Regulation of gene expression in the embryonic pancreas by Oc1 and its impact on postnatal function

By

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CHAPTER I

INTRODUCTION

Adapted from Kropp and Gannon, “Onecut transcription factors in development and disease”,

Trends in Developmental Biology, 2016

General Background

Significance

Pancreatic diseases encompass a diverse array of conditions. As will be discussed in detail later, the most significant pancreatic diseases are arguably Type 2 diabetes (T2D) and pancreatic cancer. T2D, a disease characterized by dysregulated blood glucose levels, affects over 9% of the adult population in the United States and accounts for over $250 billion in health care costs (Centers for Disease Control and Prevention, National Diabetes Statistics Report, 2017). While these numbers are already staggering, the incidence rate of T2D only continues to increase. Another serious disease of the pancreas is pancreatic cancer, the most common form of which is pancreatic ductal adenocarcinoma (PDAC). Although PDAC accounts for <3% of new cancer cases per year in the United states, it accounts for approximately 14% of cancer deaths annually (American Cancer Society, Cancer Facts & Figures, 2017). Indeed, the 5-year survival for PDAC is only 8% which clearly demonstrates the severity and tragedy of this disease. While both of these diseases are generally considered to develop in adults, it has become appreciated that adult diseases can be rooted in alterations (e.g. genetic mutations) in the development of the afflicted tissue. The concept that developmental processes impact adult disease predisposition is analogous to the Developmental Origins of Health and Disease

(DOHaD) hypothesis which states that the environmental conditions and processes occurring *in utero* can have lasting impacts on the cells, tissues, organs, and individuals. Thus, the importance of development and aberrations in organ development cannot be overstated when considering adult diseases.

Pancreas Development

The pancreas is both an endocrine and exocrine organ with dual roles in regulation of blood glucose homeostasis and production of digestive enzymes. The endocrine compartment, composed of the islets of Langerhans, makes up 2% of the adult pancreas by mass and is responsible for sensing blood glucose levels and secreting endocrine hormones to maintain glucose homeostasis. The exocrine compartment constitutes the remaining 98% of pancreatic mass and is predominantly composed of the digestive enzyme-secreting acinar cells as well as the pancreatic ducts, which transport those enzymes to the rostral duodenum. All pancreatic cell types are specified from endodermally-derived multipotent pancreatic progenitor cells (MPCs) during development. Establishment of these MPCs and their subsequent stepwise differentiation to mature cells is a highly regulated process reliant on transcription factor cascades and complex regulatory relationships. Some of these relationships and the regulatory cascades in which they reside will be detailed below.

Pancreas Specification

The pancreas is specified from the definitive posterior foregut endoderm (at approximately e8.5 in the mouse) (Figure 1-1), with the dorsal pancreatic bud emerging first.

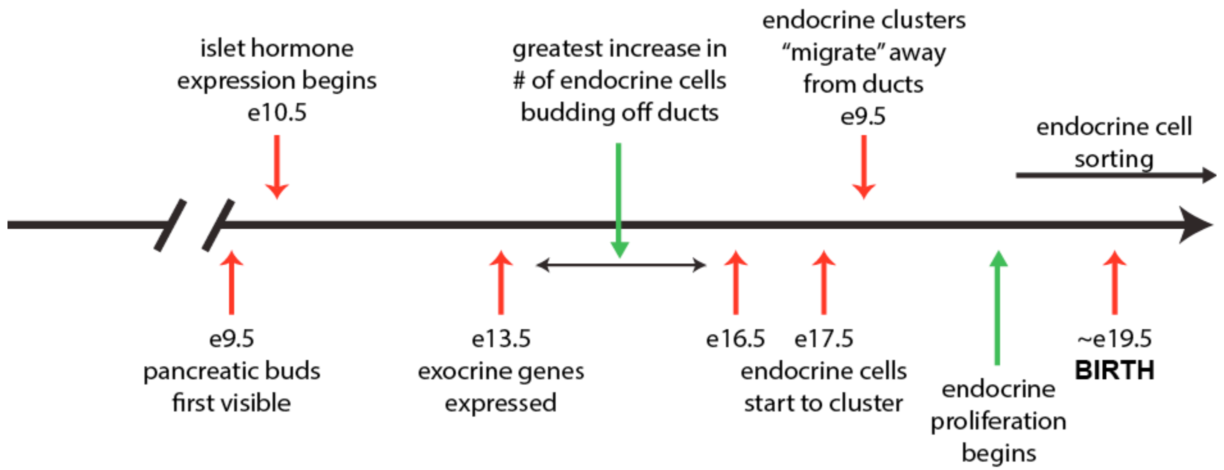

Figure 1-1: Timeline of murine pancreas development. In mice, the pancreatic buds become visible at embryonic day (e)9.5. The first wave of endocrine differentiation occurs between ~e10.5 and ~e12.5. The second wave of endocrine differentiation occurs between ~e13.5 and ~e16.5. After the second wave of endocrine differentiation the endocrine compartment the endocrine cells migrate away from the trunk epithelium, proliferate, and begin to cluster into islets of Langerhans.

Both inductive and permissive signals from the adjacent paraxial mesoderm and notochord, respectively, specify the dorsal pancreatic region of the foregut endoderm. These signals include Fibroblast growth factors (Fgfs), bone morphogenic proteins (BMPs), retinoic acid (RA), and Activin B2 which together create a permissive environment through suppression of Sonic Hedgehog (Shh) and active induction of pancreas specification (reviewed in (1)) (Figure 1-2A). Approximately 12 hours after specification of the dorsal pancreatic bud, the ventral pancreatic bud is specified from a small region of the hepatobiliary biliary bud by inductive signals from the lateral plate mesoderm (2). For both the dorsal and ventral buds, these cues induce thickening of the primitive gut tube and evagination to form the pancreatic buds. An essential part of pancreatic bud evagination is increased proliferation of the pancreatic progenitors due to proliferative stimuli (especially Fgf10) from the overlying pancreatic mesenchyme (3,4). Not only does the mesenchyme provide proliferative signals, but it also provides the signals for early definition of domains within the pancreatic buds. As will be covered in more detail below, the distal cells of the buds receive a higher concentration of mesenchymally-derived Fgfs, and follistatin that begin to specify a "tip" domain while the more central cells become a "trunk" domain. Each of these domains becomes biased to either an acinar or duct/endocrine cell fate. Prior to the establishment of these domains, the cells of both pancreatic buds are considered MPCs due to their competency to give rise to all of the pancreatic cell types. MPCs are characterized molecularly by expression of regulatory transcription factors such as *Pancreatic and duodenal homeobox 1* (*Pdx1*), *Pancreas transcription factor 1a* (*Ptf1a*), *Onecut 1* (*Oc1*, also known as *Hepatic nuclear factor 6*, *Hnf6*), and *Hepatic nuclear factor 1β* (*Hnf1β)* (Figure 1-2B). These transcription factors operate at the top of a regulatory hierarchy that establishes the

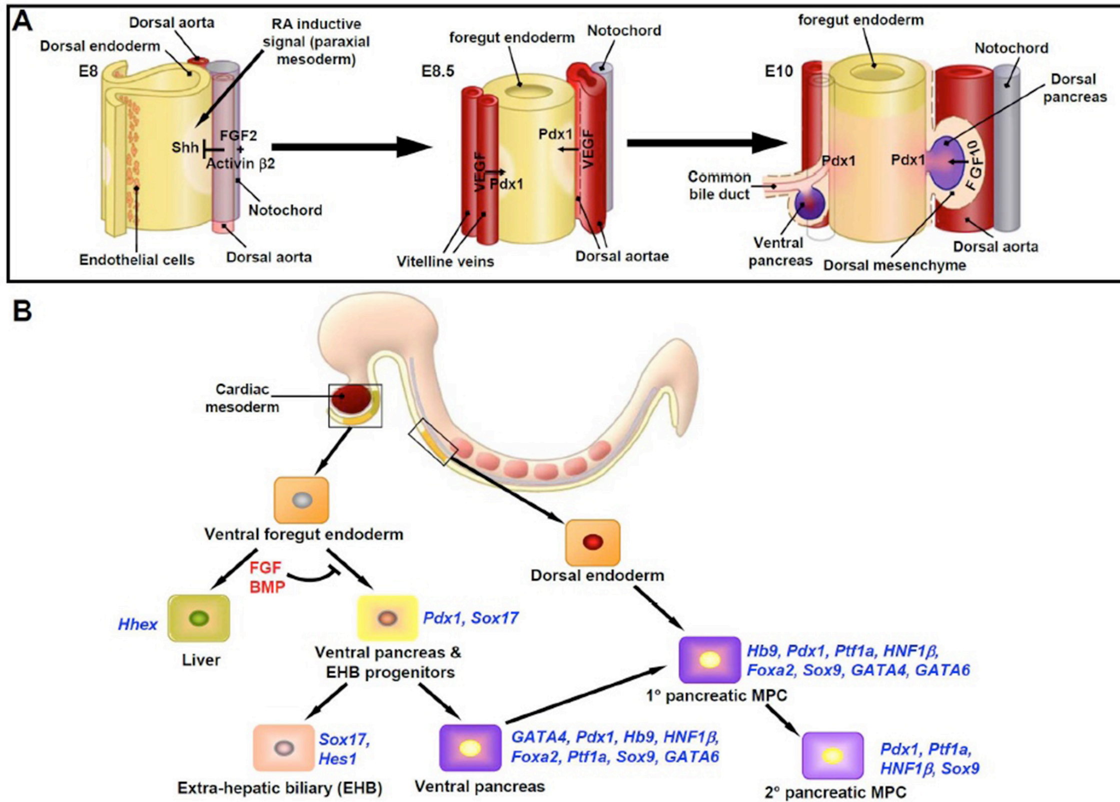

Figure 1-2: Early foregut endoderm patterning: signaling from adjacent mesodermal derivatives establishes the pancreatic domain. A: The early foregut endoderm is patterned by dynamic, distinct sets of permissive signals from the surrounding mesodermal derivatives. At E8, inductive signals (RA) from the paraxial mesoderm, together with suppression of Shh in the dorsal endoderm by FGF2 and Activinb2 from the notochord, are required to establish the dorsal pre-pancreatic domain (left). At E8.5, dorsal aortae fusion pushes notochord away from dorsal endoderm. VEGF from dorsal aortae and vitelline veins (and even their earlier precursors, as shown in the ventral open foregut region, left) induce Pdx1 and Ptf1a expression in pre-pancreatic endoderm (middle). At E10, mesenchyme condenses around the budding pancreatic anlagen. FGF10 stimulates bud outgrowth and proliferation of the pancreatic progenitor pool (right panel). B: Sagittal view of an embryo at ~E8 showing the specified dorsal and ventral pre-pancreatic endoderm (orange, top panel). Distinct sets of transcriptional regulators (blue text) and signaling molecules (red) control liver, ventral pancreas, and extrahepatic biliary (EHB) specification from common progenitors in the ventral foregut endoderm. Despite the dorsal and ventral pancreas sharing presumably highly similar genetic programs for cell differentiation, the gene activation cascade is different between these two anlagen, as depicted by the sequence of the listed transcription factors. Several of the primary (1) pancreatic mutipotent progenitor cells (MPC)-specific transcription factors are maintained in secondary (2) MPC. Figure modified and reprinted with permission from Pan and Wright, 2011 (5).

competency for MPC generation and maintains the pancreatic fate. The above-mentioned transcription factors, in addition to others acquired shortly after pancreas specification, operate within a co-regulatory network to promote pancreas specification, but are also dependent on each other for activation. Aberrations in expression of one, or many of these factors can result in impaired pancreas development or pancreatic agenesis. For example, Pdx1 is absolutely critical for pancreas development and in its absence pancreatic agenesis occurs (6-9). Likewise, inactivation of Ptf1a results in near complete pancreatic agenesis, however, a severely hypoplastic dorsal pancreatic bud remains (10,11). Ptf1a binds to and activates both the *Pdx1* and *Oc1* promoters thereby promoting the pancreatic MPC identity (12-14). Oc1 is necessary for timely activation of Pdx1 as evidenced by delayed expression of Pdx1 in the absence of Oc1 (15). This delayed Pdx1 expression contributes to the hypoplastic pancreatic epithelium observed as a result Oc1 inactivation (15,16). Together, these transcription factors contribute to the greater gene regulatory network that is essential for pancreas specification. Work from multiple groups has clarified this network and provided insight into the complicated nature of regulation of pancreas development. More information specific to Pdx1 and Oc1 is detailed below.

Pancreas Morphogenesis

As the pancreatic MPCs continue to undergo proliferation and evagination, higher order structure develops in the form of a stratified epithelium then giving way to epithelial tubules with central micro-lumens (Figure 1-3). The formation of these micro-lumens is dependent upon polarization of the MPCs, a process governed by Stromal cell-Derived Factor (SDF-1) produced in the mesenchyme and its receptor, CXCR4 which is expressed in the epithelium

(17,18). Only the innermost cells have apical polarity whereas cells on the outer edges of the buds have basal polarity (Figure 1-3). By ~e10.5 the pancreatic epithelium exists as a plexus of tubules that are not coordinated or necessarily connected, but by ~e13.5, micro-lumen fusion begins to occur resulting in condensation of tubules and a more distinct ductal tree (Figure 1-4) (17-19). The process of tubule condensation is in part regulated by signaling through ephrinB/EphB signaling in the pancreatic epithelium (18,20).The resulting structure, polarized cells surrounding a central lumen, defines the epithelial ultrastructure that persists throughout the branching morphogenetic process (covered in more detail below). Further, the condensation of the ductal tree has the additional consequence of establishing specific niches and domains that give rise to different cell types of the mature pancreas (19,21). These are the aforementioned tip and trunk domains that are biased to particular cell fates (acinar, duct, or endocrine) (Figure 1-5). The establishment of these domains is also an important aspect of the branching morphogenetic process that further develops the ductal tree with terminal acinar cell rosettes.

