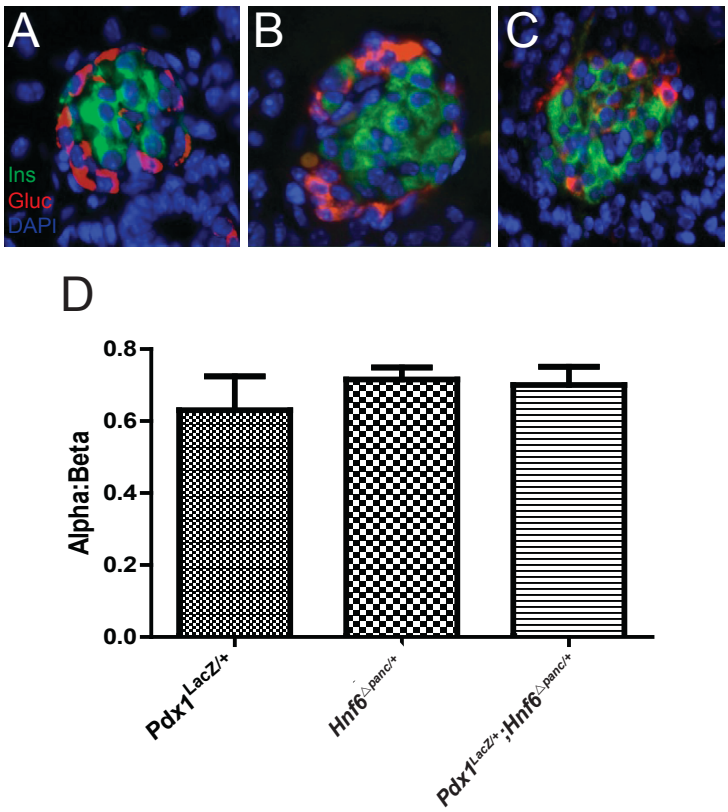


Figure 32. Islet morphology in single and double *Pdx1*$^{LacZ/+}$;*Hnf6* $^{\triangle panc/+}$ animals at P1. Distribution of α (red) and β (green) cells in *Pdx1*$^{LacZ/+}$ (A), *Hnf6* $^{\triangle panc/+}$ (B), and *Pdx1*$^{LacZ/+}$;*Hnf6* $^{\triangle panc/+}$ (C) mice at P1 reveals normal islet morphology. (D) Quantification of the ratio of α to β cells in single and double heterozygous animals at P1. For (D), *Pdx1*$^{LacZ/+}$, n = 2; *Hnf6* $^{\triangle panc/+}$, n = 2, *Pdx1*$^{LacZ/+}$;*Hnf6* $^{\triangle panc/+}$, n = 4.

relation to the overlap of *Pdx1* and *Hnf6* expression in primary MPCs (e9.5-e11.5) and the birth of endocrine progenitors from the bipotent ductal epithelium (e12.5 onward) (Figure 5). To assess recombination of the floxed *Hnf6* allele by *Pdx1-Cre*, embryos carrying one copy of the *Hnf6* floxed allele, the *Pdx1-Cre* transgene, and the *R26R-EYFP* reporter allele were harvested at e10.5 and processed for paraffin sectioning. Pancreatic tissue from whole embryo sections were isolated and PCR was performed to detect the recombined *Hnf6* allele. At e10.5, both the recombined and unrecombined *Hnf6* allele could be detected from DNA isolated from sections of pancreas (data not shown). Indeed, both the intact and recombined *R26R-EYFP* reporter allele could also be detected by PCR at this time (data not shown).

Pdx1-Cre activity was also assessed at the mRNA level. qRT-PCR was performed using RNA isolated from the epithelium-derived gut tube containing nascent pancreatic buds at e11.5 to assess a potential reduction in *Hnf6* expression. At this time, however, a significant reduction in *Hnf6* expression could not be detected (Figure 33).

The efficiency of *Pdx1-Cre* was also evaluated in the postnatal pancreas of single and double heterozygous animals. Using EYFP immunolabeling as a surrogate for *Pdx1-Cre* activity, recombination was assessed in the pancreata of single and double heterozygous animals at P1 (Figure 34). At this time, variable immunolabeling of endocrine clusters was observed in all three genotypes (Figure 34A'-A'''), whereas labeling of the acinar portion of the pancreas was relatively consistent, regardless of single or double heterozygosity (Figure 34C'-C'''). Collectively, these data indicate that the *Pdx1-Cre* transgene is active at e10.5, but does not recombine the *Hnf6* floxed allele to an extent that appreciably reduces gene expression until after e11.5. Furthermore, *Pdx1-Cre* activity as evaluated by EYFP immunolabeling suggests that recombination is not as efficient in the endocrine population as it is in the acinar population, regardless of genotype.

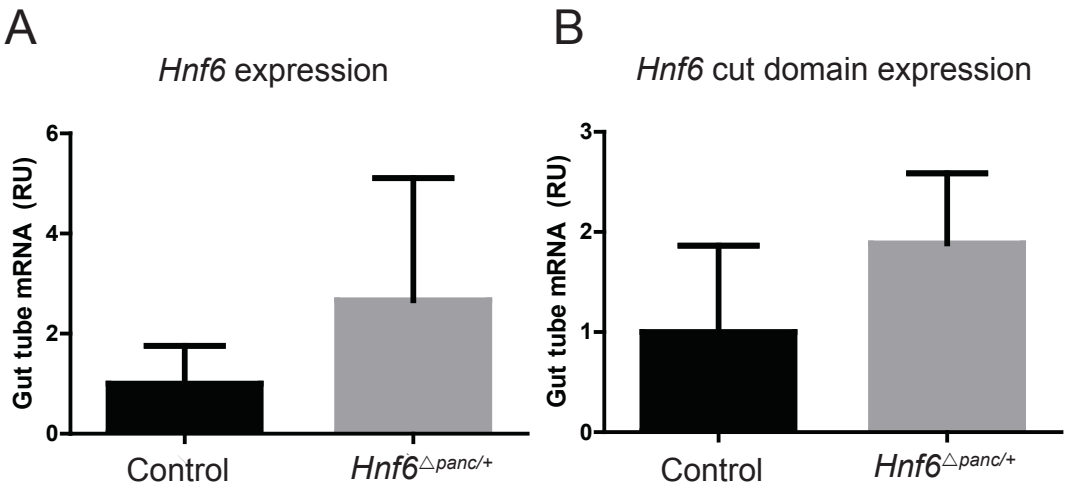


Figure 33. Recombination of the floxed *Hnf6* allele by *Pdx1-Cre* recombinase. (A) Quantification of *Hnf6* expression by qRT-PCR in control and heterozygous animals using primers spanning the first and second exons. (B) Quantification of *Hnf6* cut domain expression by qRTPCR in control and heterozygous animals using primers specific for the cut domain, which is flanked by *loxP* sites. N $\geq$ 3 for each genotype. *Hnf6* expression was normalized against the ubiquitously expressed *Hprt.*

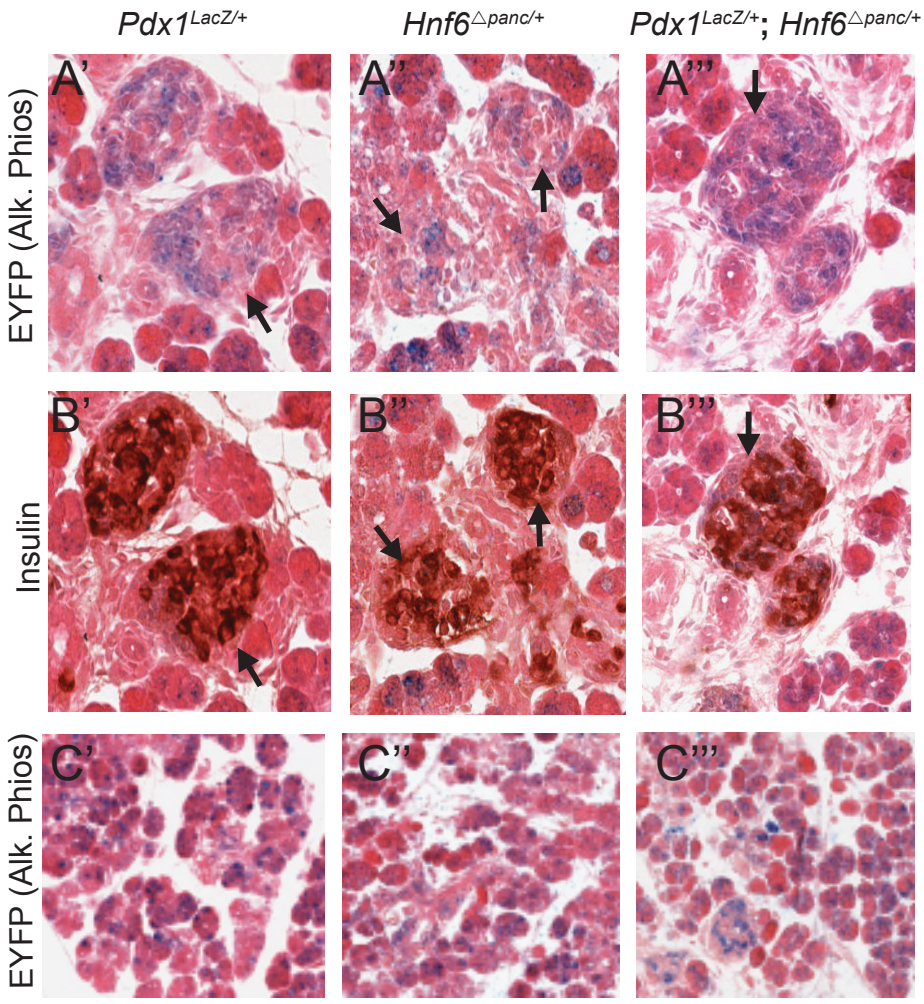


Figure 34. *Pdx1-Cre*-medicated recombination of the *EYFP* reporter allele at P1. (A) Sections of single (A' and A'') and double heterozygous (A''') pancreata immunolabeled for EYFP (blue). Arrows indicate variable *EYFP* recombination in the clusters labeled with insulin in all three genotypes (see B). (B) Identical sections from (A) immunolabeled for insulin (brown). (C) Representative images of EYFP immunolabeling in acinar tissue of pancreata from single (C' and C'') and double heterozygous (C''') animals at P1. Note consistency of labeling in acinar tissue in all images in (C) compared to endocrine clusters in (A). All images shown are from animals carrying both the *Pdx1-Cre* transgene and the *R26R-EYFP* reporter allele.

Global double heterozygosity impairs early postnatal endocrine function

To eliminate the possibility that timing or efficiency of *Pdx1-Cre* facilitated recombination is not suitable for studying the early genetic interactions between *Pdx1* and *Hnf6* in primary MPCs or bipotent precursors, we generated animals globally heterozygous for both *Pdx1* and *Hnf6.* To achieve this, *Protamine-Cre* (*Prm-Cre)*, which is expressed in the male germ line and exhibits recombinase activity at the 2- to 4-cell zygotic stage, was used to recombine the floxed *Hnf6* allele (O'Gorman et al. 1997).

To determine if global double heterozygosity affects development of the endocrine pancreas, we sacrificed control, single, and double heterozygous animals at birth and measured body weight, pancreas weight, and blood glucose levels. At this time point, there were no significant differences in the parameters examined (Figure 35A-C). However, one day later significant decreases in body and pancreas size in $Pdx1^{LacZ/=,}$ $Hnf6^{+/-}$, and double heterozygous animals were observed (Figure 35D, E). These results were unexpected, as there has been no documented developmental or early postnatal phenotype for either single heterozygous animal reported in the literature.

The reduced pancreas size seen in $Pdx1^{LacZ/+}$ and $Pdx1^{LacZ/+};Hnf6^{+/-}$ pups after birth was reminiscent of the developmental effects of intrauterine growth restriction (IUGR) due to placental insufficiency. Specifically, IUGR causes low birth weight and reduced organ size while brain size is maintained (Eleftheriades, Creatsas, and Nicolaides 2006). This situation was pertinent to our study as *Pdx1* is expressed in the placenta and is up-regulated during IUGR (Buffat et al. 2007). Thus, we were concerned about IUGR due to placental defects in the setting of *Pdx1* heteozygosity. To determine if single *Pdx1* or double *Pdx1/Hnf6* heterozygosity results in IUGR and subsequent reduced organ size, animals were sacrificed at birth and pancreas, heart, kidney, and brain weights were measured (Figure 36A-D). When assessed as a percentage of body weight, there were no differences in organ size between genotypes, signifying that single

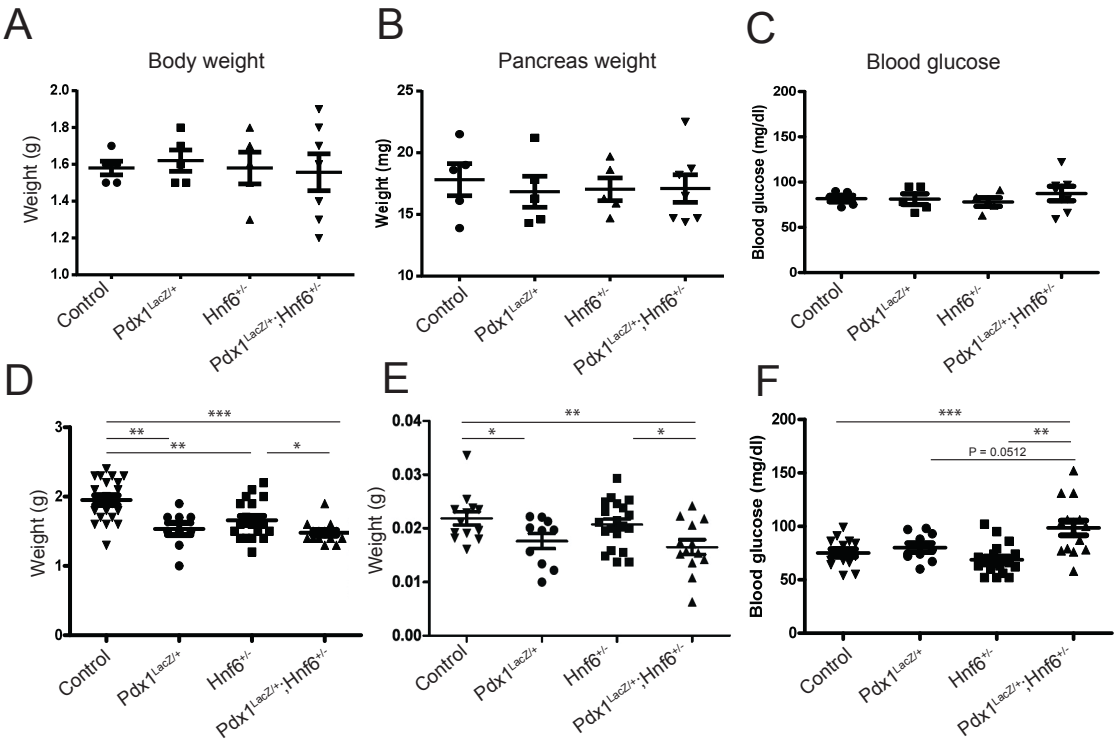


Figure 35. Assessment of weight and blood glucose changes in postnatal double heterozygous animals. At birth, double heterozygous (*Pdx1*$^{LacZ/+}$;*Hnf6*$^{+/-}$) mice have comparable body weight (A), pancreas weight (B) and blood glucose (C) compared to *Pdx1*$^{LacZ/+}$, *Hnf6*$^{+/-}$, and control animals. At P1, single and double heterozygous animals weigh less than control animals (D) and *Pdx1*$^{LacZ/+}$ and double heterozygous mice have smaller pancreata than control and *Hnf6*$^{+/-}$ animals (E). Double heterozygous animals exhibit elevated blood glucose compared to control and Hnf6$^{+/-}$ animals at P1 (F) * P = 0.01; ** P = 0.05; *** P = 0.001. Error bars represent SEM.