As the pancreatic buds expand, multiple morphogenetic processes act simultaneously resulting in a single organ with the ductal tree and lobular acinar rosettes that define mature pancreas ultrastructure (Figure 1-6). The progressive condensation of the ductal micro-lumens (see above) is the first process to affect pancreas morphogenesis. Separately, gut rotation at approximately e12.5 in the mouse brings the two pancreatic buds together. They eventually fuse resulting in a single organ (reviewed in (5)). While bud fusion is the most overt morphogenetic change, branching morphogenesis also begins ~e12.5, the process that defines pancreas ultrastructure both throughout development and in the adult organ.

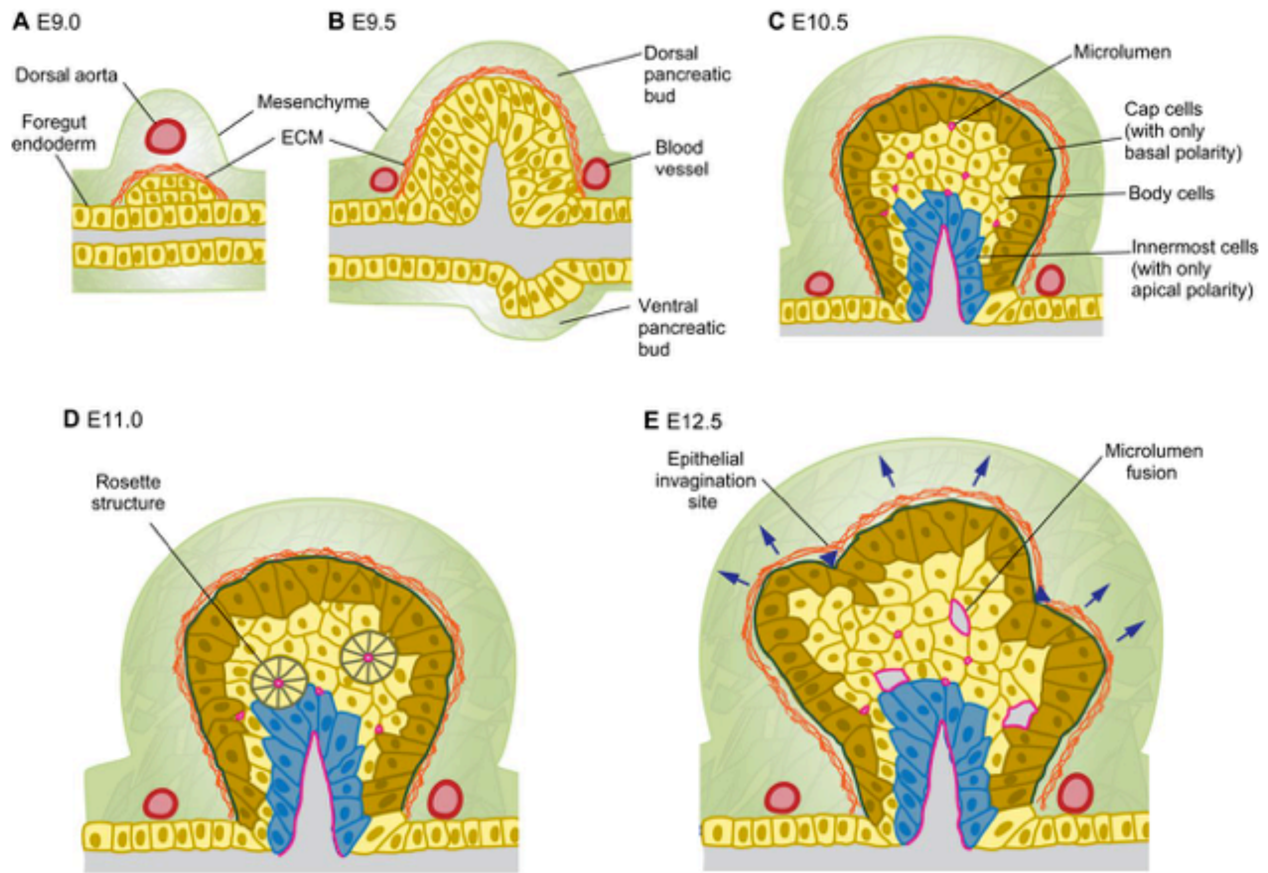

Figure 1-3: The early stages of pancreas development. (A) Evagination of the foregut endoderm drives the formation of the dorsal pancreatic bud. (B) While the dorsal pancreatic bud expands due to multipotent progenitor cell (MPC) proliferation, the ventral pancreatic bud emerges from the ventral foregut endoderm. (C) Segregation of the pancreatic buds gives rise to innermost cells that exhibit only apical polarity, inner 'body' cells that are non-polarized, and outer 'cap' cells that exhibit only basal polarity. For simplicity, only the dorsal bud is shown. (D) The establishment of cell polarity in individual body cells produces microlumen and rosette structures. (E) Microlumen fusion and tubulogenesis coincide with the initiation of branching morphogenesis, which involves epithelial invagination (blue arrowhead) and outgrowth (blue arrows). Figure modified and reprinted with permission from Bastidas-Ponce *et al*, 2017 (22).

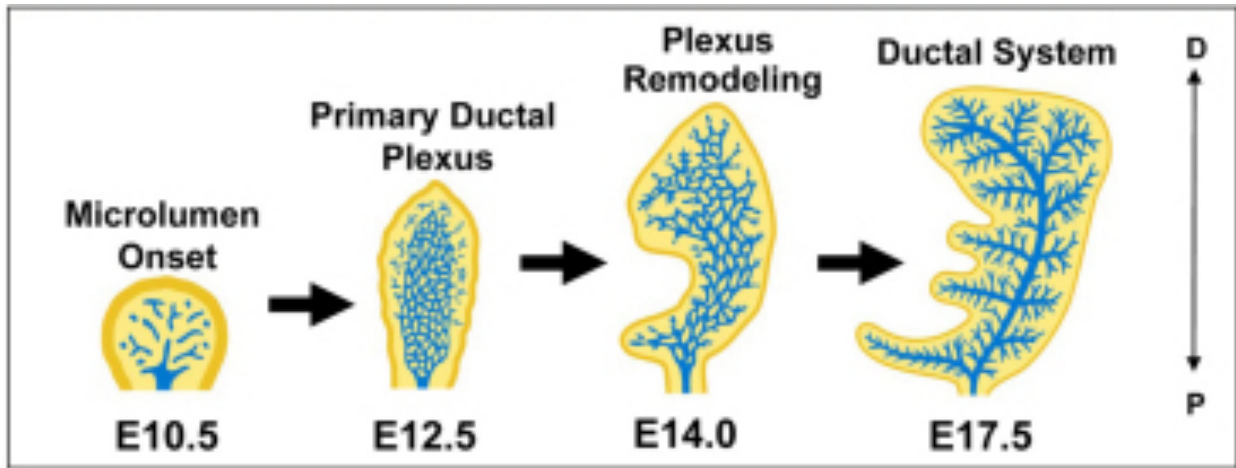

Figure 1-4: Model of pancreas ductal morphogenesis. Cartoon model of lumen network formation and remodeling into a tree-like ductal system. D: distal; P: proximal. Figure modified and reprinted with permission from Villasenor *et al*, 2010 (18).

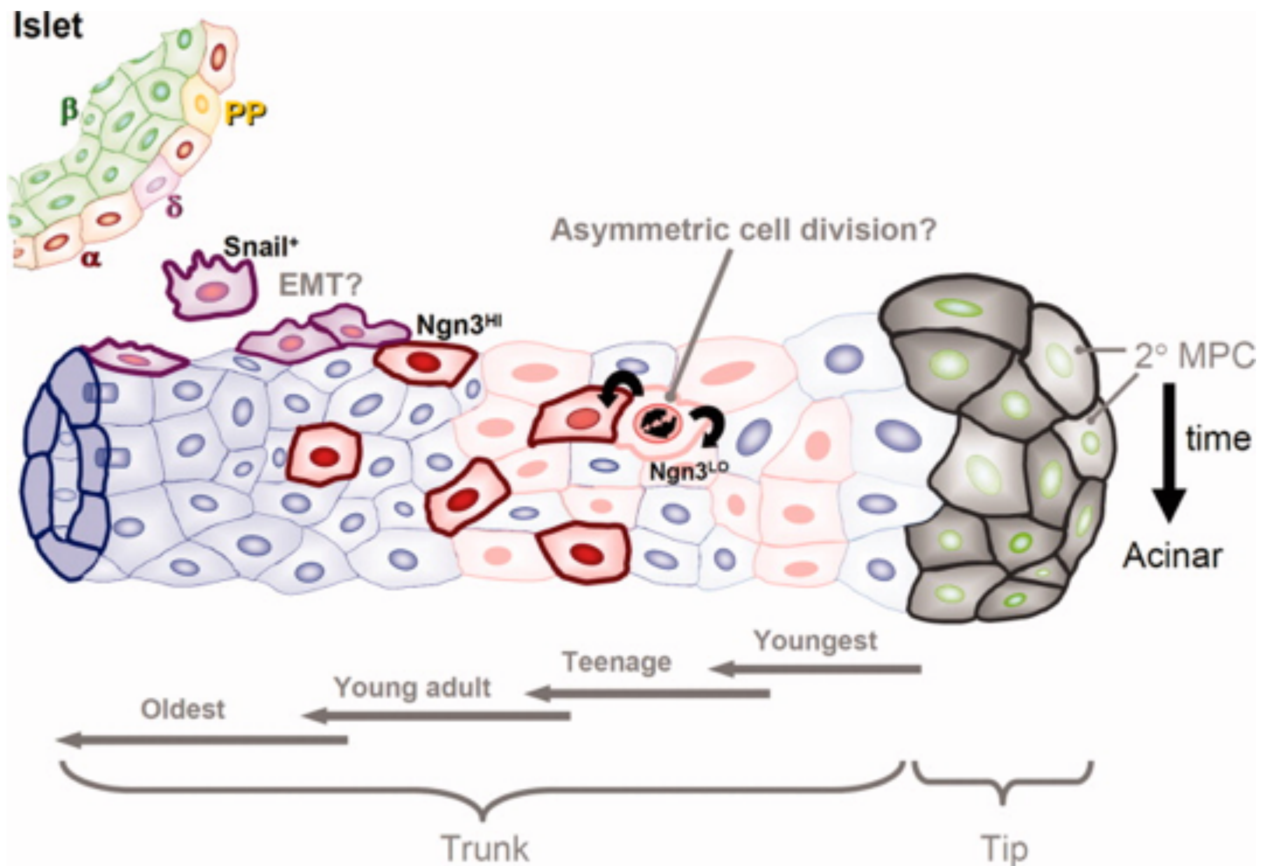

Figure 1-5: Model of epithelial organization during the secondary transition. A single piece of tip- region epithelium from the secondary transition (~E13.5) is depicted, consisting at this stage of two major domains: an MPC-containing tip domain (grey), and a trunk region harboring the endocrine/duct bipotential progenitor pool (duct progenitors are shaded blue). The trunk domain is subdivided with respect to their age after birth from tip MPC. Trunk cells nearest the tip are youngest with older cells moving back down the trunk (teenage through oldest). Scattered cells within the trunk epithelium activate Ngn3 expression, with Ngn3^{LO} cells (light pink) representing a putative metastable, relative plastic, uncommitted but endocrine-biased mitotic state. Ngn3^{LO} asymmetric cell division leads to one daughter having higher Ngn3 expression (Ngn3^{HI}: darker nucleus, red border) that becomes endocrine-committed, leaving a Ngn3^{LO} progenitor available for more rounds of endocrine birth via production of additional Ngn3^{HI} daughters. Committed Ngn3^{HI} endocrine precursors rapidly activate Snail2 (purple cells) and escape the trunk epithelium, probably via epithelial-mesenchymal transition (EMT), before clustering to form the endocrine islets of Langerhans. Figure modified and reprinted with permission from Pan and Wright, 2011 (5).

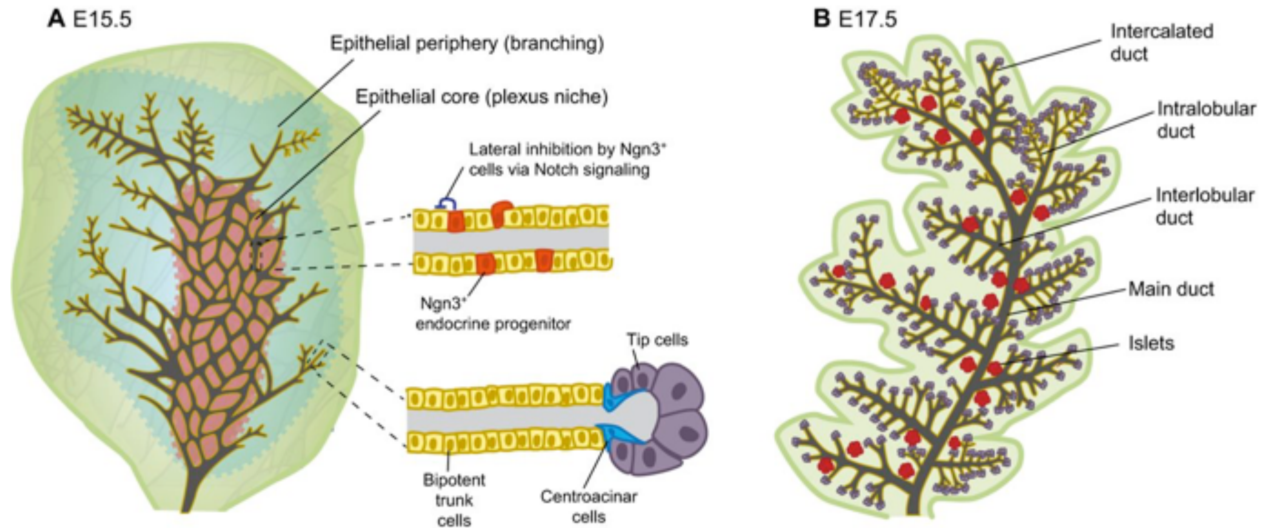

Figure 1-6: Branching morphogenesis during pancreas development. (A) At E15.5, the pancreatic epithelium consists of a central plexus (red) that serves as a niche, maintaining and harboring endocrine progenitors. Within this niche, Ngn3-expressing progenitors prohibit the differentiation of neighboring cells through lateral inhibition in a Notch-dependent manner and thereby allow epithelial expansion. The central plexus is surrounded by an epithelial periphery (blue) in which the epithelium remodels into epithelial branches, which contain bipotent trunk cells, centroacinar cells and tip cells. (B) Remodeling of the plexus gives rise (by ~E17.5) to the formation of the main duct, which branches into interlobular ducts that are connected to acini via intralobular and intercalated ducts. At this stage, the remodeled plexus also contains developing islets. Figure modified and reprinted with permission from Bastidas-Ponce *et al*, 2017 (22).

In brief, branching morphogenesis is the processes of tubule outgrowth following the condensation of the epithelial plexus prior to e12.5. Pancreatic branching morphogenesis is somewhat different from other glandular organs in two important ways: 1) the epithelial plexus exists prior to formation of a central lumen, and 2) the process is not simply the extension and branching of a single tube with terminal bifurcations, but rather lateral branching with buds coming off of one side of the growing tip (Figure 1-7) (23). As mentioned above, the overlying pancreatic mesenchyme provides permissive signals (e.g. Fgf and the activin inhibitor follistatin), for branching and outgrowth. Early work to understand this process demonstrated that the signals were indeed permissive and not instructive since replacement of pancreatic mesenchyme with salivary mesenchyme was sufficient for branching morphogenesis to take place (24). The concentration of these mesenchymally-derived factors plays a significant role in determining cell fate seeing as those cells closest to the mesenchyme become the tip acinar-biased tip domain (25,26). Thus, the mesenchyme plays a central role in providing the signals that not only induce proliferation and expansion but also create the distinct tip and trunk domains that become increasingly restricted to either an acinar or duct/endocrine fate (Figure 1-5).

A more detailed explanation of differentiation of the different pancreatic cell types is below, but it is important at this point to cover how spatial restriction of cell fate is established. The high concentration of mesenchymally-derived Fgfs and follistatin establish the acinar-biased tip domain (25,26). Additionally, TGF-β signaling early in the morphogenesis process promotes tip/acinar fates rather than trunk/duct and endocrine fates (27). Further, potentiation of Notch signaling by the pancreatic mesenchyme has a role in determining

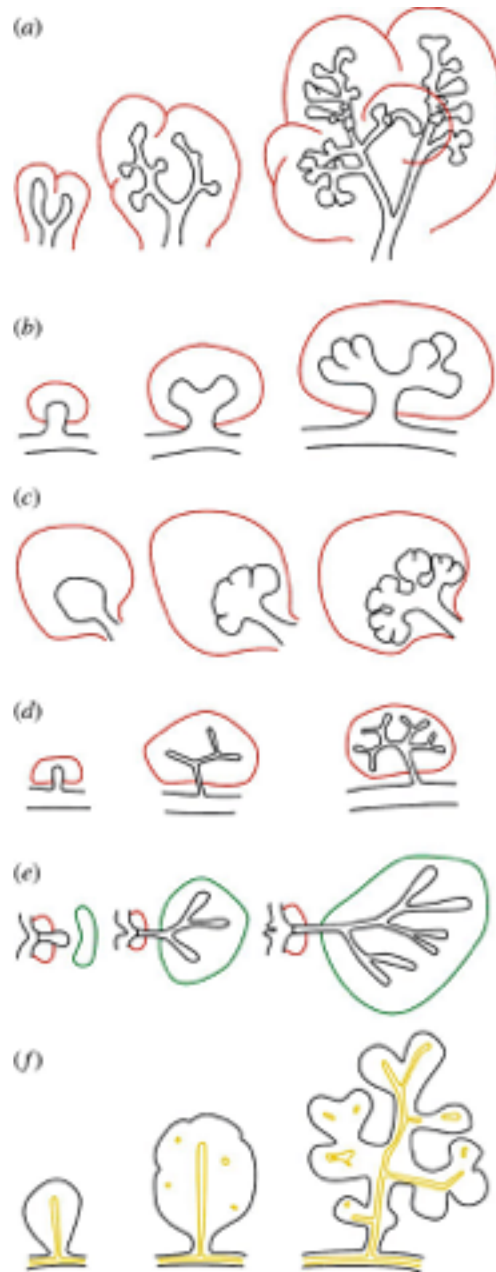

Figure 1-7: Branching morphogenesis. Typical branching pattern over develop- mental time in the (a) lung, (b) ureteric bud, (c) salivary gland, (d) prostate, (e) mammary gland and (f) pancreas. The epithelium is shown in black, the mesenchyme in red, the fat pad in the mammary gland in green and the lumen in the pancreas in yellow. Figure modified and reprinted with permission from Iber and Menshykau, 2013 (28).

tip/trunk fate. Contact between the mesenchyme and tip cells enhances Notch signaling in that domain and prevents expression of the pro-endocrine transcription factor *Neurogenin3* (discussed below) (29). The trunk cells, which are bipotent and can give rise to either endocrine cells or ductal cells, are regulated by different pathways (Figures 1-5,6) (19,21). Activin (which is inhibited by follistatin) and RA have significant roles in biasing trunk epithelial cells toward duct or endocrine fates (30-33). RA in particular has a significant role in determining tip/trunk fates and has the ability to induce endocrine and duct differentiation (34-36). Together, these findings demonstrate that a highly coordinated process of signaling between the epithelium and the mesenchyme is necessary to drive the branching morphogenetic process and establishment of niches capable of giving rise to specific adult cell types.

Endocrine Specification

As noted above, the endocrine pancreas is responsible for regulation of blood glucose. Within the pancreas are the micro-organs known as the islets of Langerhans which contain 5 endocrine cell types. The most abundant endocrine cells (accounting for ~90% of islet cells) are the insulin-secreting β cells and glucagon-secreting α cells, which function to lower or raise blood glucose levels, respectively. Additionally, there are somatostatin-secreting δ cells, ghrelin-secreting ε cells, and pancreatic polypeptide-secreting PP cells. While these 3 cell types have historically garnered less attention than α and β cells, δ cells have recently become a focal point of research thanks to the role of somatostatin as a negative regulator of both insulin and glucagon secretion. Together, the pancreatic endocrine cells work in concert to balance glucose uptake and production in peripheral tissues thereby maintaining glucose homeostasis.

During pancreas development, there are two waves of endocrine differentiation. The first wave takes place between e10.5-e13.5 while the pancreas is still largely a plexus of tubules and this wave predominantly gives rise to glucagon-expressing cells, the fate of which is unclear (37-41). The exact mechanisms that promote differentiation of these glucagon+ cells remain undefined and it is unlikely that these cells contribute in a meaningful way to adult islets. Instead, the vast majority of endocrine cells are specified and differentiate during the second wave of endocrine differentiation. The second wave of endocrine differentiation begins at approximately e13.5 and lasts until approximately e16.5. This process is far better defined than the first wave of endocrine differentiation with respect to transcriptional regulation and inductive cues. All cells that are specified to the endocrine lineage come from bipotent trunk epithelial cells (Figure 1-5,6) (19,21). The trunk cells are marked by expression of transcription factors such as Pdx1, Oc1, Hnf1β, Sox9 (SRY box 9), and others. From this pool of bipotent cells, endocrine progenitors are specified by initiation of expression of the transcription factor *Neurogenin3* (*Neurog3*, aka *Ngn3*) (42). *Neurog3* is both necessary and sufficient for initiation of endocrine specification and it is thus one of the most studied factors in pancreas development.

Neurog3 is arguably the most important transcription factor in endocrine specification and differentiation. It is absolutely essential for promoting the endocrine differentiation program as inactivation of *Neurog3* results in a complete absence of endocrine cells (43). It is also sufficient for initiating the endocrine specification program as transgenic over-expression of *Neurog3* results in significantly increased commitment of MPCs to the endocrine lineage. *Neurog3* expression must be regulated in both a spatial and temporal manner for proper

specification of the different endocrine cell types (44,45). An add-back approach in which transgenic *Neurog3* expression was induced at different time points in a *Neurog3*-null background demonstrated that early initiation (prior to e10.5) of *Neurog3* results in almost exclusive differentiation of α cells. Following e10.5, *Neurog3* expression predominantly generates β cells, and comparatively late expression (e12.5 and later) results in δ and PP cells (Figure 1-8) (46). These data paired with clonal analysis (47) suggests that Neurog3+ endocrine progenitors are unipotent and committed to a specific fate following induction of *Neurog3* expression. Following *Neurog3* induction, the resulting endocrine progenitor cells delaminate from the trunk epithelium and continue down the endocrine differentiation pathway to becoming a hormone-positive cell. This delamination likely requires an epithelial-to-mesenchymal transition although the delaminating cells never fully lose their epithelial characteristics (Figure 1-5) (48). The endocrine progenitors begin to form endocrine chords, strings of endocrine committed cells, that migrate away from the trunk epithelium and eventually coalesce into the islets of Langerhans (49).

While the canonical view of endocrine specification (described above) is that once a cell expresses *Neurog3* it becomes irreversibly committed to the endocrine lineage, recent evidence suggests otherwise. Our group has demonstrated that inactivation of Oc1 in *Neurog3+* endocrine progenitors results in reversion of some of those cells to an exocrine cell fate thereby demonstrating that their fate commitment is not irreversible (Figure 1-9) (16). Further, utilization of increasingly sensitive techniques has demonstrated that *Neurog3* expression is not binary and rather exists as gradient with cells tending to be either *Neurog3*^{Hi} or *Neurog3*^{Lo} (50). It appears that only those cells consistently maintaining *Neurog3*^{Hi} expression become fully

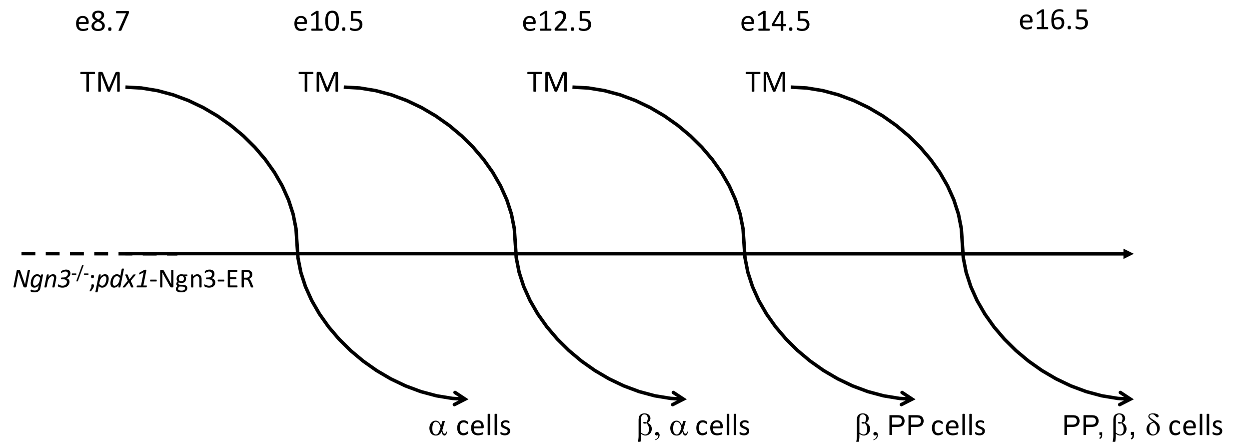

Figure 1-8: Temporally-dependent specification of Ngn3-expressing endocrine progenitors. Using the Pdx1 promoter to drive expression of a tamoxifen (TM)-inducible Ngn3-estrogen receptor (ER) fusion protein, Ngn3 expression was restored to Ngn3 null mutant embryos at different developmental time points with the addition of TM. Examination of endocrine differentiation 2 days after a single TM injection revealed that endocrine progenitors preferentially differentiate as a particular hormone-producing cell depending on when during development they were generated. α cells are formed from the earliest Ngn3-producing cells, whileβ, PP, and d cells subsequently form, in that order. Figure modified and reprinted with permission from Guney and Gannon, 2009 (51).