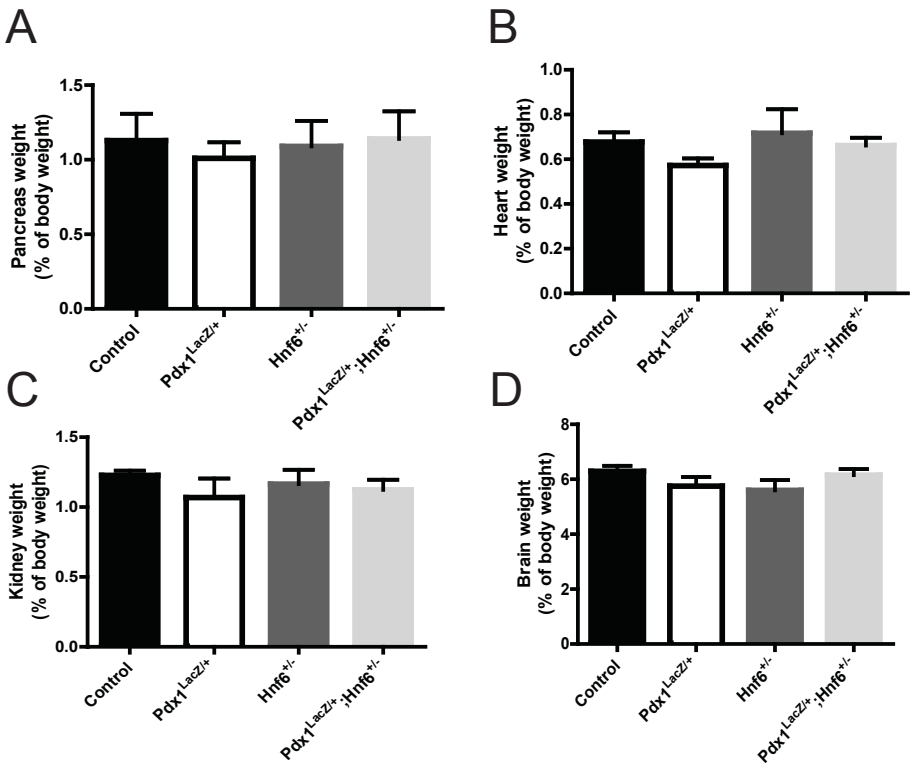


Figure 36. Organ weight in newborn double heterozygous mice. Pancreas (A), heart (B), kidney (C) and brain (D) weights were measured in control, single, and double heterozygous animals at birth and graphed as a percentage of body weight. N ≥ 5 for each genotype and each organ. Error bars represent SEM.

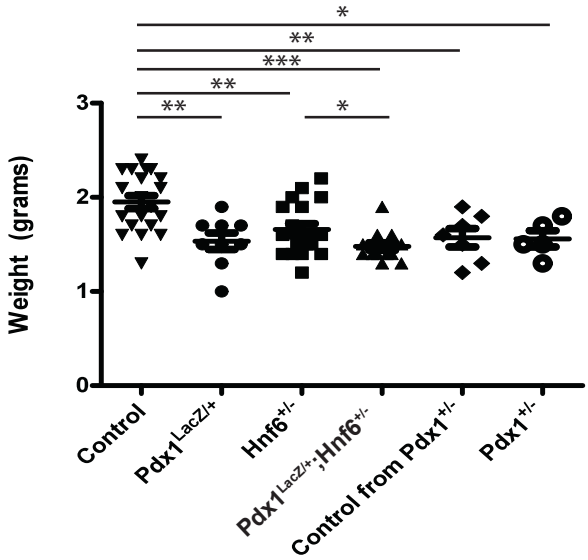


Figure 37. Analysis of body weight in models of single *Pdx1* heterozygosity. At P1, all experimental animals, including control animals from *Pdx1*$^{+/-}$ litters, weigh significantly less than control animals from *Pdx1*$^{LacZ/+}$;*Hnf6*$^{+/-}$ litters. * P = 0.05; ** P = 0.01; *** P = 0.001. Error bars represent SEM.

Pdx1 or double *Pdx1/Hnf6* heterozygosity does not result in IUGR-related reductions in organ size.

In pursuit of an explanation for phenotype seen in *Pdx1*$^{LacZ/+}$ and *Pdx1*$^{LacZ/+}$;*Hnf6*$^{+/-}$ pups after birth, we next chose to evaluate the postnatal weights of a second model of *Pdx1* heterozygosity to ensure that our phenotype was not a result of the *LacZ* knock-in allele behaving as a dominant negative. We compared P1 body weights of *Pdx1*$^{+/-}$ (*Pdx*^{XB} allele, Figure 15) and control littermates with those from our *Pdx1*$^{LacZ/+}$*;Hnf6*$^{+/-}$ litters and determined that while all experimental genotypes, as well as the control animals from the *Pdx1*$^{+/-}$ litters, were of comparable body weight and were consistent with P1 body weight reported in the literature, the body weights of control animals from *Pdx1*$^{LacZ/+}$*;Hnf6*$^{+/-}$ litters were significantly heavier (Figure 37, data not shown). We determined that the control pups born from females carrying the *Prm-Cre* transgene were significantly heavier than pups born from females without the transgene. Because these heavier pups were skewing the data, all litters born from *Prm-Cre* positive females were removed and the data were re-analyzed. In this curated data set, only *Pdx1*$^{LacZ/+}$;*Hnf6*$^{+/-}$ pups exhibited reduced body and pancreas weight at P1 compared to controls (Figure 38A, B). Double heterozygous pups exhibited elevated blood glucose compared to controls and *Hnf6*$^{+/-}$ animals at P1 (Figure 35C, 38C).

Elevated blood glucose can be caused by reduced endocrine mass or impaired endocrine function. To determine if *Pdx1*$^{LacZ/+}$*;Hnf6*$^{+/-}$ pups possess reduced endocrine mass compared to control and single heterozygous littermates, α and β cells were immunolabeled with antibodies against both glucagon and insulin (Figure 39A-D) and the mass of each cell population were quantified (Figure 40A-B). These analyses indicated that islet morphology is maintained in the setting of double heterozygosity, and, despite having smaller pancreata one day after birth, double heterozygous animals do not have evidence of reduced α or β cell mass at this time (Figure 40A, B). The mass of

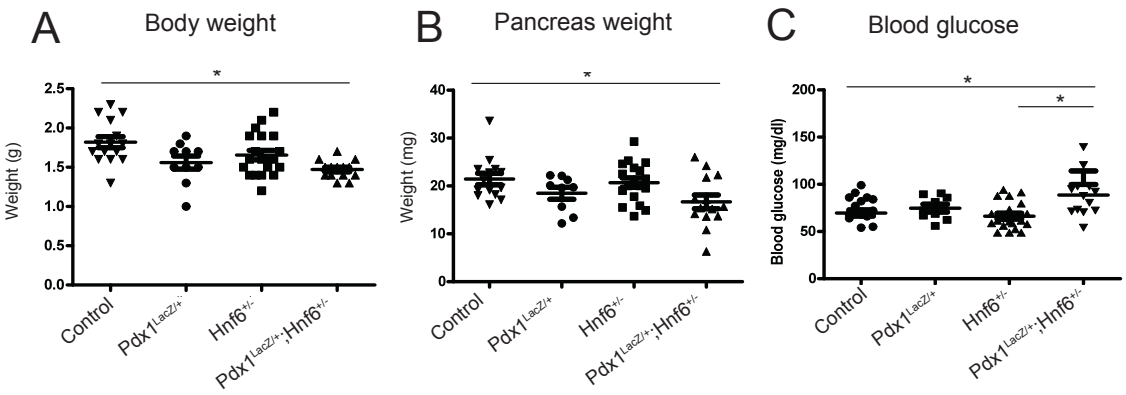


Figure 38. Assessment of weight and blood glucose changes in double heterozygous animals at P1. (A) Double heterozygous animals exhibit reduced body weight compared to controls after birth. (B) Double heterozygous animals exhibit reduced pancreas weight compared to controls after birth. (C) Double heterozygous animals exhibit elevated blood glucose compared to controls and single *Hnf6* heterozygotes. * P =0.05. Error bars represent SEM.

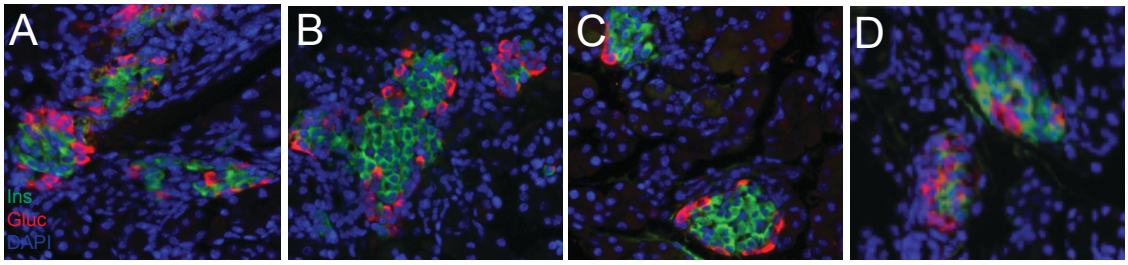


Figure 39. Islet morphology in single and double heterozygous mice at P1. Representative images of α (red) and β (green) cell immunolabeling in control (A), *Pdx1*$^{LacZ/+}$ (B), *Hnf6*$^{+/-}$ (C), and *Pdx1*$^{LacZ/+}$*;Hnf6*$^{+/-}$ (D) pancreata at P1.

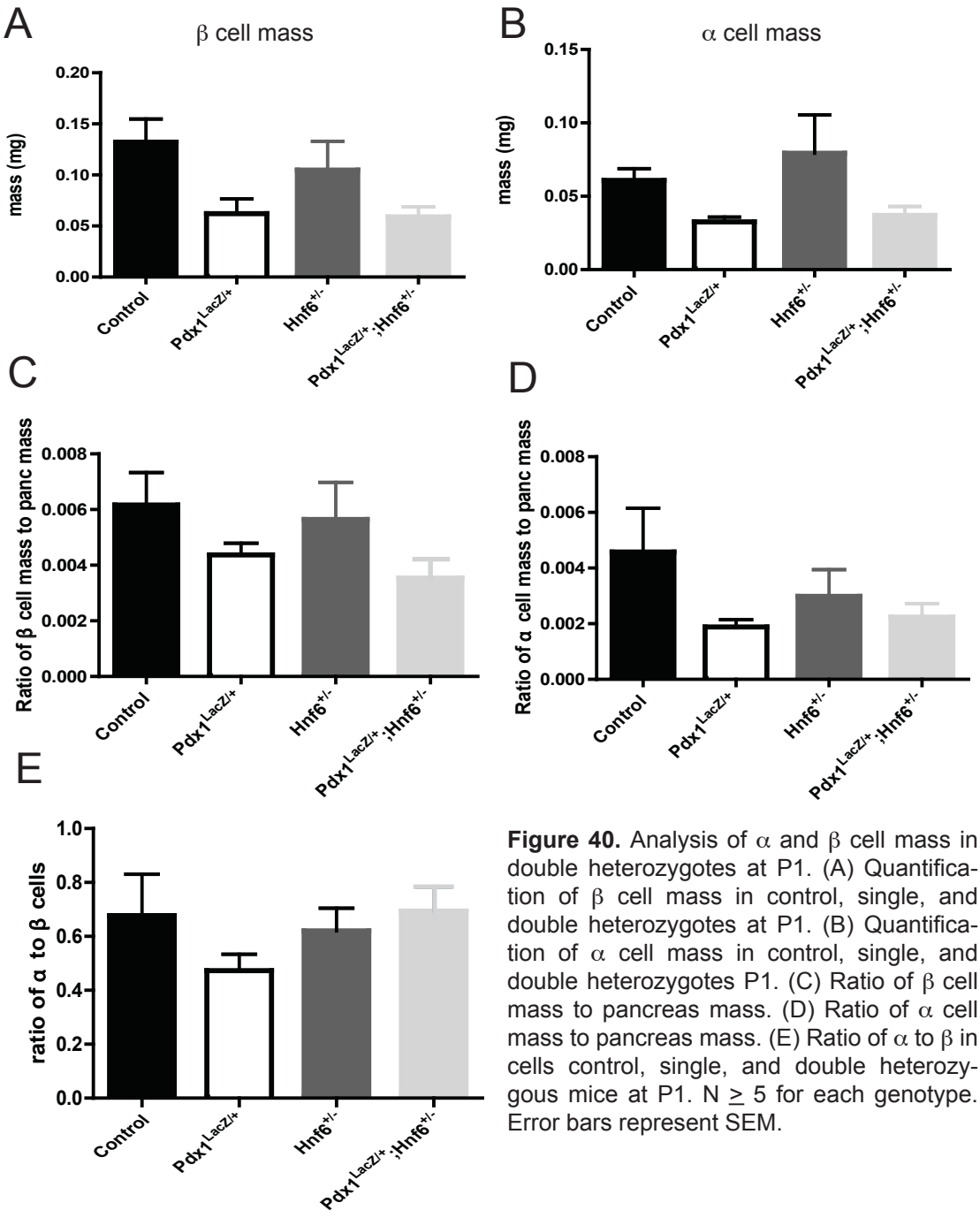


Figure 40. Analysis of α and β cell mass in double heterozygotes at P1. (A) Quantification of β cell mass in control, single, and double heterozygotes at P1. (B) Quantification of α cell mass in control, single, and double heterozygotes P1. (C) Ratio of β cell mass to pancreas mass. (D) Ratio of α cell mass to pancreas mass. (E) Ratio of α to β in cells control, single, and double heterozygous mice at P1. N ≥ 5 for each genotype. Error bars represent SEM.

α or β cells relative to pancreas mass is not statistically different between genotypes (Figure 40C-D). Furthermore, the α:β cell ratio is unchanged in *Pdx1*$^{LacZ/+}$*;Hnf6*$^{+/-}$ pups at P1 (Figure 40E). These data suggest that endocrine mass is not affected by global double heterozygosity at P1.

Glucose homeostasis is restored in double heterozygotes with age

After observing elevated blood glucose in *Pdx1*$^{LacZ/+}$*;Hnf6*$^{+/-}$ pups one day after birth, we wanted to explore whether this phenotype was maintained until and beyond weaning (3 weeks of age). Body weight was measured at weaning in control, single, and double heterozygous animals, and revealed that *Pdx1*$^{LacZ/+}$*;Hnf6*$^{+/-}$ animals no longer exhibit reduced weight (Figure 41A). Interestingly, with the exception of two mice, *Pdx1*$^{LacZ/+}$*;Hnf6*$^{+/-}$ weanlings show restored blood glucose levels at this time; however, *Pdx1* heterozygotes have elevated *ad lib* blood glucose, which is consistent with the phenotype presented in the literature (Figure 41B). When challenged with an IPGTT at 3 weeks of age, there are no striking differences in glucose clearance between genotypes (Figure 42A). At 6 weeks of age, control, single *Pdx1* heterozygotes, and double heterozygotes are glucose tolerant while *Hnf6* heterozygotes have slightly impaired glucose tolerance (Figure 42B). By 8 weeks of age, the impaired glucose clearance in *Hnf6*$^{+/-}$ animals has resolved and is comparable to control and *Pdx1*$^{LacZ/+}$*;Hnf6*$^{+/-}$ mice, while the preliminary data suggest *Pdx1*$^{LacZ/+}$ animals are glucose intolerant (Figure 42C).

Glucose homeostasis is maintained by proper endocrine response to fluctuating blood glucose levels and also by storage and release of glucose from the peripheral tissues. In particular, the liver is responsible for storing the majority of excess glucose as glycogen, which is broken down and released into the bloodstream during fasting or

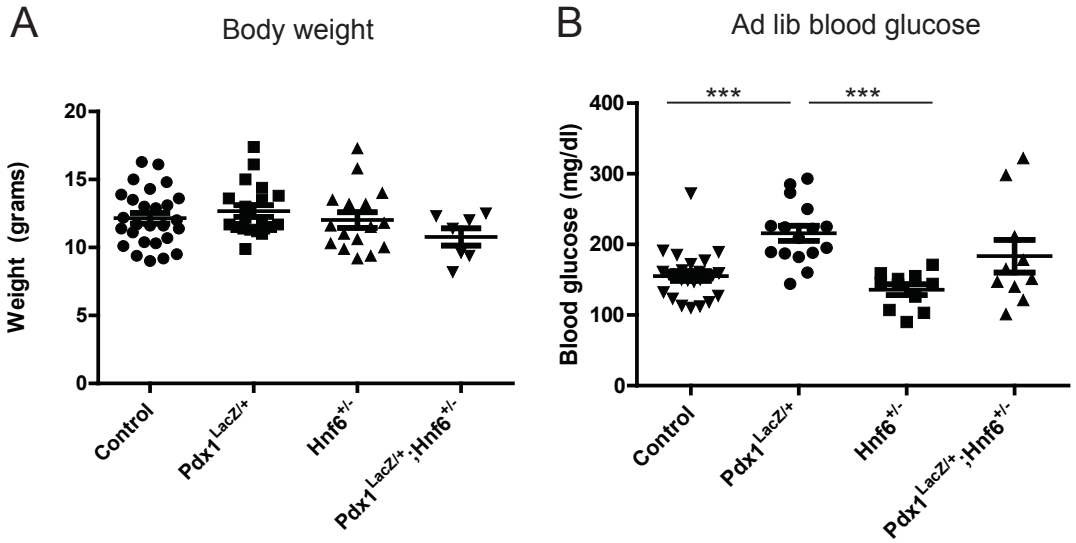


Figure 41. Body weight and *ad lib* glucose values at 3 weeks of age. (A) Body weights from control, single heterozygous, and double heterozygous animals at 3 weeks of age. (B) *Ad lib* blood glucose values for control, single, and double heterozygous animals at 3 weeks of age. *** P = 0.001. Error bars represent SEM.

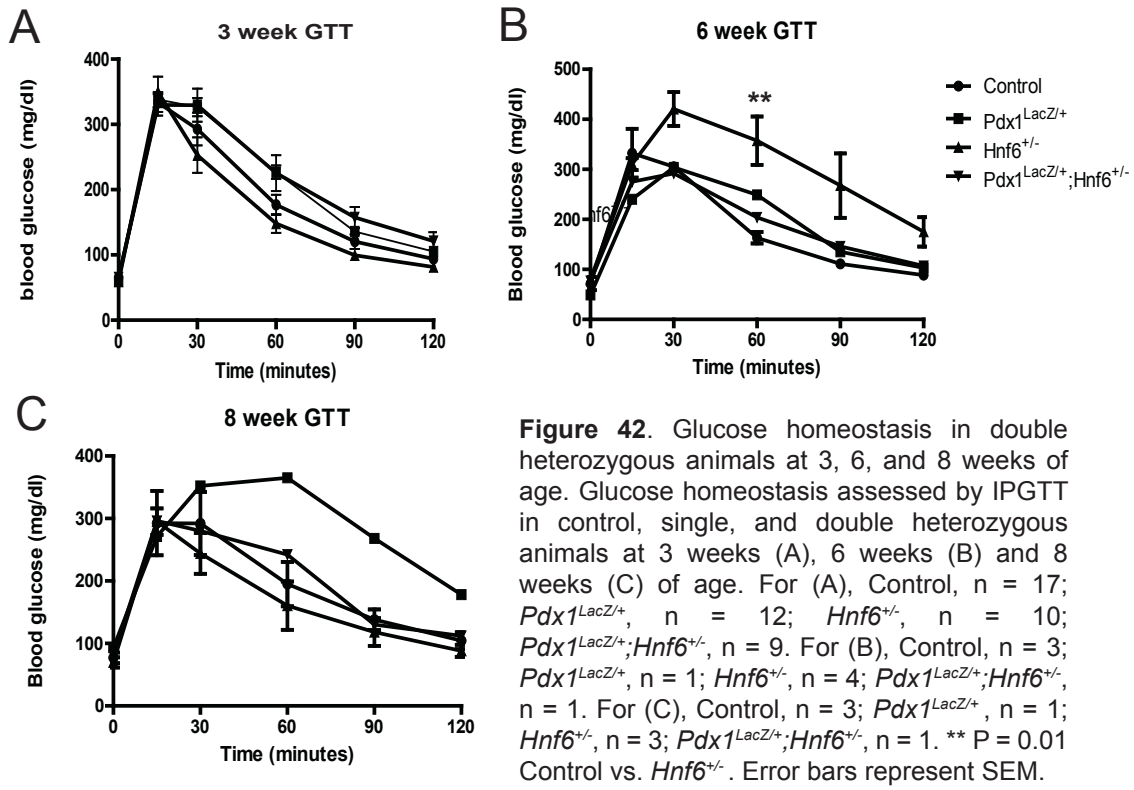


Figure 42. Glucose homeostasis in double heterozygous animals at 3, 6, and 8 weeks of age. Glucose homeostasis assessed by IPGTT in control, single, and double heterozygous animals at 3 weeks (A), 6 weeks (B) and 8 weeks (C) of age. For (A), Control, n = 17; *Pdx1*$^{LacZ/+}$, n = 12; *Hnf6*$^{+/-}$, n = 10; *Pdx1*$^{LacZ/+}$*;Hnf6*$^{+/-}$, n = 9. For (B), Control, n = 3; *Pdx1*$^{LacZ/+}$, n = 1; *Hnf6*$^{+/-}$, n = 4; *Pdx1*$^{LacZ/+}$*;Hnf6*$^{+/-}$, n = 1. For (C), Control, n = 3; *Pdx1*$^{LacZ/+}$, n = 1; *Hnf6*$^{+/-}$, n = 3; *Pdx1*$^{LacZ/+}$*;Hnf6*$^{+/-}$, n = 1. ** P = 0.01 Control vs. *Hnf6*$^{+/-}$. Error bars represent SEM.

hypoglycemia. Thus, mobilization of glucose, either to or from its stored form, is important for regulation of blood glucose. Because *Hnf6* regulates *Glut2, Gck*, and genes involved in gluconeogenesis in the liver, we wanted to assess whether glycogen deposition was altered in double heterozygous animals. We assessed glycogen deposition in livers from control, single and double heterozygous mice at 8 weeks of age (Figure 43). Control animals exhibited characteristic deposition of glycogen, with increased localization of glycogen surrounding the ducts and more sparse deposition of glycogen elsewhere (Figure 43A). *Pdx1* heterozygous animals exhibited somewhat reduced glycogen deposition surrounding the ducts compared to controls (Figure 43B), while *Hnf6* heterozygous animals showed saturation of glycogen deposition throughout liver tissue (Figure 43C). Surprisingly, we observed glycogen deposition comparable to controls in livers from $Pdx1^{LacZ/+};Hnf6^{+/-}$ animals (Figure 43D).

Islet morphology and protein localization in weaned double heterozygous animals

To determine if islet morphology is maintained after weaning in the setting of double heterozygosity, pancreatic sections were immunolabeled for insulin and glucagon and islet morphology was assessed. Compared to control animals (Figure 44A), *Pdx1* heterozygotes possessed islets with relatively normal morphology, although insulin labeling appeared less intense and some α cells were scattered throughout the β cell core (Figure 44B). Islets from *Hnf6* heterozygotes appeared mostly normal (Figure 44C), and double heterozygote islets exhibited insulin immunoreactivity comparable to control animals, but contained excess α cells scattered throughout the β cell core (Figure 44D). These data are consistent with the IPGTT results from 3 weeks of age, which showed that glucose homeostasis is maintained in $Pdx1^{LacZ/+;}Hnf6^{+/-}$ mice.

Because *Pdx1* and *Hnf6* regulate genes important to islet function, we were interested in examining localization of these regulated proteins in $Pdx1^{LacZ/+;}Hnf6^{+/-}$ mice.

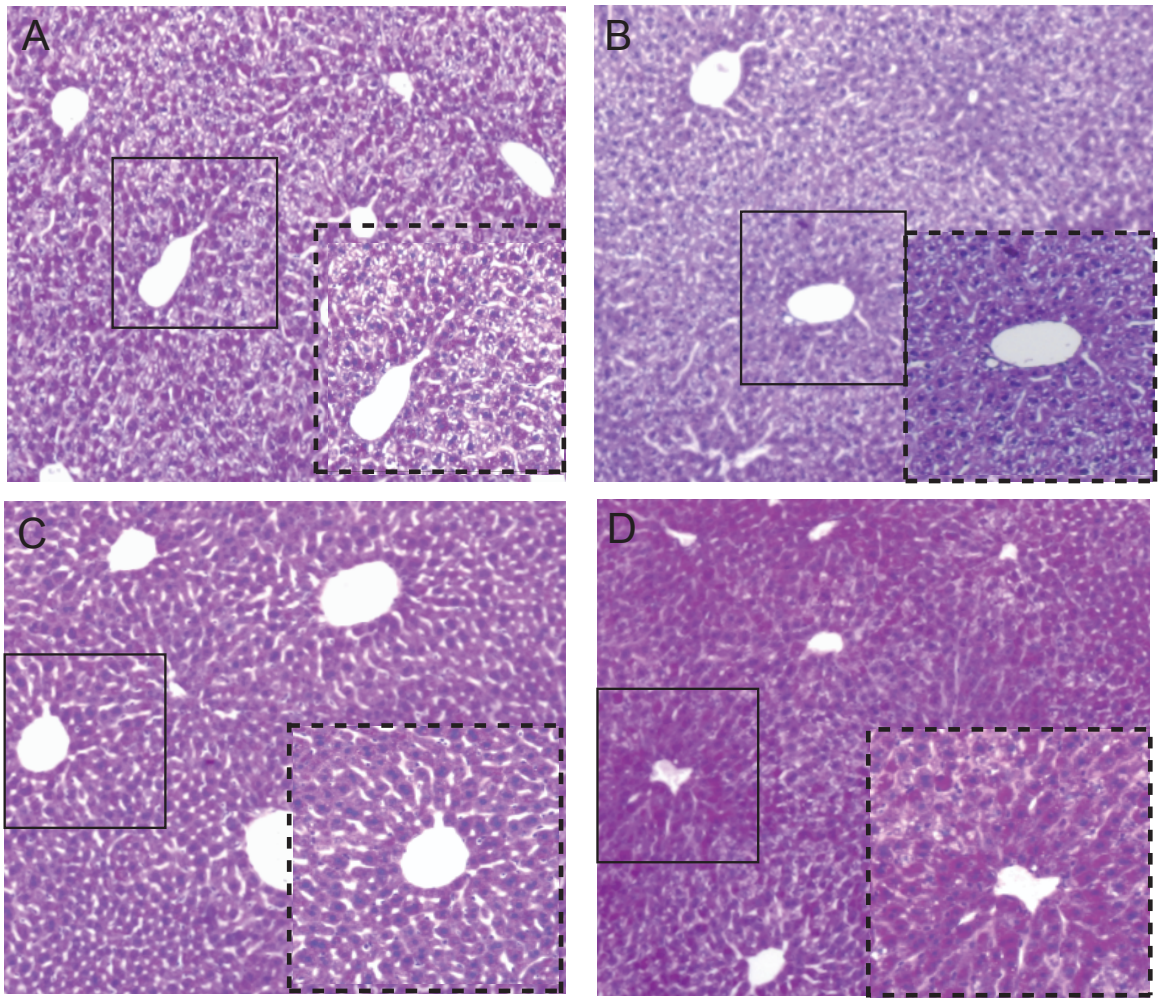


Figure 43. Glycogen deposition at 8 weeks of age. Glycogen deposition (purple) was visualized by Periodic acid-Schiff staining in control (A), *Pdx1*$^{LacZ/+}$ (B), *Hnf6*$^{+/-}$ (C), and *Pdx1*$^{LacZ/+}$;*Hnf6*$^{+/-}$ (D) livers. Dashed-line insets are enlarged representations of boxed areas.