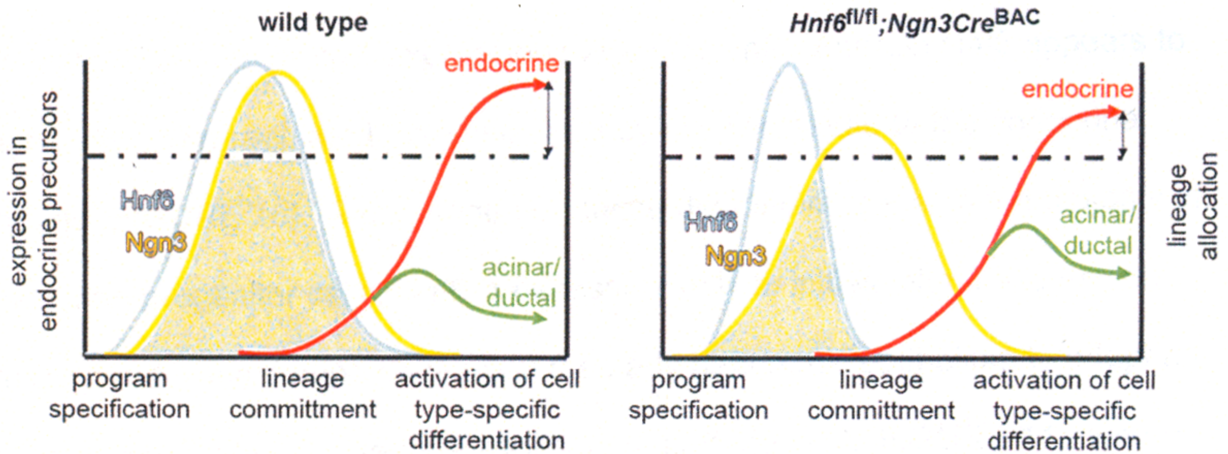

Figure 1-9: A threshold of Ngn3 is required to generate fully committed endocrine cells. Inactivation of Oc1 (Hnf6) subsequent to Ngn3 gene activation ($Hnf6^{fl/fl}$; Ngn3CreBAC) results in a reduced number of differentiated endocrine cells. In our model, in wild type mice (left graph), Hnf6 expression (light blue) precedes Ngn3 expression (yellow). Hnf6 expression must be maintained for a certain period of time to allow Ngn3 expression to exceed a critical threshold (dotted line), allowing for adequate endocrine differentiation (red). When Hnf6 expression is prematurely extinguished (right graph), Ngn3 levels reach this threshold in fewer cells, resulting in reduced endocrine mass. Figure modified and reprinted with permission from Guney and Gannon, 2009 (51).

committed to the endocrine lineage whereas *Neurog3*^{Lo} cells remain in the trunk epithelium and potentially act as a renewable pool for endocrine specification (Figure 1-10) (50,52). Indeed, *Neurog3*^{Hi} cells are much more likely to follow the canonical endocrine development pathway of delaminating from the trunk epithelium prior to initiating hormone expression ultimately producing the hormone+ cells of the islets. Conversely, *Neurog3*^{Lo} cells remaining in the trunk have higher levels of proliferation compared to *Neurog3*^{Hi} cells thus supporting the notion that they act as a niche of uncommitted but endocrine-biased cells. At this time, however, the persistence of this niche and the specific differential regulation of *Neurog3*^{Hi} and *Neurog3*^{Lo} cells remains unclear. Considering the importance for *Neurog3* in endocrine specification, the mechanisms and factors regulating its expression have been studied in great detail as have the downstream effectors that regulate endocrine cell differentiation. Together, these factors operate within a transcription factor cascade promoting the differentiation of these different endocrine cell types (Figure 1-11).

β-Cell Differentiation and Maturation

Following endocrine specification, a well-established transcription factor cascade determines the fate of each endocrine cell type (Figure 1-12). Neurog3 promotes expression of transcription factors such as *Paired box 6* (*Pax6*) and *Islet 1* (*Isl1*) which are present in all cells of the endocrine linage and maintain their commitment to the endocrine lineage (reviewed in (53)). *Paired box 4* (*Pax4*) follows expression of *Pax6* and *Isl1* and is expressed in multiple hormone+ lineages, but becomes restricted to β cells as development proceeds (54). *Pax4* and *Neuronal differentiation 1* (*NeuroD1*) are amongst the first β-cell specific factors and are

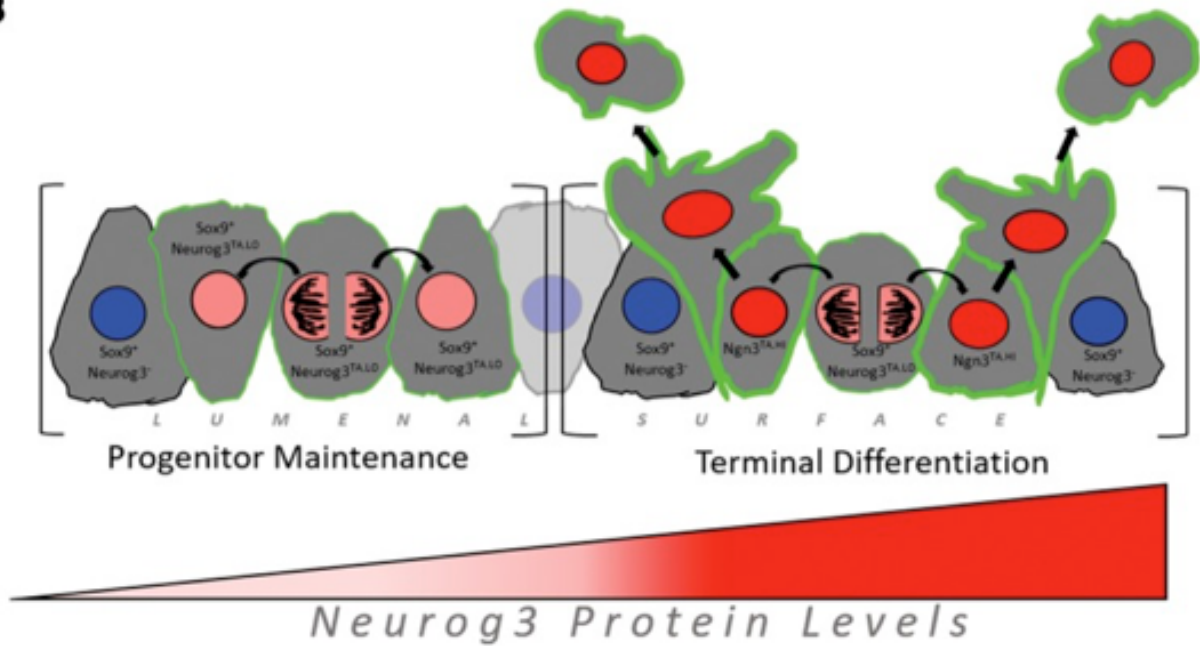

Figure 1-10: Model depicting the behavior of Neurog$^{3TA.LO}$ cells in the pancreatic epithelium. This figure illustrates the difference between proliferation of putative endocrine progenitor cells in the trunk epithelium and specification/differentiation of Neurog3^{HI} endocrine progenitors which delaminate from the trunk epithelium. Figure modified and reprinted with permission from Bechard *et al*, 2016 (50).

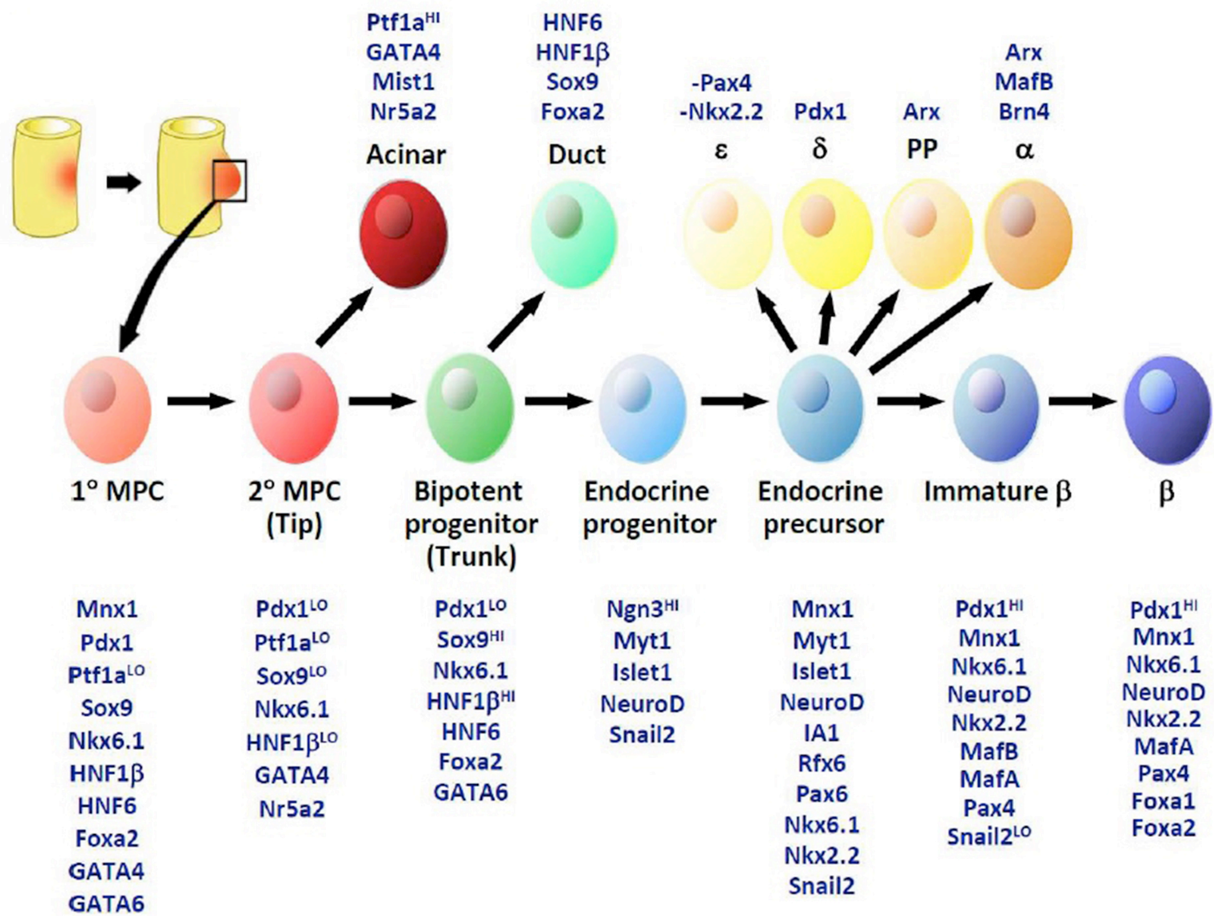

Figure 1-11: Overview model of mouse pancreas organogenesis. Important transcriptional regulators expressed at each stage of pancreas development. -Pax4 and -Nkx2.2 indicate that ε-cells develop in the absence of Pax4 and Nkx2.2. Figure modified and reprinted with permission from Pan and Wright, 2011 (5).

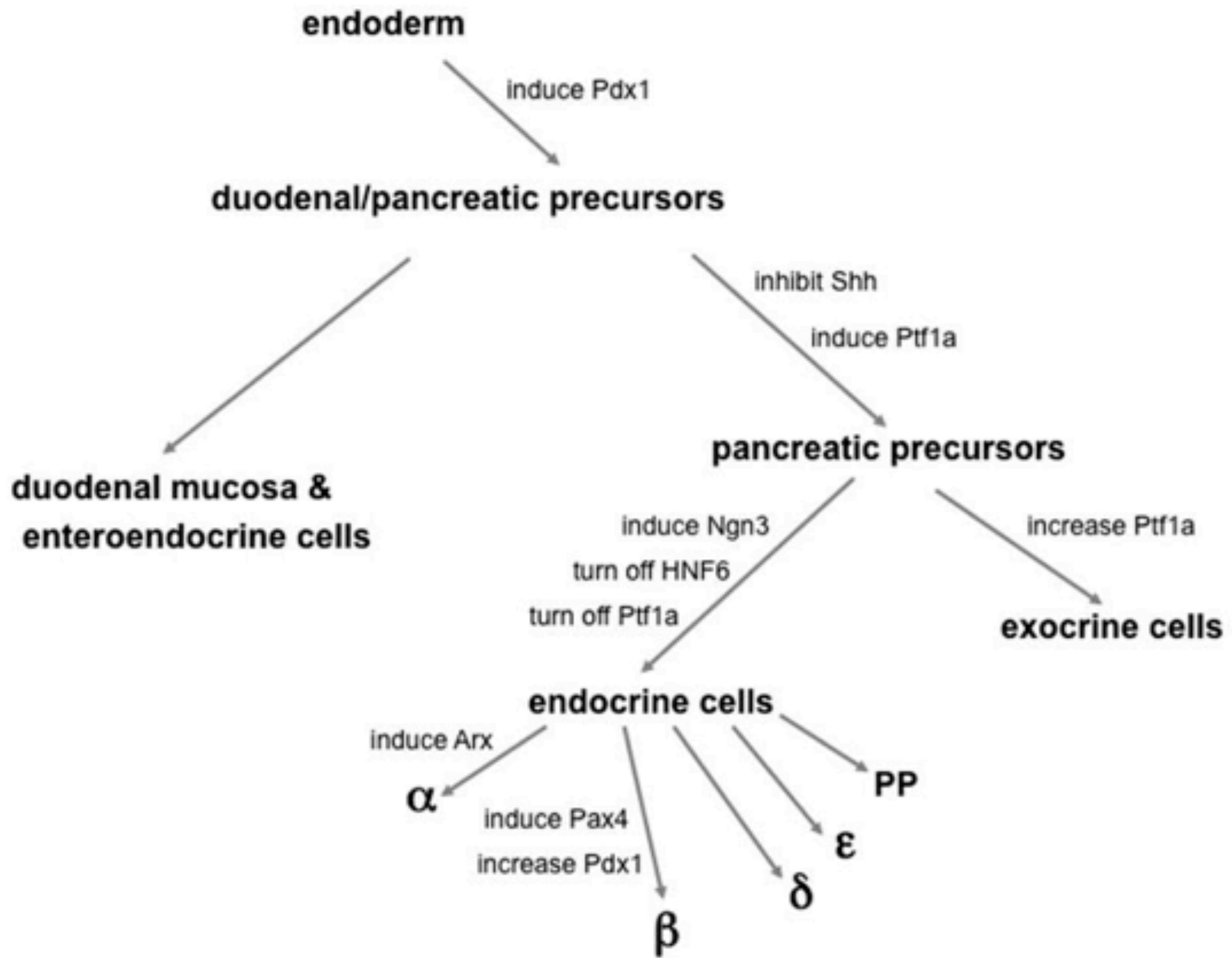

Figure 1-12: Lineage bifurcations leading to pancreatic endocrine differentiation. Beginning with definitive embryonic endoderm, cell fate specification within the posterior foregut can be thought of simply as a series of binary decisions that lead to the formation of the different cell types in the pancreas. Certain transcription factors and secreted factors are activated or inhibited along this developmental pathway, directing pluripotent cells toward one lineage or another. Although not comprehensive, this schematic highlights some of the factors critical for pancreatic cell fate specification and differentiation. See text for more details. Figure modified and reprinted with permission from Guney and Gannon, 2009 (51).

essential for proper development of β cells in addition to playing crucial roles in activation of the *Insulin* gene in mature β cells (54,55). Importantly, *Pax4* operates in a mutually inhibitory relationship with the transcription factor *Aristaless related homeobox* (*Arx*) which is essential for α cell differentiation (reviewed in (56)). Mis-expression of Pax4 or Arx in the opposing endocrine lineage has functional consequences for α or β cell identity (57-60).

Another pair of transcription factors that has an exceedingly important role in determining α- or β-cell fate are the large Maf transcription factors, MafA and MafB. MafB is expressed in both glucagon+ and insulin+ cells during development and it is maintained at a high level in mature α cells in both mouse and human, while it is gradually extinguished from postnatal β cells in mice (61,62). In contrast, MAFB expression is maintained in adult human and non-human primate β cells (63). Although it is expressed in developing insulin-producing cells, MafA primarily functions to regulate terminal differentiation of β cells and is thus considered to be a marker of β-cell maturation (62,64). MafA is a critical regulator of β-cell function through its activation of the network of functional genes necessary for glucose-stimulated insulin secretion (GSIS) and the *Insulin* gene itself (62,64-67). Together, these transcription factors operate within a highly coordinated and complex gene regulatory network that is essential for establishing and maintaining β-cell identity (reviewed in (68)).

The above-mentioned transcription factors are critical not just for promoting or inhibiting the other regulators within the β-cell differentiation cascade, but also for actively regulating components of the GSIS pathway. Transcription factors including, but not limited to, MafA, Pdx1, Hepatic nuclear factor 4α (Hnf4α) and NeuroD1 all bind to and activate the *Insulin* promoter (64,69-74). Additionally, these factors control the expression of functional genes that

are important for glucose metabolism and insulin processing and secretion. As an example, Glut2, the primary glucose transporter in β cells, is essential for glucose sensitivity and mature β-cell function (75,76). *Slc2a2* (Glut2) gene expression is regulated by Pdx1, MafA, and Hnf4α (71,77-80). Because of the importance of Glut2 for mature β-cell function, its expression and membrane-localization in β cells is frequently used as an indicator of β-cell maturity. Glut2 (Glut1 in humans) is particularly important because it, as the primary glucose transporter in β cell, operates as a gatekeeper for the first step of GSIS.

The ability of the β cell to properly sense changes in glucose concentration and secrete insulin in a regulated manner is essential for maintenance of systemic glucose homeostasis. In brief, glucose enters the β cell through the Glut2 transporter and is phosphorylated to glucose-6-phosphate (G6P) by glucokinase (Gck). G6P is metabolized in the mitochondria resulting in generation of adenosine triphosphate (ATP) which increases the ratio of ATP to adenosine diphosphate (ADP). The increased ATP:ADP ratio results in closure of the ATP-dependent potassium channel (K_{ATP}) resulting in depolarization of the cell, an influx of calcium (Ca^{2+}) to the cytosol, and fusion of insulin granules with the plasma membrane resulting in insulin release to the blood stream (Figure 1-13). Thanks to this highly-coordinated process, mature β cells have very low basal insulin secretion at low glucose conditions and very high insulin secretion at high glucose conditions. High basal insulin secretion is associated with β-cell immaturity as was well documented by the Melton group. They demonstrated that postnatal day 1 (P1) β cells secrete insulin at low (2.8 mM) glucose whereas there is no insulin secretion by P9 β cells at the same low glucose (81-84). Further, they determined that P9 is the age by which β cells acquire this control of GSIS with a tight clamp on basal insulin secretion.

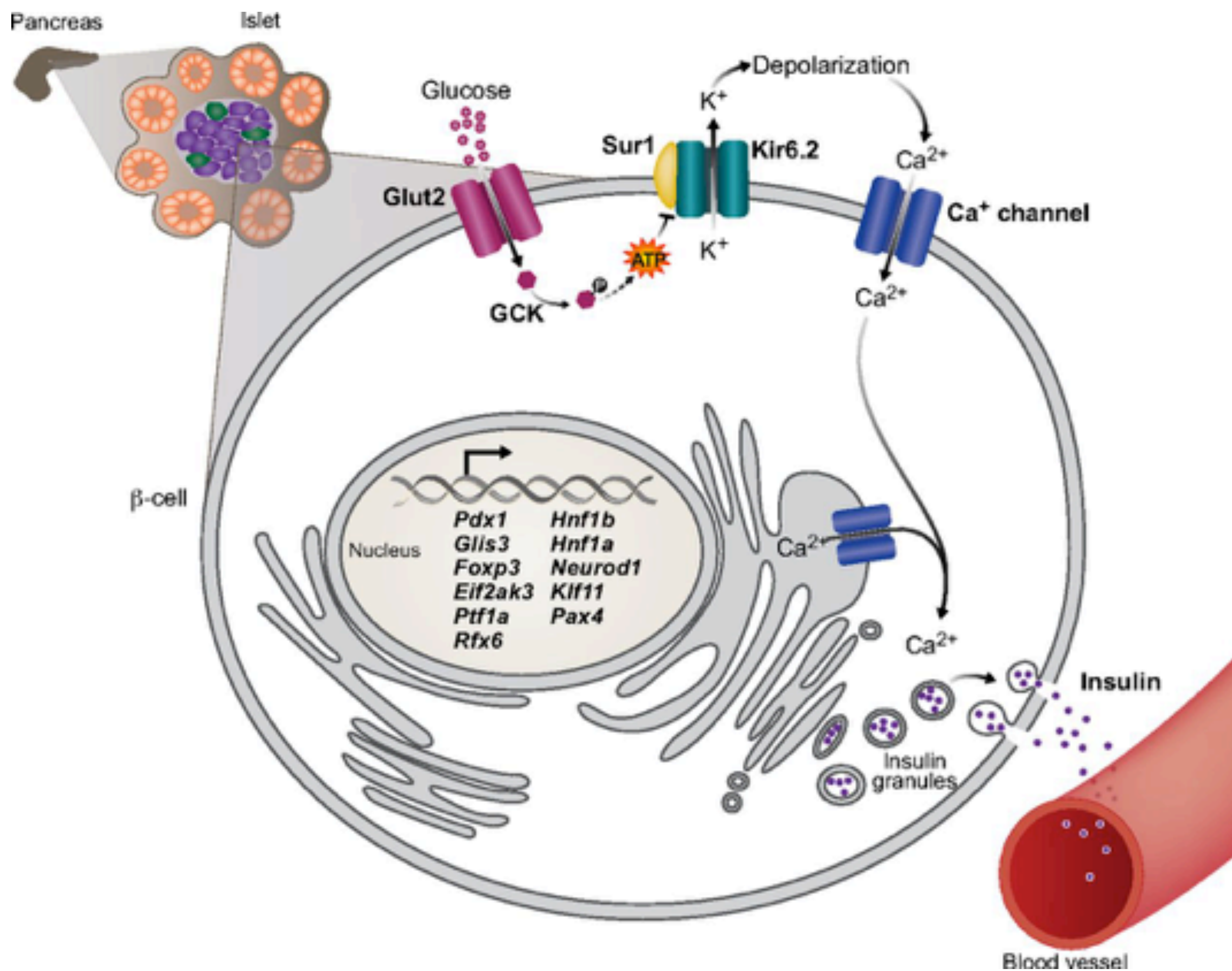

Figure 1-13: Functional β-cells respond to increasing glucose levels by increasing insulin secretion. In GSIS, glucose is transported into the cell via glucose transporters [e.g. Glut1 (Slc2a1) or Glut2 (Slc2a2), pink] where it is phosphorylated by glucokinase (GCK) and converted into ATP by subsequent metabolic reactions. Rising ATP levels (e.g. rising ATP:ADP ratios) trigger the closure of potassium channels [Sur1 (Abcc8) and Kir6.2 (Kcnj11) subunits], membrane depolarization, and the opening of calcium channels (blue). The resultant rise in intracellular calcium triggers the exocytosis of insulin-containing granules and hence leads to increased insulin levels in adjacent blood vessels. Human genetic studies of maturity onset diabetes of the young (MODY) patients have identified a number of mutations that trigger diabetes, including those in genes encoding transcription factors (depicted in the nucleus) and components of the GSIS pathway indicated in this figure. Figure modified and reprinted with permission from Pagliuca and Melton, 2013 (85).

The establishment and maintenance of β-cell maturity has become a topic of great interest and intense scrutiny in recent years. Traditionally, insulin+ cells were considered β cells even during development. Following their differentiation from endocrine progenitors, these cells were assumed to be a mostly homogenous pool capable of producing insulin for the purpose of regulating blood glucose levels. Although expression of Glut2 and MafA has often been thought to indicate a mature and thus functional β cell, it has become apparent that there is a level of β-cell heterogeneity since MafA is expressed in only ~80% of adult mouse β cells (61). This observation was amongst the first pieces of evidence that the β-cell maturation state is more nuanced than previously appreciated. Indeed, expression of certain transcription factors (e.g. MafA, Nkx6.1 [NK6 homeobox 1], Pdx1, FoxA2 [Forkhead box A2]) is important for β-cell identity. These transcription factors are necessary for the active maintenance of β-cell identity and loss of any one of these factors can result in misexpression of other hormones in β cells or impaired regulation of GSIS (67,70,77,78,86-89). Additional non-transcription factor proteins have recently been recognized as markers of β-cell maturation. The most prominent of these is the neuropeptide Urocortin 3 (Ucn3). Ucn3 is expressed in mature β cells, co-localized and secreted with insulin, and utilized as a marker of β-cell maturation. It is dispensable for the function of β cells themselves, but acts in a paracrine manner to activate somatostatin secretion from δ cells thereby activating a negative-feedback loop inhibiting further insulin secretion (81,90). While expression of Ucn3 is used as a marker of β-cell maturation, just as important for recognition of β-cell maturity is the absence of certain genes. A collection of "disallowed" genes (*hexokinase 1* and *2*, *lactate dehydrogenase a*, *aldolase B* and others) includes glycolytic genes that are absent or at very low levels in mature β cells (91,92). These

genes are subject to highly-sensitive epigenetic repression and must be kept at low levels to allow for the sensitive glucose metabolism necessary for β-cell function (82,91,93,94). De-repression of these genes in adult β cells is indicative of impaired β-cell identity and function in both humans and animal models of T2D (95,96).

With more sensitive techniques becoming available in recent years, β-cell maturity has been further complicated by increased evidence of heterogeneity and altered identity of β cells in both normal and diabetic states. Single-cell RNA-Sequencing and proteomics have revealed that there are multiple classes of β cells with varying degrees of functionality (97). It is possible that this heterogeneity is evolutionarily advantageous because it allows for pools of highly functional cells and pools of less functional cells that may be more adaptable and/or respond to proliferative stimuli. While we still have much to learn about the biological significance of β-cell heterogeneity and the different types of β cells, the information acquired from these studies has also led to the understanding that β-cell dedifferentiation or change in identity is associated with diabetes pathogenesis (98). Indeed, it is now appreciated that T2D is associated with loss of β-cell maturation markers and regulators. In β cells from T2D mouse models or humans with T2D, expression of Ucn3 is reduced or absent, while expression of the disallowed genes is increased (90,99). Additionally, β cells in the setting of T2D show reduced or absent expression of important transcription factors such as MafA, Pdx1, and Nkx6.1. Together, these data demonstrate that maintenance of the mature and differentiated β-cell state is an active process that can be disrupted in the context of disease pathophysiology. More information about the pathophysiology of T2D is below.

Exocrine differentiation

The exocrine compartment of the pancreas consists of both the duct and acinar cells. As noted in the previous section on pancreas morphogenesis, the branching morphogenesis process results in different domains within the pancreas simply due to the spatial constraints and proximity to the mesenchyme. This organization during development yields the bipotent trunk epithelial cells (from which endocrine progenitors are specified) and the tip cells also known as 2° MPCs (Figure 1-5). The trunk cells that do not become endocrine cells will ultimately give rise to the pancreatic ducts whereas the tip cells give rise to the pancreatic acinar cells. The mature versions of these cells will work in concert to produce and transport digestive enzymes to the duodenum to assist in the digestion of food.