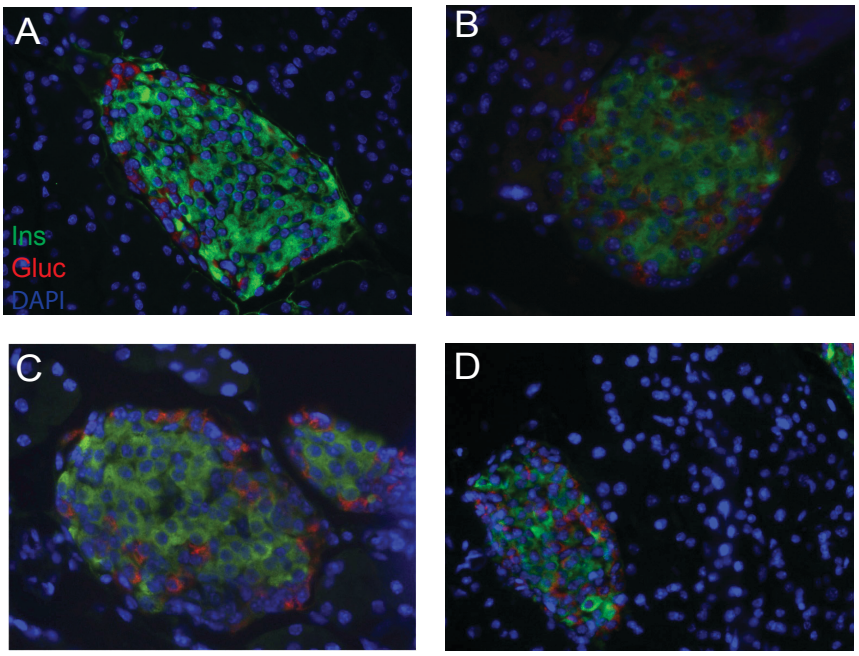


Figure 44. Islet morphology in at 3 weeks of age. Immunolabeling for insulin-positive β cells (green) and glucagon-positive α cells (red) in control (A), *Pdx1*$^{LacZ/+}$ (B), *Hnf6*$^{+/-}$ (C), and *Pdx1*$^{LacZ/+}$*;Hnf6*$^{+/-}$ (D) islets.

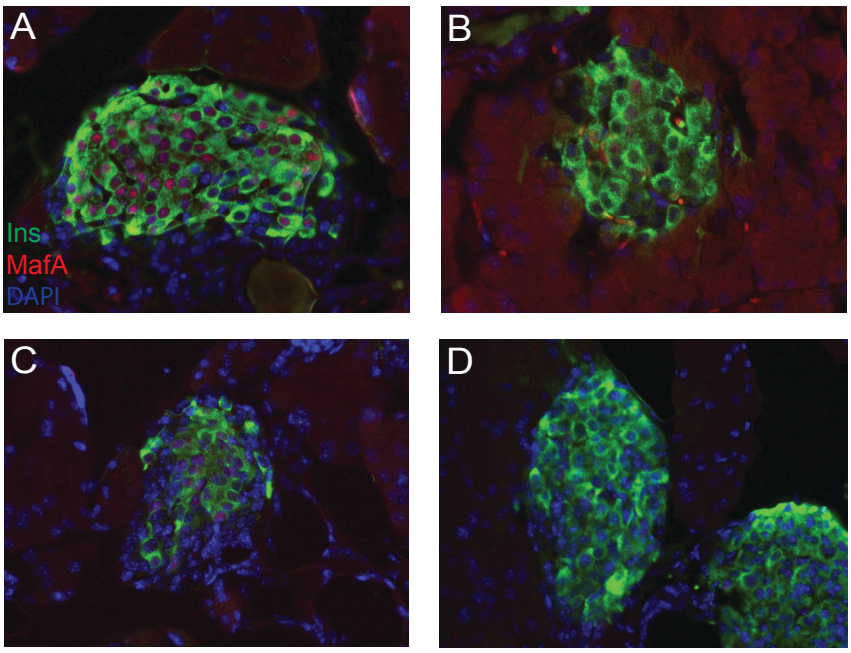


Figure 45 MafA localization at 3 weeks of age. Immunolabeling for insulin-positive β cells (green) and MafA (red) in control (A), *Pdx1*$^{LacZ/+}$ (B), *Hnf6*$^{+/-}$ (C), and *Pdx1*$^{LacZ/+}$*;Hnf6*$^{+/-}$ (D) islets.

Thus, pancreatic sections from control, single, double heterozygous animals were immunolabled for insulin and the *Pdx1*- and *Hnf6*-regulated transcription factor *MafA*. In control and *Hnf6* heterozygous animals, the majority of insulin-positive β cells were positive for MafA in nuclei (Figure 45A,C). *Pdx1* heterozygous animals also possessed MafA-positive insulin-positive cells, but fewer insulin-positive cells were labeled (Figure 45B). Of important note, double heterozygotes with elevated *ad lib* glucose at weaning (Figure 41B) exhibited very few MafA-positive β cells at this time (Figure 45D); MafA localization in double heterozygous animals with normal *ad lib* glucose levels has not yet been assessed. Control, single and double heterozygous animals were also immunolableled for Glut2, the glucose transporter regulated by *Pdx1.* Control and *Hnf6* heterozygous animals exhibited bright, membrane-localized Glut2 immunolabeling (Figure 46A, C). $Pdx1^{LacZ/+}$ islets displayed weak, somewhat diffuse Glut2 immunolabeling, as did $Pdx1^{LacZ/+};Hnf6^{+/-}$ animals with elevated *ad lib* blood glucose (> 175 mg/dl) (Figure 46B, D). Interestingly, islets from double heterozygous animals with normal *ad lib* blood glucose (< 175 mg/dl) exhibited slightly brighter Glut2 localization, although not comparable to controls (Figure 46E). These data suggest a possible connection between *ad lib* blood glucose levels and proper Glut2 localization/protein expression in $Pdx1^{LacZ/+};Hnf6^{+/-}$ animals at 3 weeks of age.

Gene expression changes at 4 weeks of age

After observing changes in protein immunolabeling in double heterozygous mice, we wanted to assess whether our results could be attributed to changes in gene expression. To this end, we examined changes in gene expression by custom TaqMan Low-Density Array (TLDA). Candidate genes for the TLDA were chosen based on involvement in β cell maturity, islet function, or because they were regulated by *Pdx1*. Islets were isolated from control, $Pdx1^{LacZ/+}$, $Hnf6^{+/-}$, and $Pdx1^{LacZ/+}; Hnf6^{+/-}$ animals at 4

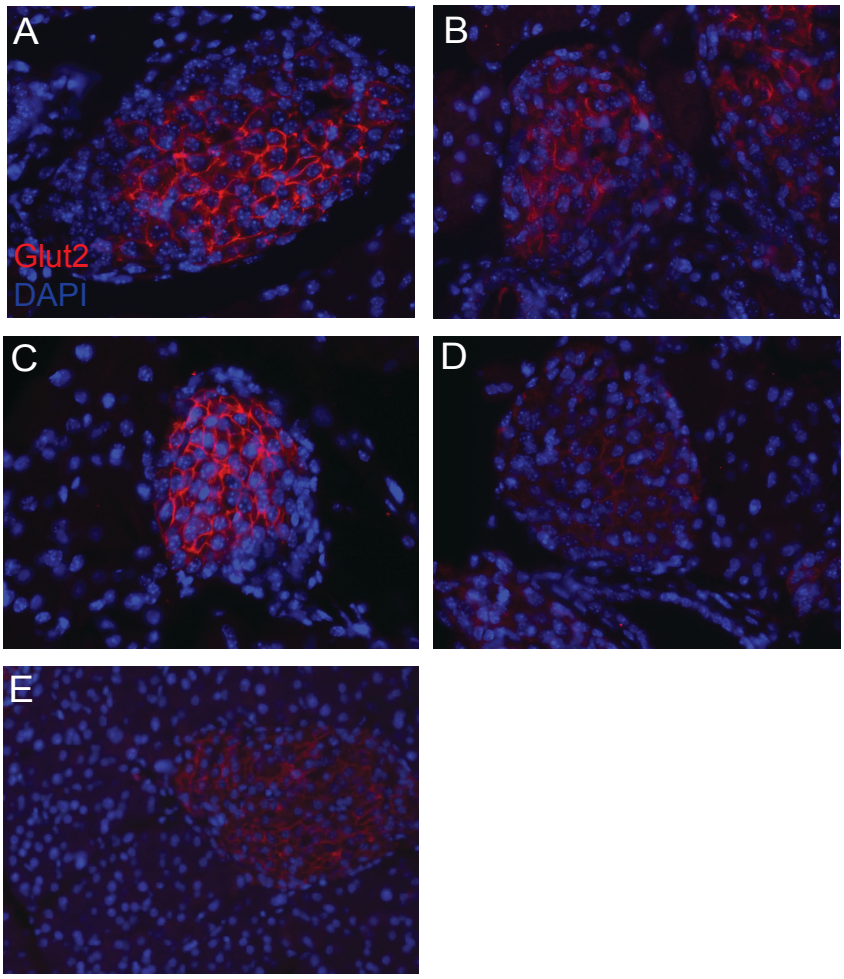


Figure 46. Glut2 immunolabeling at 3 weeks of age. Localization of the glucose transporter Glut 2 (red) in control (A), *Pdx1*$^{LacZ/+}$ (B), *Hnf6*$^{+/-}$ (C), *Pdx1*$^{LacZ/+}$;*Hnf6*$^{+/-}$ with high (> 175 mg/dl) *ad lib* glucose (D) and *Pdx1*$^{LacZ/+}$;*Hnf6*$^{+/-}$ with low (< 175 mg/dl) *ad lib* glucose (E).

weeks of age and RNA was extracted for TLDA analysis. Preliminary data revealed trends in expression of certain genes. As expected, expression of *Pdx1* was reduced in *Pdx1*$^{LacZ/+}$ islets compared to control islets (Figure 47). *Pdx1* expression was unchanged between control and *Pdx1*$^{LacZ/+}$*; Hnf6*$^{+/-}$ islets, but slightly elevated in islets from *Hnf6*$^{+/-}$ animals. *Hnf6* was not detected in single or double heterozygous islets (Figure 47), which is consistent its down-regulation upon endocrine cell differentiation. *MafA* was detected at a higher level in *Pdx1*$^{LacZ/+}$*;Hnf6*$^{+/-}$ islets than *Pdx1*$^{LacZ/+}$ islets (Figure 47), indicating that *Hnf6* heterozyosity may somehow rescue reduced *MafA* expression in the *Pdx1*$^{LacZ/+}$ background. These data are not surprising considering the recent evidence indicating that *Hnf6* negatively regulates *MafA* expression *in vitro* (Yamamoto et al. 2013). Expression of *Glut2,* the glucose transporter regulated by *Pdx1*, is unchanged in *Hnf6*$^{+/-}$ islets compared to controls, but is reduced comparably in *Pdx1* heterozygotes and double heterozygotes (Figure 47). Surprisingly, *Ctgf* expression was elevated in double heterozygous islets compared to all other genotypes. This is of particular interest considering that *Ctgf* is not normally maintained in postnatal endocrine cells, and consistent with published data that suggest that *Hnf6* negatively regulates *Ctgf* in the postnatal murine pancreas (Wilding Crawford et al. 2008). Additionally, we also observed decreased expression of *Ins1* and *Ins2* in *Pdx1*$^{LacZ/+}$*;Hnf6*$^{+/-}$ islets compared to controls (Figure 47).