The pancreatic ducts play a vital role in transport of digestive enzymes from the pancreas to the duodenum. An essential aspect of this function is the structure of the pancreatic ductal tree. The branching morphogenic process results in four progressively smaller ductal categories: 1: the main pancreatic duct, 2: interlobular ducts, 3: intralobular ducts, and 4: intercalated ducts (Figure 1-6). Ducts terminate at a rosette of acinar cells which are all polarized so as to allow for secretion of digestive enzymes, or zymogens, into the lumen of the ducts (reviewed in (100)). The lobular nature of the ducts with each intercalated duct paired with an acinar rosette is important for the functionality of the mature exocrine pancreas, and is established during the branching morphogenic process during development. Regulation of the pancreatic ducts is somewhat less complicated than the previously described endocrine compartment even though these lineages both derive from the bipotent cells of the trunk epithelium. A simplistic view is that the ducts arise from the cells left behind following

endocrine specification, but in fact the ducts are actively maintained and require regulation by certain factors to promote their structure and function.

Transcription factors such as *Sox9*, *Hnf1β*, *Prox1* (Prospero homeobox 1), and *Oc1* all play vital roles in directing the ducts to a mature state. While the ductal structure defined by a simple stratified epithelium with cells polarized toward the lumen is vital to normal function, it appears that one of the most important aspects of the pancreatic ducts is the maintenance of primary cilia. Most polarized epithelial cells have a primary cilium, a single projection from the apical surface of the cell that functions most significantly in cell signaling (Shh, Fgf, Wnt pathways) and sensing of flow, rather than motility. The primary cilia are established very early in the developing pancreas and possibly even prior to pancreas specification from the foregut endoderm (Figure 1-14) (101). Of the above mentioned transcription factors, *Hnf1β* in particular is known to have a role in regulating the expression of functional genes necessary for primary cilium establishment and maintenance (102). Indeed, inactivation of *Hnf1β* results in lost or impaired cilia and subsequent ciliopathies (103,104). These ciliopathies often present with tortuous and cystic ducts that fail to transport zymogens to the duodenum. As a consequence of the impaired transport, the pancreas can begin to auto-digest leading to pancreatitis. Sox9 also has a vital role in duct development and homeostasis. It becomes restricted to the ducts during the second wave of endocrine differentiation where it continues to act as an important regulator of duct cell identity (105,106). The role of Sox9 in pancreatic duct disease will be covered below.

Pancreatic acinar cells derive from the tip cells (aka 2° MPCs) and receive the highest levels of mesencymally-derived factors such as Fgf, follistatin, and Wnt. These 2° MPCs rapidly

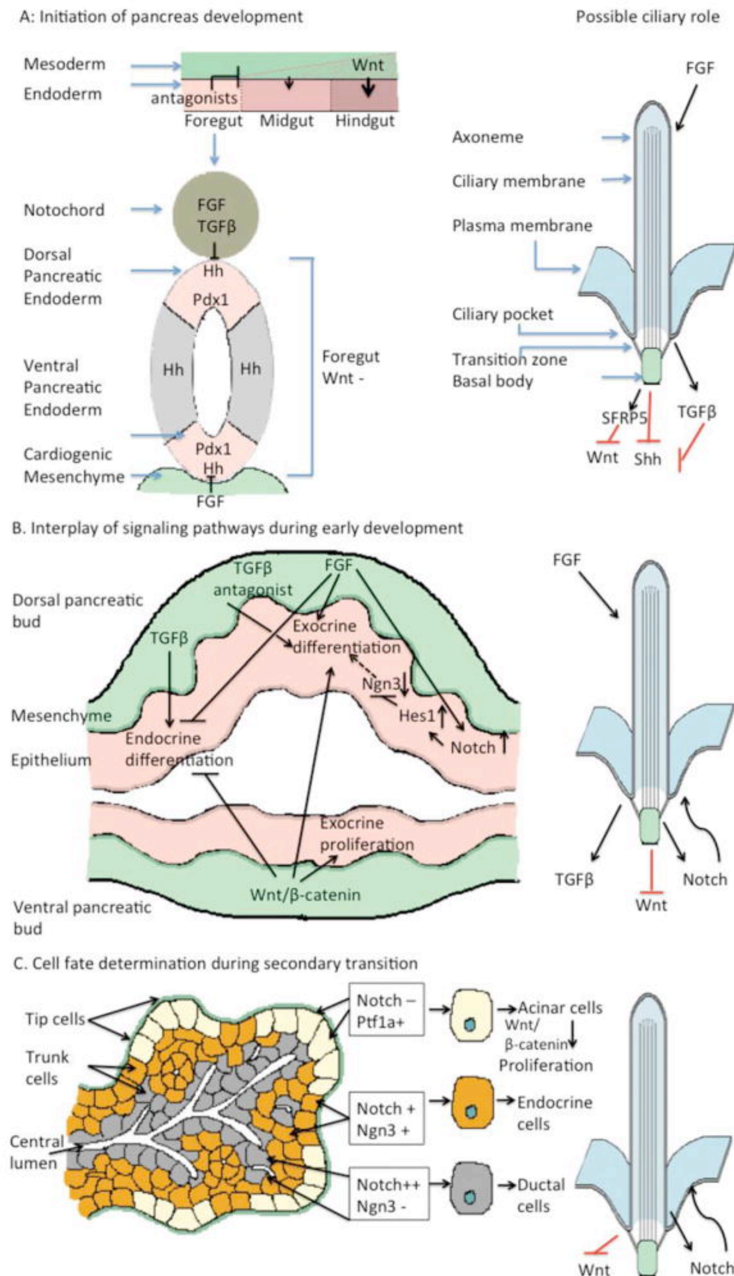

Figure 1-14: Regulation of pancreatic development by cilia-dependent pathways. A. In early endodermal patterning, Wnt/β-catenin signaling is excluded from the foregut endoderm by foregut expression of antagonists (107). The dorsal and ventral pancreatic endoderm of the foregut are subsequently specified by suppression of Shh signaling, as a result of FGF and TGF-β signaling from the notochord (108) and FGF signaling from the cardiogenic mesenchyme (109). Expression of the pancreatic progenitor marker Pdx1 is upregulated throughout the pancreatic endoderm, specifying progenitor cells that will contribute to the mature organ. Primary cilia prevent improper activation of both canonical Wnt signaling (110,111), as well as Shh (112), and are required for proper TGF-β signaling (113), implicating proper ciliary function in the

patterning of the gut endoderm and the specification of pancreatic progenitor cells. In addition, FGF signaling regulates the length of primary cilia (114-117), which blocks inappropriate activation of Hh signaling in pancreatic epithelium (112), suggesting that FGFs may repress Hh in pancreatic endoderm by maintaining cilia. B. The pancreatic epithelium, defined by Pdx1 expression, evaginate to form dorsal and ventral pancreatic buds. Pdx1+ cells that lack Notch signaling transiently express Ngn3 and eventually give rise to endocrine cells. Those that retain Notch signaling express Ptf1a, repress Ngn3 expression, and give rise to exocrine cells (42,118,119). Notch signaling is maintained by mesenchymal FGFs (120-122), which favors exocrine differentiation over endocrine differentiation (3,120,121,123). Canonical Wnt signaling is required for the proliferation of pancreatic progenitor cells (124-128), as well as for the exocrine acinar cells (125,128). Primary cilia in the pancreas are necessary to preclude over-activation of Notch signaling, and therefore may regulate the balance between endocrine and exocrine fates (112). TGF-β signaling from the surrounding mesenchyme is also important to drive production of endocrine fates from the pancreatic epithelium and inhibit exocrine fates. In contrast, canonical Wnt signaling is essential for the proliferation of acinar cells and preventing endocrine differentiation. Given the role of cilia in inhibiting Wnt signaling and promoting TGF-β signaling, cilia may block improper Wnt signaling and promote TGF-b in endocrine precursor cells. The absence of cilia in the main exocrine lineage, acinar cells, however, may serve the opposite function, providing an environment conducive to high levels of Wnt/β-catenin signaling. C. During the secondary transition of pancreatic development, tip cells at the termini of ducts give rise to exocrine acinar cells and trunk cells lining the ducts give rise to endocrine and ductal cells. Notch signaling regulates the balance between tip and trunk fates; active Notch promotes trunk identity and represses tip identity, and tip cells expressing low levels of Notch proliferate rapidly under the control of canonical Wnt signaling, ultimately producing acinar cells. Additionally, trunk cells maintain dual potential: high levels of Notch activity lead to the formation of duct cells through repression of Ngn3 and low levels of Notch enhance Ngn3 expression, driving differentiation of endocrine cells (129). Primary cilia may regulate these processes by coordinating regulation of Notch. Figure modified and reprinted with permission from Lodh *et al*, 2014 (101).

become distinct from the trunk at both the structural and molecular level. The tip cells continue to express MPC-associated transcription factors such as *Pdx1*, *Oc1*, and most importantly *Ptf1a*, but they lack expression of *Hnf1β* and *Sox9* (106,130-132). These cells are acinar-biased, but they do still retain the potential to differentiate into most cell types of the pancreas (21). Notably, this process of acinar-biasing begins just prior to the second wave of endocrine specification but continues to overlap with the second wave as both of these processes are continuous and occur over a window of time rather than at a distinct instance. A hallmark of acinar cell differentiation is the progressive reduction in expression of certain MPC transcription factors concurrent with the selective increased expression of others. *Pdx1* and *Oc1* are maintained at low levels in the acinar cells whereas *Ptf1a*, *Nr5a2* (*Nuclear receptor subfamily 5 group A member 2*), and *Mist1* (*Muscle, intestine, and stomach expression 1*) all become selectively expressed in the presumptive acinar cells (10,133-135).

Ptf1a is arguably the most important regulator of acinar cell development; its absence results in very severe loss of acinar cells and pancreatic hypoplasia (12,68). Ptf1a functions within a heterotrimeric transcription factor complex that includes Rbpj (Recombinant signal binding protein for immunoglobulin kappa J region) to promote expression of acinar-cell functional genes. Nr5a2 is an orphan nuclear receptor that is expressed in MPCs but during the window of acinar specification becomes restricted to the tip cells and ultimately the acinar cells (134). Mist1, a general regulator of zymogen-secreting cells, is activated in the tip cells and also has an important function in mature acinar cells (135). Together, these transcription factors operate within a network to promote and maintain acinar cell identity.

Production of the pancreatic digestive enzymes is an essential aspect of acinar cell identity. The transcription factors that are necessary for initiating acinar differentiation are also involved in maintaining expression of the zymogens during pancreas development and adult function. The first zymogen to be expressed, and a marker of tip cells, is *Carboxypeptidase A* (*Cpa1*). *Cpa* can first be detected at approximately e13.5 in mice, which is concurrent with the beginning of the acinar cell differentiation process from tip cells (21). Following initiation of *Cpa* expression, other zymogens begin to be expressed including *Elastase1* and *2*, *Prss1* (trypsin), and *Amy1* (amylase). Equally important is the production and secretion of peptidase inhibitors that prevent the premature activation of these zymogens. Indeed, the pancreatic zymogens, with the exception of amylase and select lipases, are secreted as pro-enzymes that must be cleaved in order to achieve their active form. The peptidase inhibitors are secreted with the zymogens from the acinar zymogen granules to ensure that the zymogens are not cleaved and activated within the pancreatic ducts and are instead transported to the duodenum. In the duodenum, the acidic environment permits activity of the intestinal peptidase enterokinase which converts typsinogen (the trypsin pro-peptide) to active trypsin. Trypsin in turn activates most of the other pancreatic zymogens by cleaving the pro-peptides to their active forms. Zymogen activity in the duodenum is essential for digestion, and reductions in zymogen production or activity can lead to pancreatic insufficiency thereby upsetting the nutrient balance in the body. Additionally, premature activity of the zymogens can result diseases such as pancreatitis which will be discussed below.

Onecut 1: Characterization and Significance

During studies of liver-enriched transcription factors, a protein activity was identified that could bind the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase promoter with high affinity. It was named Hepatocyte nuclear factor 6 (Hnf6) based upon its expression pattern and unique DNA-binding characteristics, which separated it from previously identified hepatocyte nuclear factors (Hnfs) such as Hnf1α and β, Hnf3α and β (subsequently renamed as FoxA1 and 2, respectively), and Hnf4α. Characterization of the Hnf6 protein revealed that it contained a single domain homologous to the *Drosophila* cut domain at the N-terminus and a novel, divergent homeodomain at the C-terminus (136,137). Hnf6 has since been renamed Onecut 1 (Oc1). Based on homology to Hnf6, a second and third factor with one cut domain were identified in the liver: Onecut 2 (Oc2) and Onecut 3 (Oc3), respectively establishing the Onecut family of transcription factors (138,139). The expression patterns of Oc2 and Oc3 frequently overlap with Oc1 and they have some of the same transcriptional targets, but the relationship between these factors is context-dependent.

Further investigation into the function of the Oc factors revealed that the homeodomain was dispensable for binding to the DNA of some, but not all, transcriptional targets in the liver. Conversely, binding to some targets of Oc1 does not require the cut domain and instead relies upon the homeodomain. In many circumstances, the non-DNA bound DNA binding domain participates in recruitment of transcriptional co-factors such as the CREB-binding protein (CBP) or CCAAT/enhancer-binding protein α (C/EBPα) for transcriptional activation (140,141). Interestingly, acetylation of Oc1 protein by CBP is necessary for increased Oc1 protein stability and transcriptional activity, so this recruitment of CBP by Oc1 is necessary for its function (142).

Together, these data provide evidence that Oc1 function is complex and its role at a given target gene is promoter context-specific (143).