Discussion

Studying the cooperative roles of transcription factors during pancreas development is a straightforward means of better understanding the complex genetic interactions that take place during pancreatogenesis. Evaluating the effect of double *Pdx1/Hnf6* heterozygosity developmentally and postnatally provides insight regarding

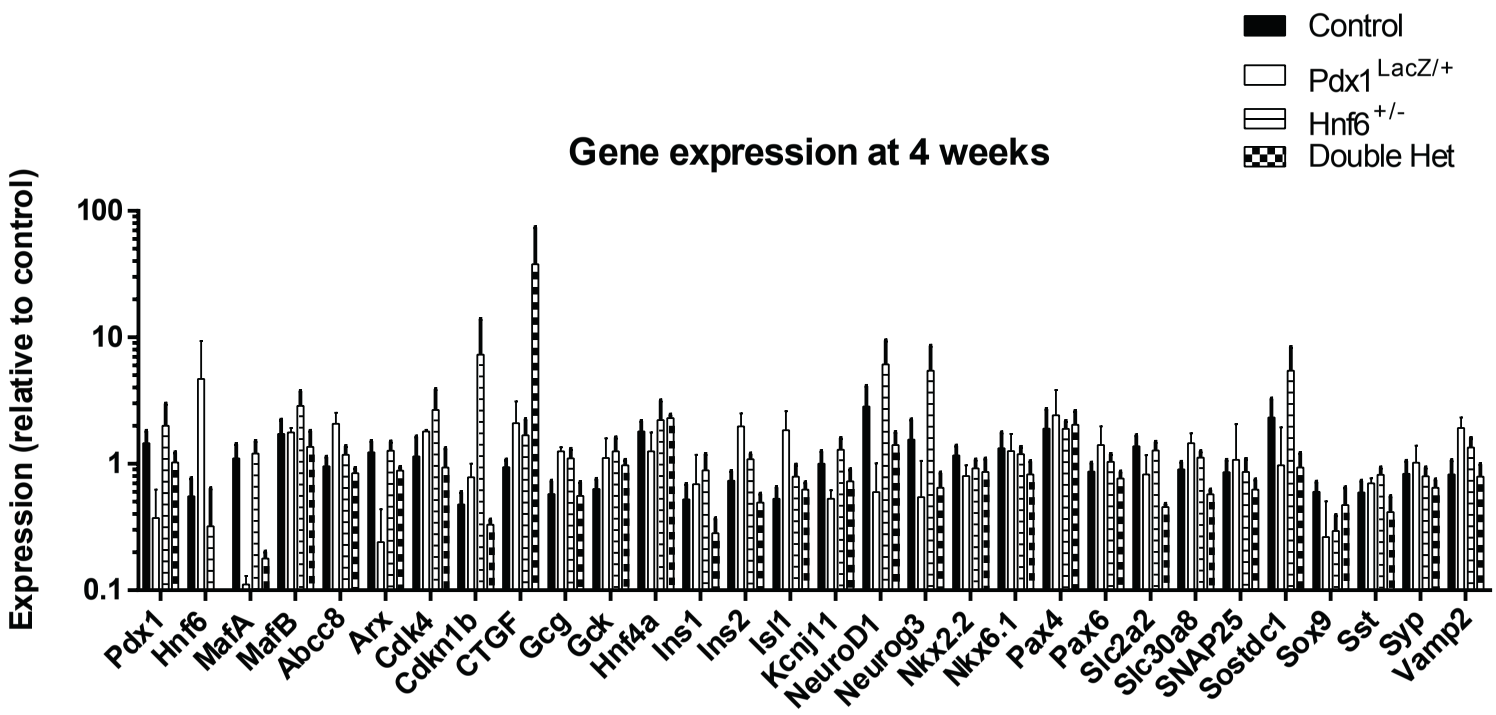


Figure 47. Gene expression at 4 weeks of age. Analysis of selected gene expression by TLDA in control (black), *Pdx1*$^{LacZ/+}$ (white), *Hnf6*$^{+/-}$ (horizontal stripe), and *Pdx1*$^{LacZ/+}$;*Hnf6*$^{+/-}$ (checkered) islets. N ≥ 3 for control, *Hnf6*$^{+/-}$, and *Pdx1*$^{LacZ/+}$;*Hnf6*$^{+/-}$. N = 2 for *Pdx1*$^{LacZ/+}$. Expression levels were normalized against ubiquitously expressed *Hprt*.

the role of these transcription factors in establishing endocrine identity and preserving endocrine function. It has been well documented that both *Pdx1* and *Hnf6* regulate genes required for the specification and postnatal function of endocrine cells; indeed, these two transcription factors cooperatively regulate *Ngn3 in vitro*.

We first examined how double heterozygosity affects pancreas development and postnatal function using a model of global *Pdx1* heterozygosity and conditional *Hnf6* heterozygosity (*Pdx1*$^{LacZ/+}$;*Hnf6*$^{\Delta panc/+}$). Our preliminary results indicated that double heterozygosity using this model did not affect endocrine development or function despite the established roles that both genes have in regulation of endocrine specification genes and genes involved in β cell function (Figure 30, 31). Indeed, neither *Pdx1* or *Hnf6* single heterozygotes have documented developmental phenotypes, but this does not preclude the possibility of a genetic interaction-driven phenotype in double heterozygotes. The absence of phenotypical change in *Pdx1*$^{LacZ/+}$;*Hnf6*$^{\Delta panc/+}$ pups at P1 motivated us to examine our model of *Pdx1-Cre*-driven *Hnf6* heterozygosity. Upon analysis, we determined that efficient recombination of the *Hnf6* allele does not take place, if at all, until after e11.5. (Figure 33). It is possible that, using *Pdx1-Cre*, deletion of *Hnf6* may not occur during the overlap with *Pdx1* expression in MPCs to affect that specific cell population of interest. If deletion occurs after the period of *Pdx1/Hnf6* co-expression, it may be too late during pancreatogenesis to effectively examine cooperation of these two transcription factors in the progenitor population. Although it is still being determined exactly when *Hnf6* is recombined, we suspect that it occurs after the birth of endocrine progenitors, but concurrent with the onset of acinar differentiation. This notion is supported by EYFP immunolabeling at P1, a surrogate for *Pdx1-Cre* activity. At this time, we observed efficient and consistent EYFP immunolabeling in the acinar population, but less consistent labeling in the endocrine clusters (Figure 34). These results suggest that *Cre* efficiently recombines the *EYFP* allele as acinar cells are differentiating, which

occurs after endocrine differentiation. Although we cannot assume that *Pdx1-Cre* recombines the floxed *Hnf6* and *EYFP* alleles with the same efficiency or at the same time, the available data indicate that recombination of the latter allele does not take place as early as we had anticipated.

To avoid the issues of tissue-specific *Cre* timing and efficiency, we employed the *Prm-Cre* transgene to generate *Hnf6* heterozygotes at the 2- to 4-cell zygotic stage (essentially, a globally heterozygous model). Using this model, we were confident that *Hnf6* heterozygosity would occur well before initiation of pancreatogenesis. Indeed, we observed a phenotype in $Pdx1^{LacZ/+};Hnf6^{+/-}$ pups shortly after birth (Figure 35). We were surprised, however, to also observe a phenotype in single *Pdx1* heterozygote pups at this time. These results prompted us to identify the cause of reduced postnatal weight and organ size in experimental animals. It has been well established that decreased body weight and organ size in newborn animals may result from placental insufficiency and IUGR (Eleftheriades, Creatsas, and Nicolaides 2006). Interestingly, *Pdx1* has been isolated from the murine placenta and is up-regulated during placental insufficiency (Buffat et al. 2007). We hypothesized that reduced body and pancreas weight in single *Pdx1* and double *Hnf6/Pdx1* heterozygous animals may result from decreased expression of *Pdx1* in the placenta and subsequent insufficiency. When organ weights were measured and compared to body weight, they were directly proportional to the body size, indicating that reduced pancreas size manifests postnatally and is most likely not a result of IUGR (Figure 36).

We also explored the possibility that the $Pdx1^{LacZ}$ knock-in allele was behaving as a dominant negative to explain our unexpected P1 results. In both *Pdx1* mutant alleles, a portion of the second exon, including the homeodomain, is absent, and partial Pdx1 protein generated from $Pdx1^{LacZ}$ allele is more stable than the $Pdx1^{+/-}$ allele because it is fused to *β galactosidase*. The first exon of *Pdx1* is intact in both mutant alleles (Figure

15). This exon contains an FPWMK motif that enables interaction with the cofactor *Pbx1,* which is required for proliferation and morphogenesis of the embryonic pancreas (Kim et al. 2002). The *Pbx1:Pdx1* interaction is necessary for proliferation of the endocrine portion of the developing pancreas (Dutta 2001). Furthermore, adult animals heterozygous for both *Pbx1* and *Pdx1* develop normally, but exhibit overt diabetes by 10 weeks of age due to impaired endocrine function (Kim et al. 2002). This research indicates that N-terminal interactions between *Pdx1* and other factors are important for development and function, and suggest a possible explanation for the observed phenotype in pups from $Pdx1^{LacZ/+};Hnf6^{+/-}$ litters.

To determine whether the postnatal changes were related to *Pdx1* heterozygosity or the stability of the β-galactosidase fusion protein, we examined a second model of *Pdx1* heterozygosity. The P1 pups generated from $Pdx1^{+/-}$ litters were indistinguishable from those generated from $Pdx1^{LacZ/}$-containing litters in terms of body weight (Figure 37) However, this experiment revealed that control animals from $Pdx1^{LacZ/+};Hnf6^{+/-}$ litters weighed significantly more than all other experimental and control animals (Figure 37). We determined that the heavier control pups from $Pdx1^{LacZ/+};Hnf6^{+/-}$ litters were born to females carrying the *Prm-Cre* transgene. This situation has not been reported in the literature, but it is possible that the insertion site of the *Prm-Cre* transgene somehow influences pup size. Indeed, females that undergo hyperglycemia or gestational diabetes give birth to macrosomic offspring (Oh, Gelardi, and Cha 1988). Thus, if an as yet undefined characteristic of the *Prm-Cre* transgene causes elevated blood glucose during pregnancy, the pups may be larger as a result. We removed all pups born to *Prm-Cre* positive females, re-analyzed the curated data, and observed that only double heterozygous animals exhibited reduced body/pancreas weight and elevated blood glucose at P1 (Figure 38).

After removal of macrosomic pups from the data set, one characteristic of

Pdx1$^{LacZ/+}$;*Hnf6*$^{+/-}$ postnatal animals remained consistent: in both data sets, global double heterozygous animals exhibited hyperglycemia after birth. Considering that endocrine mass and ratios are unchanged in *Pdx1*$^{LacZ/+}$;*Hnf6*$^{+/-}$ animals (Figure 40), it is most likely that elevated blood glucose is a reflection of impaired endocrine function. This idea becomes more interesting when one considers the expression pattern of *Pdx1* and *Hnf6,* as these two factors initially overlap, but are not co-expressed in mature β cells, or any other endocrine cell type. It is possible that *Pdx1* and *Hnf6* overlap in postnatal endocrine cells that are still undergoing differentiation and have not yet activated hormone expression (Dr. Diana Stanescu, personal communication). However, it is interesting to consider the mechanism by which decreased expression of two transcription factors affects a cell population in which the factors are not normally co-expressed. It is possible that the initial co-expression of *Pdx1* and *Hnf6* in MPCs and, to a lesser extent, endocrine progenitors, primes the cells for postnatal function. This notion is not novel, as deletion of *Hnf4α* in the embryonic liver affects gene expression in differentiated hepatocytes long after expression would normally be down-regulated (Kyrmizi et al. 2006). Similarly, deletion of the transcription factor *Foxd3*, which is not expressed in islets during pregnancy, causes decreased β cell proliferation and glucose intolerance during pregnancy (Plank, Frist, et al. 2011). In line with this, it is possible that co-expression of *Pdx1* and *Hnf6* in MPCs has a priming affect that later influences islet function. To address this, we are currently assessing changes in gene expression in double heterozygous animals by RNA sequencing (RNA-Seq) and TLDA at e15.5 and P1, respectively.

Given that *Pdx1*$^{LacZ/+}$;*Hnf6*$^{+/-}$ animals exhibit elevated blood glucose at P1, we anticipated that glucose homeostasis would worsen over time and eventually result in glucose intolerance. We were surprised to observe that the majority of double heterozygous animals restore glycemic control by 3 weeks of age and maintain glucose

homeostasis more effectively than *Pdx1* heterozygotes (Figure 41, 42). These results suggest that double heterozygous animals somehow compensate for their initial defects in glucose homeostasis. The majority of double heterozygotes exhibit normalized *ad lib* blood glucose by weaning, with the exception of two animals. Assessment of glycogen deposition in the livers of adult double heterozygous mice revealed no obvious changes compared to controls, suggesting appropriate storage and mobilization of glucose in these animals. These data are consistent with our 8 week GTT results. We were surprised to observe reduced glycogen deposition in *Pdx1* heterozygous livers, as *Pdx1* is not expressed hepatic tissue and a *Pdx1*-related liver phenotype has not been reported previously. However, it is possible that reduced insulin signaling in the setting of *Pdx1* heterozygosity may affect glucose uptake in the liver, resulting in decreased glycogen storage (and possibly impaired glucose homeostasis). However, this does not explain why $Pdx1^{LacZ/+};Hnf6^{+/-}$ mice have normal glycogen deposition, as double heterozygosity results in decreased *Ins1* and *Ins2* expression at 4 weeks of age (Figure 47). *Hnf6* heterozygosity could cause decreased glycogen breakdown and glucose mobilization in the liver, thus 'cancelling out' the reduced glycogen deposition that results from *Pdx1* heterozygosity in double heterozygous animals. The consequence of *Pdx1* heterozygosity, alone or in combination with *Hnf6* heterozygosity, on liver function is a topic that requires more attention, and cannot be fully explained by the data presented here.

Preliminary assessment of gene expression in double heterozygotes revealed trends that will require additional samples to confirm. However, we observed *MafA* expression in $Pdx1^{LacZ/+};Hnf6^{+/-}$ islets that was reduced compared to controls, but elevated compared to *Pdx1* heterozygous islets, suggesting that compound heterozygosity rescues this reduction in *MafA* expression. *Pdx1* positively regulates *MafA* expression in islets, while *Hnf6* indirectly negatively regulates *MafA* (Raum et al.

2006, Vanhoose et al. 2008, Tweedie et al. 2006, Yamamoto et al. 2013). We hypothesize that reduced expression of *Hnf6* relieves its inhibition or otherwise promotes expression of *MafA* in the double heterozygous background. Our lab has demonstrated that maintained expression of *Hnf6* in endocrine cells results in absence of MafA protein in β cells (Tweedie et al. 2006). Therefore, reduced *Hnf6* expression may, in fact, promote increased levels *MafA* in double heterozygous islets. We also observed decreased expression of *Ins1* and *Ins2* in compound heterozygote islets. These results are perplexing, as *Pdx1*$^{LacZ/+}$*;Hnf6*$^{+/-}$ mice are glucose tolerant at all times examined (Figure 42). Reduced levels of *Ins1/Ins2* expression compared to controls may reflect compensatory mechanisms that have not yet been completed. For example, we may observe restored expression of *Ins1* or *Ins2* at 6 or 8 weeks of age. Indeed, analysis of glycogen deposition at 8 weeks of age in *Pdx1*$^{LacZ/+}$*;Hnf6*$^{+/-}$ livers suggests normal insulin signaling and glucose uptake (Figure 43), indicating that *Ins* expression may be normal at that time. The idea of gradually increasing gene expression to compensate for early postnatal β cell dysfunction could also explain reduced *Glut2* (*Slc2a2)* expression in *Pdx1*$^{LacZ/+}$*;Hnf6*$^{+/-}$ islets at 4 weeks of age (Figure 47). It is also possible that genes related to β cell function are reduced in double heterozygotes because islet function has been restored and expression no longer required at high levels. The ongoing characterization of gene expression changes in compound heterozygotes will provide answers to these remaining uncertainties.

The trend in elevated *Ctgf* expression observed in *Pdx1*$^{LacZ/+}$*;Hnf6*$^{+/-}$ islets is intriguing, and could possibly be explained by reduced *Hnf6* expression during embryogenesis. Maintenance of *Hnf6* in endocrine cells results in a 2.5-fold reduction of *Ctgf* expression in neonatal pancreata, indicating that *Hnf6* negatively regulates *Ctgf* (Wilding Crawford et al. 2008). However, neither factor is expressed in mature endocrine cells under normal conditions. Perhaps *Ctgf* expression is up-regulated in double

heterozygous islets to promote proliferation of β cells and improve glucose homeostasis. Assessment of gene expression at P1 when *Pdx1*$^{LacZ/+}$;*Hnf6*$^{+/-}$ islets are dysfunctional will determine whether this is the case.

Assessment of protein localization in 3 week old animals revealed reduced immunolabeling of Glut2, a *Pdx1*-regulated gene necessary for glucose homeostasis, in a *Pdx1*$^{LacZ/+}$;*Hnf6*$^{+/-}$ animal with elevated blood glucose (Figure 41B, 46). Glut2 immunolabeling was modestly restored in a *Pdx1*$^{LacZ/+}$;*Hnf6*$^{+/-}$ animal with normal blood glucose (Figure 46E) This suggests that Glut2 protein expression may be linked to glucose homeostasis, but we have yet to determine if Glut2 down-regulation precedes or follows elevated *ad lib* blood glucose. These data also suggest that some double heterozygous animals do not activate the compensatory mechanisms that restore blood glucose by weaning. These precise mechanisms and the means by which they are activated have not yet been elucidated, but we expect that forthcoming data from RNA-Seq and TLDA analysis will provide meaningful information regarding changes in gene expression that influence islet function. Furthermore, we are also exploring whether regulated over-expression of *Pdx1* and *Hnf6*, together, in the developing pancreatic epithelium can expand the endocrine compartment or enhance postnatal function *in vivo*. This system will use the *Pdx1-tTA* allele to drive expression HA- and Myc-tagged versions of *Pdx1* and *Hnf6,* respectively, throughout embryogenesis (Figure 18, 19).

CHAPTER V

OVER-EXPRESSION OF PDX1 AND HNF6 AS A MEANS TO EXPLORE THE PLASTICITY OF HUMAN DUCT CELLS

Introduction

Efforts to generate insulin-producing β cells from non-endocrine cell sources have increased substantially in recent years. Re-establishing functional β cell mass is a primary goal for treating both TID and T2D, though different approaches may be required depending on the mechanism of initial β cell death or dysfunction. Indeed, endocrine cells have been derived from human induced pluripotent stem cells (hiPS) and human embryonic stem (hES) cells (Tateishi et al. 2008, Jiang et al. 2007, Shim et al. 2007, Phillips et al. 2007, D'Amour et al. 2006, Kroon et al. 2008), and the resultant cells have possessed β cell-like qualities (Jiang et al. 2007, Shim et al. 2007, Phillips et al. 2007, D'Amour et al. 2006, Kroon et al. 2008). Caveats of established differentiation protocols include low yield of β cells after differentiation, the length of time to establish maturity, and poor glucose-responsiveness of differentiated β-like cells. Despite these setbacks, the field continues to move forward in the production of β cells from a variety of cell types.

Differentiating β cells from other sources of pancreatic origin, including exocrine tissue, has proved somewhat effective. Exploring the potential of exocrine tissue to transdifferentiate into endocrine tissue holds promise, as both of these cell populations originate from the same primary MPCs during embryogenesis (Figure 5) (Gu, Dubauskaite, and Melton 2002, Kopp, Dubois, Schaffer, et al. 2011, Kopp, Dubois, Hao, et al. 2011). The viral over-expression of *Pdx1, Ngn3,* and *MafA* together in the acinar component of the mouse pancreas results in transdifferentiation of acinar cells into β-like

cells that are structurally and functionally similar to endogenous β cells and express some appropriate β cell markers (Zhou et al. 2008). Furthermore, administration of the *Pdx1/Ngn3/MafA* virus and induction of acinar transdifferentiation improves blood glucose in mice treated with the β cell-specific toxin streptozotocin (STZ), indicating that the induced β cells can respond appropriately to glucose (Zhou et al. 2008). When a similar experiment was repeated in an acinar cell line, comparable results were obtained, including the down-regulation of acinar genes, up-regulation of endocrine genes, and amelioration of elevated blood glucose in STZ-treated mice (Akinci et al. 2012). However, these induced cells lack the glucose-sensing mechanism that is characteristic of a functional β cell. Additional experiments have showed that after combined pancreatic duct ligation (PDL) and STZ treatment, Ptf1a-positive acinar cells have the ability transdifferentiate into β cells after transitioning through a CK19/Hnf1β/Sox9-positive ductal and Ngn3-positive intermediate (Pan et al. 2013). These data suggest that multiple models of injury and gene manipulation can stimulate acinar cells to activate the endocrine program.

Pancreatic duct cells have also been explored as a potential source of β cells (Inada et al. 2008, Solar et al. 2009, Kopinke and Murtaugh 2010, Furuyama et al. 2011, Kopp, Dubois, Schaffer, et al. 2011, Xiao et al. 2013). Depending on the model of lineage tracing, results regarding the contribution of duct-derived cells to regenerating endocrine tissue have been inconsistent. Indeed, *in vivo* studies using *Hnf1β-Cre* as a lineage marker suggest that bipotent trunk progenitors only give rise to endocrine cells until e15.5 (Solar et al. 2009). However, this specific *Cre* line labeled less than ten percent of cells in adults, potentially permitting *Hnf1β*-positive cells with endocrine potential to escape *Cre* labeling. Additional studies in mice revealed that *Sox9*+ cells contribute to all three pancreatic lineages through embryogenesis, but lose the potential

to contribute to β cells shortly after birth, even after stimulation with PDL (Furuyama et al. 2011, Kopp, Dubois, Schaffer, et al. 2011). Furthermore, although *Ngn3* is activated in adult murine pancreatic duct cells following PDL or treatment with alloxan, another β cell-specific toxin, lineage tracing of these presumed endocrine progenitors does not indicate that they become β cells (Xiao et al. 2013). The conflicting results described above, as well as others, contribute to the controversial nature of facultative progenitor cells in the pancreas and confirm that more research is required for complete understanding.

To explore the potential of duct cells to transdifferentiate into endocrine cells, we employed the HPDE-6 human ductal epithelial cell line. These cells were derived from healthy human donor tissue and are non-tumorogenic in mice, express CK8/18 and CK19, and are polarized with surface microvilli, (Furukawa et al. 1996, Ouyang et al. 2000). The goal of these studies is to virally over-express *Pdx1* and *Hnf6* in HPDE-6 cells to determine if the endocrine program can be activated. *In vitro*, *Pdx1* and *Hnf6* have an additive affect on *Ngn3* reporter activity, and bind to a conserved region of the *Ngn3* promoter (Oliver-Krasinski et al. 2009) Viral vectors to over-express each transcription factor were generated (Figure 20, 21). Analysis of gene expression in HPDE-6 cells revealed that both *Pdx1* and *Hnf6* are expressed endogenously. Future directions will include the regulated over-expression of *Hnf6* and constitutive over-expression of *Pdx1* individually and in combination, followed by analysis of endocrine-specific gene and hormone expression.

Results

Generation of *Pdx1* and *Hnf6* over-expression viral vectors

The construction of the *pLVX-Pdx1-mCherry* and *pLVX-TRE3G-myc-Hnf6* vectors was described previously (see 'Materials and Methods'). These viral vectors were designed to over-express *Pdx1* and *Hnf6* in a constitutive and regulated manner, respectively, in HPDE-6 cells. *mCherry* and *myc* tags were added to *Pdx1* and *Hnf6* cDNA, respectively, to enable differentiation between endogenous and exogenous protein expression by immunolabeling. Additionally, the presence of the fluorescent tag to the *Pdx1* construct will facilitate fluorescence-activated cell (FAC) sorting in future experiments.

HPDE-6 cells express genes required for *Ngn3* activation

Prior to viral infection of HPDE-6 cells with *Pdx1* and *Hnf6* (Figure 20, 21), we wanted to asses endogenous expression levels of these two factors. To quantify endogenous expression of *Hnf6* and *Pdx1* in HPDE-6 cells, qRTPCR was performed on RNA isolated from the cells. Both *Pdx1* and *Hnf6* were expressed HPDE-6 cells (Figure 48).

Discussion

This project is designed to test the potential of human duct cells to activate the endocrine program when *Pdx1* and *Hnf6*, two factors critical to endocrine development, are over-expressed. Pancreatic duct cells have been examined for their potential to generate endocrine cells in previous studies with varying results (Gasa et al. 2004, Solar

et al. 2009, Kopinke and Murtaugh 2010, Furuyama et al. 2011, Kopp, Dubois, Schaffer, et al. 2011, Xiao et al. 2013). Previous work explored the ability of HPDE-6 cells to

differentiate into endocrine cells by adenoviral infection of *Ngn3* alone, and found that this method activates expression of *Neurod1*; however, investigation of whether this cell line can activate hormone expression was not completed (Gasa et al. 2004). Our approach will instead use lentivirus to generate a stable cell line that over-expresses *Pdx1* and *Hnf6,* which will potentially activate *Ngn3.* We opted to address the experiment this way, rather than lentiviral over-expression of *Ngn3* itself, as forced expression of *Ngn3* causes precocious differentiation of endocrine progenitors to the α cell fate (Johansson et al. 2007). Furthermore, recent studies have revealed that adenoviral infection of human duct cells with *Ngn3* alone initiates the neuro-endocrine program, but does not fully commit cells to the pancreatic endocrine lineage (Swales et al. 2012). These data suggest that other factors are needed to establish the commitment to the pancreatic endocrine lineage, and that *Ngn3* alone is not sufficient to enforce this decision.

Our approach to *Pdx1* and *Hnf6* over-expression also differs from previously published models using human ductal cells. As mentioned above, we will utilize lentivirus to establish stable cell lines. Furthermore, we plan to regulate *Hnf6* expression using a doxycycline-inducible system of lentiviral gene expression. *In vivo,* maintained expression of *Hnf6* in the endocrine component of the pancreas has detrimental effects on lineage allocation and postnatal gene expression and β cell function (Gannon et al. 2000, Tweedie et al. 2006, Wilding Crawford et al. 2008). We have chosen to transiently induce *Hnf6* over-expression to avoid these potential issues. As *Pdx1* is expressed in the progenitors that give rise to ductal epithelium and is maintained at low levels in the adult, we will maintain constitutive expression of *Pdx1* in our model. Additionally, *Pdx1* is

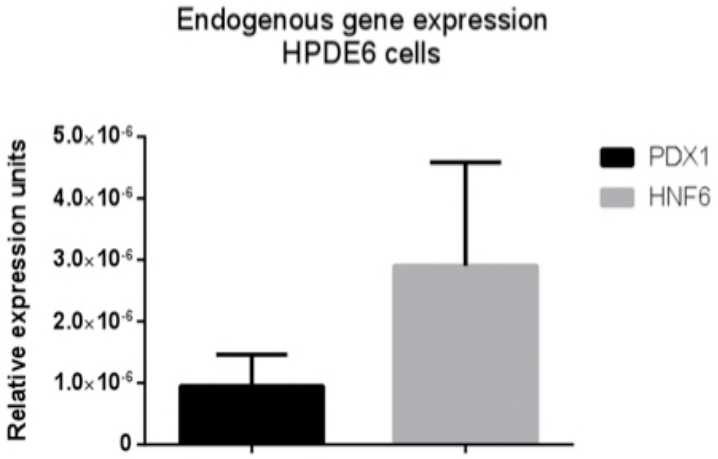


Figure 48. Endogenous expression of *Pdx1* and *Hnf6* in HPDE-6 cells. qRT-PCR was performed on RNA isolated from HPDE-6 cells. Gene expression was normalized to *18S* expression.

maintained at high levels in adult β cells, and thus we expect no deleterious consequences of continued *Pdx1* expression.

The goal of this project is to determine whether simple gene activation can activate the endocrine program in human ductal cells. Ultimately, the information learned from these studies will not only enrich the current transdifferentiation protocols and create new avenues for the generation of β cells, but will provide a better understanding of endocrine development *in vivo.*

CHAPTER VI

SUMMARY AND FUTURE DIRECTIONS

Hnf6 is a one-cut domain containing transcription factor required for specification, differentiation, maintenance of cell and tissue identity, and regulation of genes involved in adult functional processes in the murine liver, central nervous system (CNS), and pancreas (Landry et al. 1997, Clotman et al. 2002, Hodge et al. 2007, Chakrabarty et al. 2012, Espana and Clotman 2012a, b, Roy et al. 2012, Stam et al. 2012, Audouard et al. 2012). In the pancreas, *Hnf6* regulates specification and differentiation of endocrine tissue, maturation of β cells, and maintenance of exocrine differentiation (Gannon et al. 2000, Jacquemin et al. 2000, Jacquemin, Lemaigre, and Rousseau 2003, Tweedie et al. 2006, Zhang et al. 2009). Thus, *Hnf6* plays diverse roles in various stages of pancreas development and function, and is therefore an ideal factor to investigate in the quest for cell-based T1D and T2D therapies. good

The focus of this dissertation is the function of *Hnf6* and its downstream effectors and coregulators in postnatal pancreatic endocrine function. We chose to examine a gene regulated by *Hnf6*, *Sostdc1,* and its role in the endocrine pancreas after observing a near 2-fold up-regulation of *Sostdc1* expression in a transgenic mouse model of diabetes in which *Hnf6* is over-expressed in endocrine cells. The results in Chapter III demonstrated that loss of *Sostdc1*, a BMP and Wnt inhibitor, provides protection against diet-induced glucose intolerance and impaired insulin secretion. Autocrine BMP signaling promotes insulin secretion, and we showed that HFD induces expression of BMP inhibitors. In the absence of *Sostdc1*, expression of other BMP and Wnt antagonists are not increased as they are in control animals. Thus, in *Sostdc1* mutant

animals BMP/Wnt signaling is not inhibited and is able to maintain islet function. These findings support the therapeutic potential for blocking BMP or Wnt antagonist activity in adult islets to enhance insulin secretion. Although we observed consistent changes in gene expression in *Sostdc1* mutant islets from a mixed genetic background, the changes in insulin secretion and glucose tolerance were detected in only a subset of mutants. Because the phenotype could not be attributed to percent contribution of a particular genetic background, the data suggest that other, presently undefined factors may influence endocrine function in animals with the improved phenotype. It is possible that specific modifier genes that are controlled by *Sostdc1* influence islet function. In addition, negative regulation of genes involved in endocrine function, including *MafA* or genes pertinent to insulin exocytosis that have not yet been assessed, may be ameliorated in the absence of *Sostc1.* Recent studies have indicated that over-expression of *Sostdc1* enhances proliferation and cell cycle progression in a gastric adenocarcinoma cell line (Gopal et al. 2013). In β cells, it is thought that insulin secretion capacity is reduced while cells undergo replication. Thus, reduced proliferative capacity in combination with expression of genes that positively regulate insulin secretion may enhance function in *Sostc1*null islets. Future endeavors will include the analysis of global gene expression changes in *Sostdc1* mutant islets to identify potential modifiers and comprehensively characterize the mechanism of improved islet function observed in these animals.