As alluded to above, Oc1 has a significant role in promotion of pancreas specification and development. Oc1-null animals have a severe embryonic pancreas defects (discussed in more detail below), but die shortly after birth due to liver defects (15,143,144). Oc1 has an important role in the gene regulatory network of MPCs. In addition to regulating *Pdx1*, Oc1 positively regulates several transcription factors involved in pancreas development including *Hnf1β*, *Hnf4α*, and *FoxA2* (Figure 1-15) (145-147). While Oc1 is not essential for the expression of these factors, loss of Oc1 expression results in their reduced expression, which has functional consequences for pancreas development in no small part due to the role of Oc1 in regulation of a regulatory transcription factor network (Figure 1-15). Some of these regulatory relationships will be covered in more detail below. The significance of Oc1 in pancreas development is further underscored by the fact that the two other members of the Onecut family, Oc2 and 3, have minor and apparently inconsequential roles in pancreas development. Oc2 expression largely overlaps with Oc1 during pancreas specification, but its expression within the whole pancreas progressively decreases following e12.5 (148). Oc3 expression completely overlaps with Oc1 in the developing pancreas and liver, and its expression appears to be entirely dependent upon activation by Oc1 as *Oc1*-null animals do not express Oc3 at any stage. The reliance of *Oc3* on Oc1 for expression is in contrast to *Oc2* expression, which is independent of Oc1 (149). Importantly, Oc2 and Oc3 are not fully redundant with Oc1 as these two factors cannot compensate for the loss of Oc1 during development, and combined inactivation of Oc2/Oc3 does not impair pancreas development (150). Additionally, the pancreatic phenotype

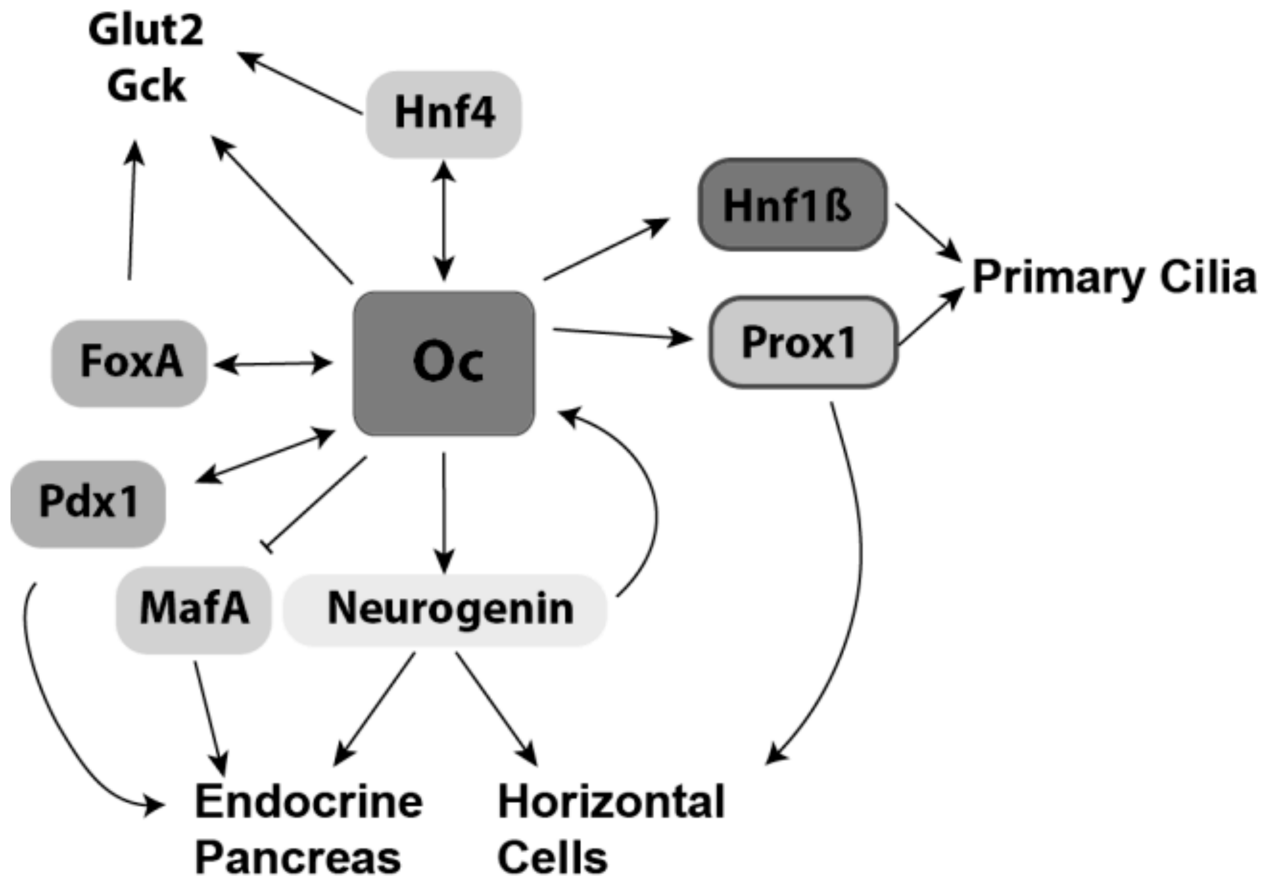

Figure 1-15: Network of Oc factor targets and associated processes**.** Oc factors regulate many other transcription factors during development to promote differentiation of multiple different mature cell types. This regulation carries over to function of mature cells in the liver through regulation of glucose-processing enzymes. Figure modified and reprinted with permission from Kropp and Gannon, 2016 (151).

in Oc1 null mutants is not exacerbated by the additional inactivation of either Oc2 or Oc3, indicating that those factors contribute a less significant role to pancreas specification (16,138,150,152).

A specific role for Oc1 during the branching morphogenesis process is unclear. However, it is necessary for proper expansion of the pancreatic epithelium in this early stage. Global Inactivation of Oc1 early in the pancreatic epithelium using a *Pdx1*-Cre driver results in pancreatic hypoplasia due to failure of the epithelium to expand and fill the capsule generated by the pancreatic mesenchyme (16). A direct role for Oc1 in regulation of MPC proliferation is unknown. Oc1 is one of the essential factors necessary for proper induction of *Neurog3,* thus initiating endocrine specification; *Oc1* inactivation results in a near complete loss of Neurog3+ cells (16,153). Oc1 directly binds the *Neurog3* promoter suggesting that it is one of the few known direct activators of the endocrine specification program (153,154). Although Oc1 alone is capable of activating *Neurog3* transcription, it acts cooperatively with Pdx1 to increase *Neurog3* transcript levels *in vitro* suggesting the importance of these two factors working together to specify the endocrine lineage (Figure 1-16) (154). In pancreata from *Oc1* null mice, a few hormone-positive cells persist; however, these cells do not express markers of mature endocrine cells suggesting that Oc1 is also required for endocrine maturation (discussed below) (16,153).

Following endocrine specification, the role of Oc1 becomes more nuanced as continued Oc1 activity is required to ensure endocrine differentiation. Deletion of Oc1 from committed endocrine cells using a *Neurog3*-Cre driver results in some endocrine progenitor cells being diverted to the exocrine lineage (Figure 1-9) (16). Yet, Oc1 expression is silenced later in the

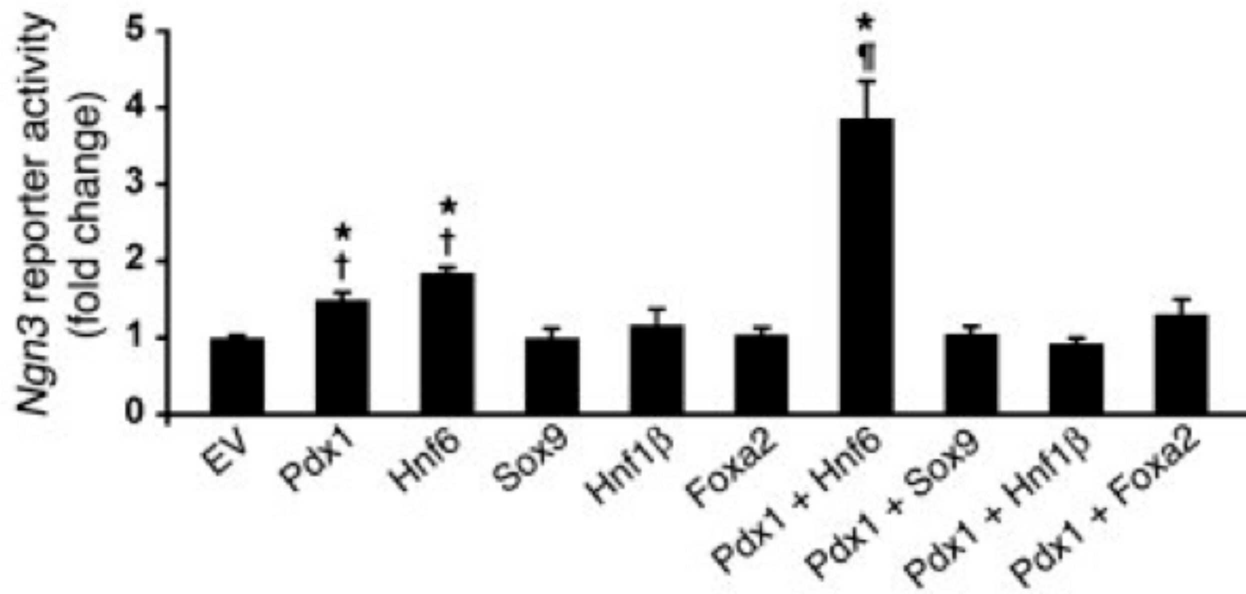

Figure 1-16: Pdx1 cooperates with Oc1 (Hnf6) to regulate *Ngn3*. HepG2 cells transfected with enhancer reporter Ngn3(–3379 to –3227)-tkluc and expression vectors for Pdx1, Hnf6, Sox9, Foxa2. **P* < 0.05 versus empty vector; †*P* < 0.01 versus Pdx1 plus Hnf6; ¶*P* < 0.01 versus Pdx1; n=4. Figure modified and reprinted with permission from Oliver-Krasinski *et al*, 2009 (154).

endocrine lineage and is not detected in hormone-positive cells at any time (Figure 1-17) (16,146). Indeed, our group has shown that this down-regulation of Oc1 is necessary for proper differentiation and maturation of β cells (155-158). Maintenance of *Oc1* expression in the endocrine lineage results in increased expression of *Neurog3* and increased numbers of endocrine cells, but defects in β-cell maturation, leading to diabetes. Sustained Oc1 expression in the β-cell lineage represses the expression of the β-cell maturity markers *MafA* and *Glut2*, leading to impaired β-cell function as indicated by impaired insulin granule biosynthesis and GSIS. Activation of *Oc1* in differentiated β cells using the *insulin* promoter also results in decreased insulin production and development of diabetes. However, in this model there was increased β-cell apoptosis and decreased β-cell mass that was not observed when *Oc1* expression was maintained earlier in the endocrine lineage (159). These data indicate that Oc1 is critical for endocrine specification, but that it acts only in the initial stages of specification and commitment and in fact becomes detrimental to endocrine cells at later stages of differentiation.

Oc1 expression is maintained at a high level in ducts and a low level in acinar cells throughout development and adulthood (Figure 1-18) (16,102). Although the role of Oc1 in differentiation of the acinar cells is not fully elucidated, it clearly plays a role in proper differentiation of ducts. Oc1 promotes the duct cell fate by acting upstream of the definitive duct marker Hnf1β. Indeed, loss of *Oc1* results in a greater than 2-fold reduction in *Hnf1β* transcript levels during early duct differentiation; a partial recovery of *Hnf1β* occurs later in gestation. The increase in *Hnf1β* later in development in the absence of Oc1 is possibly due to up-regulation of Oc2 in an attempt to compensate for the loss of Oc1 (146).

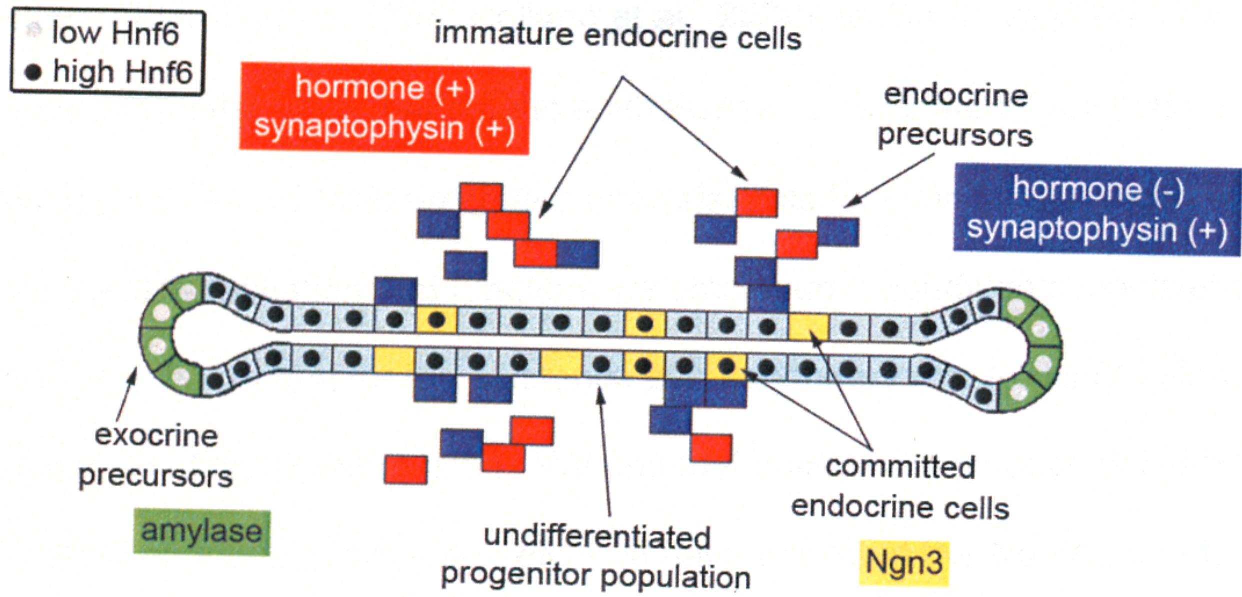

Figure 1-17: Schematic representation of Oc1 (Hnf6) expression in the developing pancreas. Oc1 is expressed at high levels (black circles) in the undifferentiated pancreatic stalk epithelium (light blue), and at lower levels (gray circles) in differentiating acinar cells (green) at branch tips at the secondary transition. Oc1 is not expressed in definitive endocrine cells (blue and red), but is detected in a sub-population of Ngn3-positive endocrine progenitors (yellow). Figure modified and reprinted with permission from Guney and Gannon, 2009 (51).