The above-mentioned *Hnf6* transgenic model was generated to investigate the programmed down-regulation of *Hnf6* and its effect on islet development. *Hnf6* expression is maintained throughout pancreas development within primary MPCs, bipotent precursors, and endocrine progenitors, during which it is co-expressed with *Pdx1*, and is down-regulated in endocrine cells as they differentiate. Chapter IV of this dissertation investigates the cooperative function of *Hnf6* and *Pdx1* during

pancreatogenesis and in the postnatal islet using a model of double heterozygosity for both genes. These studies have revealed that *Pdx1/Hnf6* co-expression during embryogenesis is required for early postnatal islet function, as evidenced by elevated blood glucose observed in double heterozygous animals shortly after birth. Although these changes are not evident until one day of age, we suspect that gene expression changes, or priming of specific genes, takes place prior to the manifestation of the phenotype. Thus, these studies provide evidence that *Hnf6* is required, briefly, during late gestation and the early postnatal period to ensure proper endocrine function (Figure 49). Perhaps *Hnf6* plays a role in activating, recruiting, or identifying targets of the islet-enriched methyltransferase *Set7/9*, which maintains the structure of euchromatin at genes required for islet function and also synergizes with *Pdx1* to activate *insulin* transcription (Deering, Ogihara, et al. 2009, Francis et al. 2005). In this situation, reduced *Hnf6* expression during pancreatogenesis could negatively influence *Set7/9* activity in the postnatal islet and result in impaired endocrine function. Future studies to explore this possibility could include analysis of the *Set7/9* promoter for *Hnf6* binding sites and assessment of *Set7/9* occupancy of target genes in the setting of *Hnf6* heterozygosity.

By weaning, *Pdx1/Hnf6* double heterozygotes show restored blood glucose levels, suggesting the activation of a compensatory response that improves function. Identifying the precise mechanism by which function is re-established is a prospective study that will further characterize the double heterozygous phenotype. Additionally, determining specific genes that are co-regulated by *Pdx1* and *Hnf6* using chromatin immunoprecipitation (ChIP) and re-ChIP assays will be immensely informative. Currently, we are exploring changes in gene expression during embryogenesis using

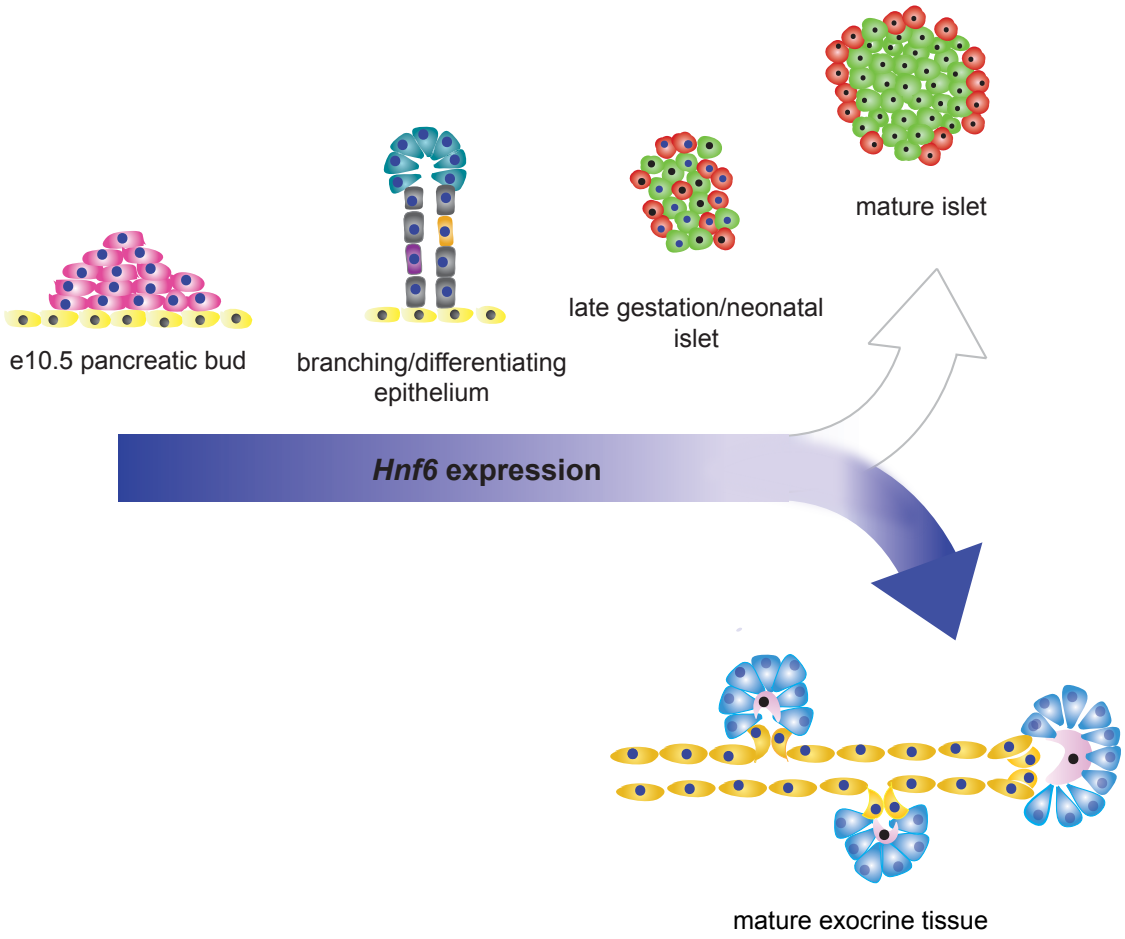


Figure 49. Model of required *Hnf6* expression in the developing and postnatal pancreas. *Hnf6* is expressed in the primary multipotent pancreatic progenitors (MPC, pink cells with blue nuclei) at e10.5. Expression is maintained in the bipotent endocrine/ductal progenitors (grey), endocrine progenitors (purple), ductal cells (gold), and to a lesser extent in the secondary MPCs (teal). *Hnf6* persists in a subset of postnatal endocrine cells (red and green cells with blue nuclei), at late gestation and in the early postnatal pancreas, but is down-regulated in adult endocrine cells (red and green cells with black nuclei). In the adult duct cells (gold with blue nuclei) and acinar cells (light blue with blue nuclei), *Hnf6* expression is maintained. It has not yet been investigated whether *Hnf6* is expressed in centroacinar cells (light pink).

RNA sequencing (RNA-Seq), and we anticipate expression of *Pdx1, MafA,* and other genes related to β cell maturation to be decreased in the double heterozygous state. These experiments will provide a more clear understanding of the temporal and functional relationship between *Hnf6* and *Pdx1,* which will guide future endeavors involving the generation of fully functional β cells *in vitro* from stem or progenitor cells or *in vivo* from facultative progenitors or closely related lineages.

The role that *Hnf6* plays in specification to the endocrine lineage during development makes it an excellent candidate to study in the differentiation of β cells from non-β cell sources *in vitro*. Chapter V presents preliminary data and planned experiments designed to explore the cooperative relationship between *Hnf6* and *Pdx1* in the activation of the endocrine program in human duct cells. Ongoing investigations are determining whether viral-mediated simultaneous over-expression of *Hnf6* and *Pdx1* can stimulate *Ngn3* expression and its downstream endocrine program in a healthy human duct cell line. This simple scientific inquiry will surely prompt additional studies that require further investigation, including the timing and regulation of *Pdx1/Hnf6* over-expression, the requirement of additional factors, the efficiency of transdifferentiation, and the functionality of newly-differentiated cells. We can also identify *Pdx1/Hnf6* target genes in human duct cells by ChIP, and assess epigenetic modifications of these genes during the transdifferentiation process. The information gained from this set of experiments will prove useful for differentiation of endocrine cells from a variety of sources, and move the field closer to a cell-based therapy for T1D.

These studies have continued the exploration of *Hnf6* and its downstream effectors in the postnatal endocrine pancreas. Because *Hnf6* is down-regulated in hormone-positive cells, much focus has been placed on its role during pancreatogenesis. Our lab was the first to examine the effect of maintained expression

of *Hnf6* in the postnatal endocrine lineage (Gannon et al. 2000, Tweedie et al. 2006, Wilding Crawford et al. 2008). The research presented in this thesis reveals novel roles for *Hnf6* in postnatal islet function. Specifically, these studies show that loss of *Sostdc1*, a gene activated in response to *Hnf6,* improves endocrine pancreas function and affects expression of other, similarly related genes in islets; these data reinforce the importance of the temporal expression pattern of *Hnf6.* Furthermore, data presented here illustrate a cooperative relationship between *Hnf6* and *Pdx1* that extends beyond the window of co-expression in progenitor cells and indicates that, together, these two factors prepare islets for postnatal function (Figure 49). Additionally, preliminary data and proposed experiments are presented in this document that will determine whether combined over-expression of *Hnf6* and *Pdx1* can initiate the endocrine program in human duct cells. Together, these studies have furthered our knowledge of *Hnf6* and its influence on islet function in the murine and human pancreas.

APPENDIX

Appendix A: Strain-specific SNP genotyping for Sostdc1 null animals

Chromosome-bp	B6	##	DBA	KFD 5 KO	KGJ 1 KO	KFM3 KO	KFW3KO	KFL8 KO	KEY1 KO	KFD9 KO	KFL4 KO	KEY2 KO	KFO2 KO	KGJ2 KO	KFX6 WT	KFO3 WT	KGK2 WT	KEW 7 WT	KGJ 4 WT	KEW 8 WT	KFN2 WT
01-005230167-M	C	T	T	het	C	het	T	het	het	het	het	C	C	C	T	C	T	C	het	C	C
01-021004950-M	G	C	C	het	G	G	C	het	het	het	het	G	G	G	het	G	het	G	G	G	G
01-035000687-N	T	C	C	T	T	het	het	het	T	het	het	het	T	T	T	T	het	T	T	het	T
01-058997955-M	G	A	A	G	G	A	het	A	het	het	A	A	G	G	G	G	G	het	het	A	het
01-077241287-M	C	A	A	A	C	A	het	A	het	het	A	A	het	C	C	A	het	het	het	A	A
01-089168561-M	A	G	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
01-102716312-G	G	T	T	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
01-116988763-M	A	C	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
01-134214449-N	G	C	C	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
01-150091142-M	A	G	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
01-162977516-M	G	A	A	G	G	G	G	G	het	G	G	het	G	G	G	G	het	G	G	G	G
01-184035421-M	C	T	T	het	C	C	C	het	het	het	C	het	het	C	C	C	het	het	C	het	het
01-193173300-M	G	A	A	het	het	het	het	het	het	het	G	A	het	het	A	het	A	het	het	A	G
02-008298278-G	G	G	A																		
02-020414303-M	G	C	C	het	C	C	G	C	C	C	C	het	C	het	het	het	G	C	C	het	het
02-038092910-M	C	T	T	het	T	T	het	T	T	T	T	het	T	T	het	C	het	T	T	T	T
02-054004198-M	G	A	A	G	A	het	het	het	G	G	het	G	A	A	het	G	het	G	A	G	A
02-076977189-M	T	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
02-096383071-G	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
02-113220302-M	T	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
02-136425886-G	T	C	C	C	C	C		C	C	C	C	C	C	C	C	C	C	C	C	C	C
02-145828186-N	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
02-161858323-M	C	A	A	A	het	A	A	A	A	A	het	A	het	A	het	het	het	A	A	A	C
02-172943830-M	G	A	A	A	het	A	het	A	het	A	het	het	het	het	het	het	het	het	A	het	A
03-019363137-G	C	T	T	C	het	het	het	C	C	C	het	C	het	het	C	het	C	C	C	C	het
03-033933315-N	T	C	C	T	T	T	T	T	T	T	T	T	T	het	T	T	T	T	T	T	T
03-051281956-M	A	A	C	A	A	het	het	A	A	A	A	A	A	het	A	het	A	A	A	A	het
03-053161686-M	T	C	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
03-071243972-M	A	T	T	A	het	T	T	het	het	T	A	het	T	T	T	het	T	T		het	T

Chromosome-bp	B6	##	DBA	KFD 5 KO	KGJ 1 KO	KFM3 KO	KFW3KO	KFL8 KO	KEY1 KO	KFD9 KO	KFL4 KO	KEY2 KO	KFO2 KO	KGJ2 KO	KFX6 WT	KFO3 WT	KGK2 WT	KEW 7 WT	KGJ 4 WT	KEW 8 WT	KFN2 WT
03-086182591-M	T	G	G	T	het	G	G	het	het	het	T	het	G	G	G	het	G	G	het	het	G
03-099146375-M	A	G	G	A	A	het	het	A	het	A	A	het	het	het	het	A	het	A	A	het	het
03-114333031-N	A	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
03-133183187-M	C	T	T	T	T	T	het	het	het	T	T	T	T	het	T	T	het	het	T	T	T
03-151125653-M	A	T	T	A	het		het	A	A	A	A	het	A	A	A	A	A	A	het	het	A
04-003163167-M	G	T	T	het	het	het	T	het	het	het	het	het	G	het	T	G	T	het	het	het	het
04-017970077-M	A	T	T	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
04-032923355-M	C	T	T	het	C	C	het	het	het	het	C	het	T	C	het	het	het	het	het		C
04-043070534-M	A	G	G	het	A	het	G	G	het	het	het	G	G	A	het	het	het	het	G	G	A
04-058850394-M	C	T	T	C	het	het	het	T	C	C	het	T	het	C	het	T	het	C	T	het	het
04-072075941-M	C	T	T	C	T	het	het	het	het	C	C	C	het	het	het	T	het	het	T	C	
04-088959560-M	C	A	A	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
04-106314110-M	C	A	A	het	C	C	C	C	het	C	C	het	het	C	C	C	het	C	C	C	C
04-122948823-M	A	G	G	het	A	A	A	A	het	A	A	het	het	A	A	A	het	A	A	A	A
04-141084977-M	A	G	G	G	A	het	het	G	G	G	G	G	G	A	G	G	G	G	A	G	G
04-151168886-M	G	T	T	het	het	het	het	het	het	het	T	het	T	G	het	het	T	het	G	G	het
05-015030133-M	G	T	T	G	G	G	G	G	G	G	G	G	G	G	het	G	het	G	G	G	G
05-021194830-M	C	T	T	C	C	C	C	C	C	het	C	C	C	C	het	C	het	C	C	C	C
05-039017415-M	C	A	A	C	C	C	C	C	C	het	C	C	C	C	het	C	het	C	C	C	C
05-065734378-N	A	G	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
05-082050497-M	C	A	A	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
05-094897264-N	T	C	C	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
05-113995253-M	C	T	T	T	C	C	T	T	T	het	T	het	C	C	C	het	het	T	C	het	het
05-142017413-M	A	G	G	G	G	het	G	het	G	G	het	G	A	G	G	G	het	het	G	G	het
06-003167392-M	C	T	T	C	C	C	C	C	C	C	C	C	het	C	C	C	C	C	C	C	het
06-017592278-N	G	T	T	G	het	het	G	G	G	G	G	G	het	G	G	G	G	G	het	G	G
06-032993330-M	C	G	G	C	G	het	C	C	het	het	het	het	het	G	C	C	C	het	G	C	C
06-045126062-M	A	G	G	A	A	het	A	A	het	A	A	het	G	het	A	A	het	het	A	A	het
06-060887613-G	A	G	G		G	A	A	A	A	het	het	het	A	het	A	A	A	het	G	het	A
06-076285738-M	T	A	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T

Chromosome-bp	B6	##	DBA	KFD 5 KO	KGJ 1 KO	KFM3 KO	KFW3KO	KFL8 KO	KEY1 KO	KFD9 KO	KFL4 KO	KEY2 KO	KFO2 KO	KGJ2 KO	KFX6 WT	KFO3 WT	KGK2 WT	KEW 7 WT	KGJ 4 WT	KEW 8 WT	KFN2 WT
06-091998787-M	A	G	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
06-112199886-M	G	T	T	G	G	G	het	G	G	G	G	G	het	G	het	G	het	G	het	G	G
06-122941044-M	T	A	A	T	T	T	het	T	T	T	T	T	het	T	T	T	het	T	het	T	T
06-135955068-M	C	A	A	A	het	het	A	A	het	A	A	het	A	het	het	A	A	A	het	A	A
06-149052281-M	C	G	G	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
07-004201219-N	A	G	G		het	A	het	A	A	A	A	A	het	A	het	het	het	A	A	A	A
07-014982749-M	T	C	C	T	het	T	het	T	T	T	T	T	het	T	het	het	het	T	T	T	T
07-022058953-N	G	A	A	G	het	G	het	G	G	G	G	G	het	G	het	het	het	G	G	G	G
07-036014647-M	G	A	A	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
07-048028246-M	G	T	T	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
07-063201365-M	A	G	G	het	het	het	het	het	A	het	het	het	A	het	G	het	het	het	het	A	A
07-078044642-M	G	C	C	het	het	het	het	het	G	het	G	het	G	G	het	G	G	het	het	G	G
07-093235239-M	A	C	C	A	C	het	het	het	A	A	A	het	A	A	het	A	A	C	het	A	A
07-108090524-M	G	T	T	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
07-122982432-M	T	G	G	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
07-135345950-N	A	G	G	A		A	A	A	A	A	A	A	A	A	A	A		A	A	A	A
08-015199792-M	T	C	C	C	C	C	het	C	C	het	het	C	C	C	het	C	C	C	C	C	C
08-028926710-M	G	A	A	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G		G
08-039133894-M	G	A	A	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
08-054093936-M	C	G	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
08-055226913-M	T	T	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
08-067470102-N	G	C	C	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
08-080610993-N	T	C	C	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
08-098079794-M	G	A	A	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
08-109943429-M	C	T	T	het	C	C	het	C	C	het	C	het	het		het	het	C	C	C	C	C
08-119264649-G	C	T	T	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
09-008979920-M	G	T	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
09-010567705-G	T	T	C	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
09-021337884-G	T	C	C	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
09-034961903-M	G	A	A	G	G	G	G	G	G	G	G	G	G	G		G	G	G	G	G	G
09-052052234-M	T	C	C	T	het	T	T	het	het	T	T	het	T	T	T	T	T	T	het	het	T
09-065820797-M	A	G	G	het	het	G	A	G	het	het	het	het	G	het	A	G	A	A	het	het	G
09-082788952-M	C	T	T	het	C	het	het	T	het	C	het	het	T	het	C	T	C	C	C	het	T
09-098865563-M	T	C	C	het	T	het	het	C	het	T	het	het	C	het	T	C	T	T	T	het	C
09-110057634-M	A	G	G	A	A	het	het	het	het	A	A	het	A	het	het	A	A	A	het	A	A
10-006360048-M	G	A	A	G	G	G	G	G	G	G	G	het	G	het	het	G	A	het	het	het	G
10-015140823-M	G	C	C	G	G	G	G	G	G	G	G	het	G	G	het	G	het	het	G	het	G
10-034328321-G	C	A	A	het	A	A	A	het	A	C	het	A	het	A	A	A	A	A	A	A	het
10-053898997-M	G	C	C	G	het	C	C	het	C	G	het	C	het	C	G	C	C	C	C	C	het
10-067317776-G	A	G	G	het	het	het	A	A	G	het	A	G	A	A	A	A	G	G		G	A
10-072471838-G	A	G	G	A	A	het	A	A	A	A	A	A	A	A	A	A	het	A	A	A	A
10-093476458-N	T	C	C	T	het	het	T	T	T	T	T	T	T	T	T	T	het	T	het	T	T
10-108166251-G	T	C	C	het	het	het	T	T	het	het	T	het	het	T	T	het	T	het	het	het	C
10-118769726-M	C	A	A	C	C	het	C	A	het	C	het	het	het	het	A	het	het	het	A	het	het
11-004367508-M	G	A	A	G	G	G	het	G	G	het	G	G	G	G	het	G	G	G	G	G	G
11-019956728-M	A	G	G	A	A	A	het	A	A	het	A	A	A	A	het	A	het	A	A	A	A
11-024515722-M	G	A	A	G	G	G	het	G	G	het	G	G	G	G	het	G	het	G	G	G	G
11-044333996-M	A	G	G	G	het	A	het	het	G	het	het	het	het	het	G	G	G	het	het	het	het
11-059502265-N	T	C	C	het	T	T	het	T	het	het	T	T	T	T	het	T	het	het	T	T	T
11-076223449-M	T	C	C	T	T	T	T	T	het	T	T	T	T	T	T	T	T	T	T	T	T
11-091287860-M	A	G	G	A	A	A	het	A	het	A	A	het	A	A	A	A	het	A	A	A	A
11-107678903-M	G	T	T	het	T		het	T	het	G	T	het	T	T	het	het	het	G	T	het	het
11-118804416-N	G	A	A	G	het	G	G	A	het	G	het	het	het	het	het	het	het	het	het	A	het
12-011234798-M	A	G	G	het	het	het	het	G	het	A	G	het	G	het	het	G	A	G	G	G	het
12-019201035-M	G	A	A	het	A	A	A	A	het	G	A	het	A	A	het	A	G	A	A	A	het
12-039674614-M	G	A	A	A	A	A	het	A	A	A	A	A	A	A	het	A	het	A	A	A	A
12-054303300-M	C	A	A	het	C	C	C	het	A	het	A	A	C	C	het	C	het	het	C	het	het
12-070670478-M	A	G	G	het	het	het	het	A	het	het	het	het	G	het	G	het	G	G	het	G	G
12-084289638-M	C	A	A	het	het	het	A	C	het	het	A	het	A	het	A	het	A	A	het	A	A
12-099072867-M	T	C	C	het	C	het	C	T	T	T	het	T	T	C	het	T	C	het	C	het	T
12-113315003-M	C	G	G	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
13-015609597-M	A	G	G	A	A	A	A	A	het	A	het	het	A	A	A	A	het	A	A	het	A
13-030913320-M	T	C	C		C	het	C	C	het	C	C	het	het	C	T	het	het	het	C	het	het

Chromosome-bp	B6	##	DBA	KFD 5 KO	KGJ 1 KO	KFM3 KO	KFW3KO	KFL8 KO	KEY1 KO	KFD9 KO	KFL4 KO	KEY2 KO	KFO2 KO	KGJ2 KO	KFX6 WT	KFO3 WT	KGK2 WT	KEW 7 WT	KGJ 4 WT	KEW 8 WT	KFN2 WT
13-046957715-M	A	G	G	G	het	A	het	G	het	G	A	A	het	het	A	A	A	het	G	het	het
13-065339791-N	C	T	T		het	C	C	C	C	het	C	C	C	C	C	C	C	C	het	C	C
13-077818021-M	T	A	A																		
13-091042598-M	G	A	A	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
13-107993079-M	A	G	G	G	het	het	A	het	A	het	het	het	A	A	het	A	A	A	het	A	het
13-117094028-M	G	A	A	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
14-005055006-M	T	A	A	T	T	T	het	het	het	T	T	het	T	het	A	T	A	A	A	het	T
14-017693796-G	G	A	A	G	G	G	het	het	het	G	G	het	G	het	A	G	A	A	A	het	G
14-037395207-M	T	G	G	het	T	T	het	het	het	het	T	het	G	het	G	het	G	het	G	het	T
14-054014360-M	C	G	G		het	het	het	het	het	het	het	het	G	het	G	G	G	het	G	het	het
14-068974190-M	C	G	G	G	het	G	het	G	het	G	het	het	G	het	G	G	G	G	G	het	het
14-072865727-G	C	C	T	het	het	het	het	het	het	het	het	het	T	het	T	T	T	T	T	het	het
14-074468555-M	G	A	G	het	G	het	het	het	het	het	G	het	G	G	G	G	G	G	G	het	het
14-095233920-G	T	C	C																		
14-108203728-M	C	T	T	het	het	het	T	het	het	het	het	het	T	het	C	T	T	het	het	het	het
15-003094890-M	A	G	G	het	het	G	het	G	het	G	G	G	G	G	het	G	G	het	het	G	G
15-020953071-M	T	C	C	het	het	C	het	C	C	C	C	C	C	C	T	C	het	C	het	C	C
15-033125499-M	A	G	G		het	G	het	G	G	G	G	G	G	G	A	G	het	G	het	G	G
15-052940678-M	A	T	T																		
15-071604153-N	G	T	T		het	G	G	het	G	G	het	het	het	G	G	het	T	G	het	het	het
15-086076093-M	C	G	G	C	C	het	C	het	C	C	het	C	het	C	C	het	G	C	C	C	het
15-096231715-M	T	G	G	T	T	het	T	het	T	het	T	T	het	T	T	het	G	T	T	T	het
16-005053446-C	G	T	T		G	G	G	G	G	het	G	G	G	G	het	G	het	G	G	G	G
16-019621494-C	A	G	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
16-037994236-C	G	A	A	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
16-055156188-G	G	A	A	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
16-070833511-G	C	T	T	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
16-084296159-G	T	G	G	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
16-091028945-C	C	G	G	het	C	C	C	C	het	het	het	het	C	C	G	C	het	het	C	het	C
17-007230139-M	C	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
17-023157746-M	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
17-039842179-N	T	C	C	het	C	T	C	het	het	T	T	het	T	het	C	T	het	het	het	het	T
17-052179489-M	A	G	G	het	G	A	G	het	het	A	het	het	A	G	het	A	het	G	het	G	A
17-068191393-M	T	C	C	C	C	T	C	C	C	T	C	C	T	C	T	T	het	C	het	C	T
17-086110517-N	G	A	A	het	het	G	het	het	het	G	het	het	G	A	A	G	A	A	het	A	het
17-092673068-N	T	G	G	het	het	T	T	T	het	T	het	het	T	het	G	T	het	het	het	het	T
18-014248154-G	T	C	C	T	het	T	C	het	het	C	het	T	T	het	T	T	het		het	T	T
18-033822734-M	A	G	G	A	het	G	G	G	G	G	G	het	het	G	A	G	het	het	G	G	G
18-047863056-M	A	T	T	T	het	T	T	het	T	T	T	het	T	T	het	T	T	het	T	T	T
18-065947946-M	T	G	G	het	het	T	het	T	T	het	T	T	het	T	T	het	T	T	T	T	het
18-075832307-M	A	C	C	het	het	A	het	A	A	het	A	A	C	A	A	C	A	A	A	A	C
18-086980249-M	G	G	A	het	G	G	A	G	G	G	het	G	A	G	het	het	A	G	G	G	het
18-088017394-M	C	T	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
19-020257763-M	C	T	T	C	C	C	het	T	C	C	het	het	T	het	het	het	het	T	C	C	het
19-031809029-G	G	A	A	G	G	G	het	het	G	G	het	het	A	G	G	het	G	het	G	G	het
19-054115869-M	G	C	C	C	G	G	het	het	het	het	het	het	het	G	G	het	het	het	G	het	het
19-060030696-N	A	G	G	G	het	het	het	het	G	G	het	G	het	het	G	G	G	het	het	G	G
X-016114535-G	C	A	A	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
X-025132696-G	C	G	G	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
X-041569348-G	C	A	A	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
X-055512473-M	T	C	C	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
X-071927400-G	A	T	T	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
X-091976871-G	C	T	T	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
X-109677875-M	A	G	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
X-115589667-M	C	A	A	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
X-133535206-G	T	C	C	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
X-143466659-M	A	C	C	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A

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