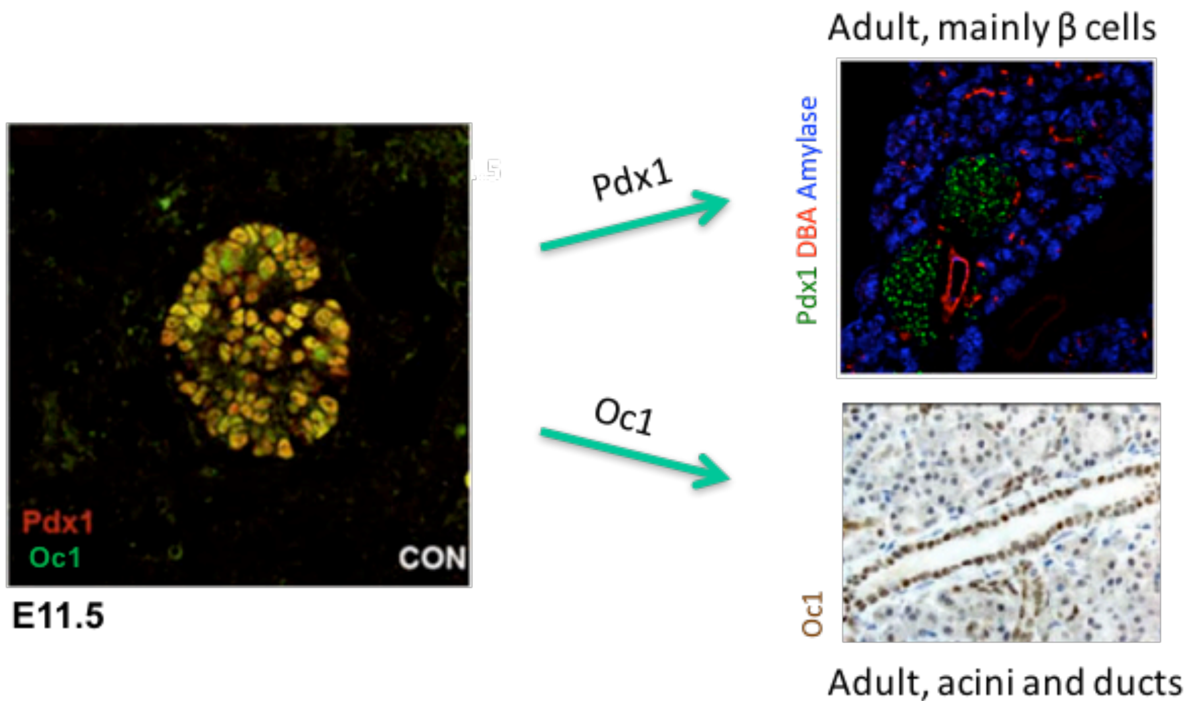

Figure 1-18: Divergent expression patterns of Oc1 and Pdx1 in the pancreas. Left: immunofluorescence image of an e11.5 mouse pancreatic bud labeled for Pdx1 (red) and Oc1 (green). Upper right: immunofluorescence image of adult mouse tissue section labeled for Pdx1 (green) amylase (blue) and DBA (red) demonstrates that Pdx1 is expressed at a high level in adult β cells. Immunohistochemical staining of adult human pancreas labeled for OC1 (brown) and counterstained with hematoxylin to label nuclei. OC1 is expressed at a high level in the ducts and a lower, more variable level in acinar cells. Figures modified and reprinted with permission from Zhang *et al*, 2009 (16), Boyer *et al*, 2006 (160), and Pekala *et al*, 2014 (161).

Although Oc1 is important for duct development, it does not affect differentiation of all types of pancreatic ducts equally. Loss of *Oc1* does not affect intercalated ducts (the smallest ducts within the pancreas), but impairs interlobular and intralobular ducts. As early as e12.5 ductal branching is impaired in *Oc1* mutants and dilated ductal lumens as well as ductal cysts are apparent by e15.5 (102). Proliferation is increased in the ductal epithelium in *Oc1*-null mutant mice and the normal cuboidal squamous architecture is lost, resulting in a multilayered epithelium that has lost its polarity (16,102). The exact mechanism of the ductal dysmorphogenesis is not yet fully elucidated, but it is likely due in part to the loss of primary cilia in duct cells that have lost *Oc1* expression. Just as in the hepatobiliary system, Oc1 is part of a transcriptional regulatory pathway that includes *Hnf1β* and *Prox1,* and regulates the transcription of genes involved in formation of primary cilia, such as *Pkhd1* (*Polycystic kidney and hepatic disease 1*) and *Cys1* (*Cystin 1*). Expression of both *Hnf1β* and *Prox1* is reduced in the Oc1-null pancreatic ductal epithelium (16,102). Oc2 and Oc3 cannot compensate for Oc1 with respect to primary cilia formation, as at no point during development do those structures develop in the ductal epithelial cells. Additionally, *Oc2*-null animals have normal duct and cilia formation indicating that Oc1 is the primary Oc factor regulating exocrine development. Interestingly, these results and regulatory networks are very similar to those of the developing intrahepatic bile duct suggesting commonalities in function.

Pdx1 in Pancreas Development and Function

As mentioned above, Pdx1 is essential for pancreas development with pancreatic agenesis occurring in its absence (6-9). *Pdx1* expression is regulated in a complex manner

through distinct areas in its promoter termed Areas I,II, III, and IV (Figure 1-19) (162,163). Whereas Area IV appears to be dispensable for pancreas development, Areas I-III are capable of generating sufficient levels of *Pdx1* for pancreas development in the absence of Area IV (7,160). Areas I-II are necessary for islet-specific gene expression and Area III confers β cell-specific *Pdx1* expression (164). Importantly, these regions are highly regulated by other MPC transcription factors such as Pdx1 itself, FoxA2, Oc1, and Hnf1α (15,163,165-168) further highlighting the importance of gene regulatory networks in establishing and promoting MPC identity. During the second wave of endocrine differentiation, Pdx1 expression becomes reduced in Neurog3+ endocrine progenitors while it concurrently becomes increasingly restricted to β cells. By the end of development Pdx1 is expressed at a high level in mature β cells and much lower levels in acinar cells (Figure 1-18). Pdx1 expression is essential for maintaining the functionality of adult β cells where it has a vital role in promoting *Insulin* gene expression as well as other factors that are necessary for acquirement of β-cell identity (e.g *MafA*, *Nkx6.1*) and glucose-stimulated insulin secretion (e.g. *Slc2a2*, *Gck*) (61,62,78,169-176). Pdx1 mediates its effects in part by recruiting co-regulators that modify the chromatin environment to be permissive for β-cell function (86,177). In this way, Pdx1 is capable of mediating long lasting effects that promote β-cell identity. While the importance of Pdx1 in β-cell identity and function is well established, its role in acinar cells has only recently become appreciated and remains unclear. It has been demonstrated that Pdx1 is necessary for acinar cell development and that it is important for maintaining acinar cell identity and preventing metaplasia in disease models (178,179). Collectively, it is clear that Pdx1 plays a vital role in the establishment and maintenance of multiple cell types in the pancreas.

Figure 1-19: Diagram of highly conserved areas within the *Pdx1* promoter/enhancer region. Area I (bp 2852 to 2547), area II (bp 2247 to 2071), area III (bp 1973 to 1694), and area IV (bp 6422 to 5931) are conserved regions among species. Figure modified and reprinted with permission from Wiebe *et al*, 2007 (13).

Pancreatic Disease

Diabetes Mellitus

Diabetes mellitus represents a collection of conditions with two major forms. Type I diabetes (T1D) is an autoimmune disease where pancreatic β cells are destroyed by immune cells and is associated with generation of autoantibodies against insulin or other β-cell components. T1D is believed to be initiated in genetically susceptible individuals when an infection causes some beta cell destruction and an inappropriate immune response resulting in aberrant generation of autoantibodies and T-cell activation (Figure 1-20) (180). T1D is most often diagnosed in children, but it can develop in adulthood. Currently, there is no cure for T1D and the best option for treatment remains exogenous insulin. T1D only accounts for ~10% of cases of diabetes in the United States whereas type 2 diabetes (T2D) accounts for the other ~90% of cases. T2D is strongly associated with obesity and the metabolic syndrome as well as many other comorbidities such as cardiovascular disease and stroke.

T2D is a disease of insufficient β-cell mass and/or function in which β-cells cannot produce enough insulin to meet increased demands imposed by insulin resistance (the impaired efficacy of insulin action on peripheral tissues thereby prohibiting the uptake of glucose from the blood stream). Insulin resistance is a complex and heterogeneous condition but is closely linked to obesity. Increased adiposity is associated with increased adipose tissue inflammation, impaired fatty acid (FA) storage, and accumulation of FA as triglycerides in other peripheral tissues (e.g. muscle, liver) which impairs insulin signaling (181,182).

The β cells of the pancreas respond to insulin resistance by increasing insulin output through a number of mechanisms. β cells can increase insulin production and secretion in

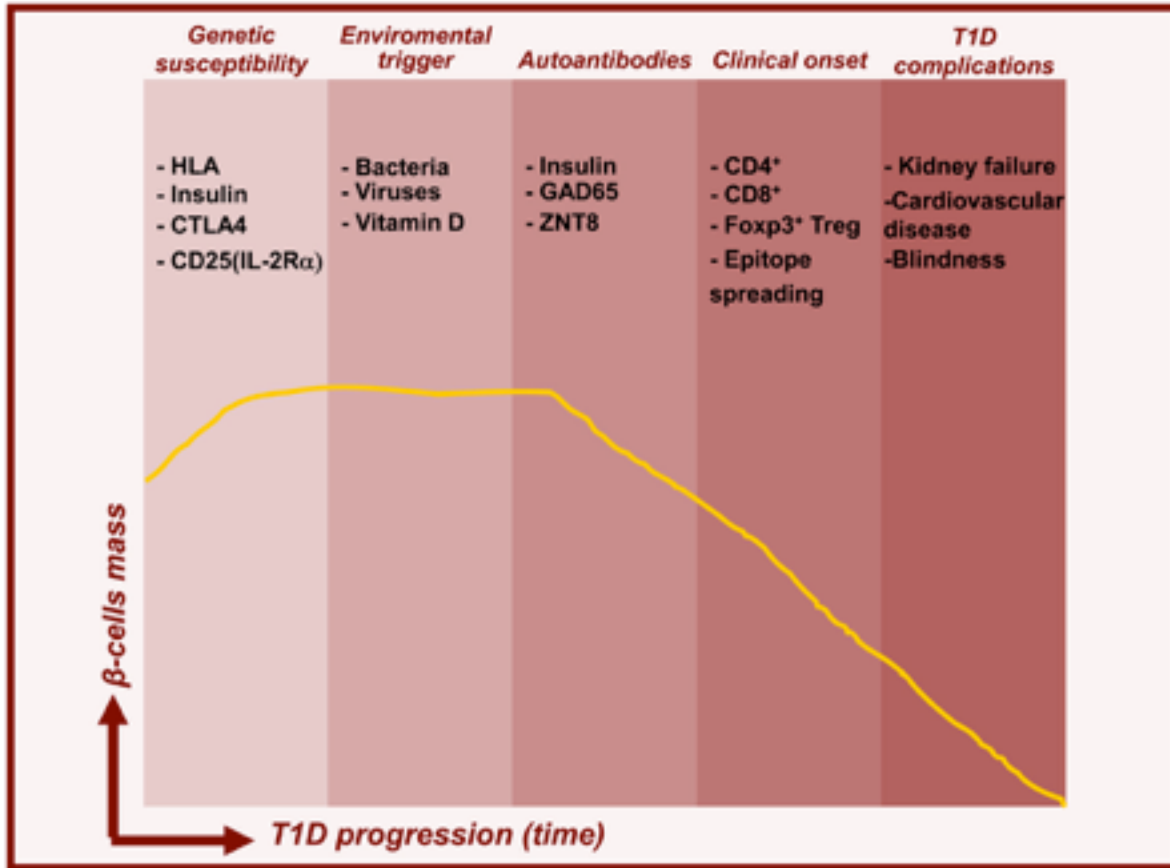

Figure 1-20: Timelines for type 1 diabetes. Model for temporal relationship between β-cell mass decline and features of T1D pathogenesis. In addition to genetic predisposition, environmental triggers induce islet autoimmunity and β-cell death leading to prediabetes and subsequent clinical onset and complications. Figure modified and reprinted with permission from Kornete *et al*, 2013 (183).

response to insulin resistance, but the increased translation of insulin protein results in endoplasmic reticulum stress and increased glucose metabolism causes oxidative stress (174,184). Another mechanism by which functional β-cell mass can be increased is increasing β-cell proliferation. Adult basal β-cell proliferation is very low (~0.5-1.0% in mice, <0.5% in humans) which suggests that β cells are largely post-mitotic, however, in times of metabolic stress and increased insulin demand β cell proliferation can increase (185). The degree to which this increase happens is variable, and seems to be more robust in mouse than human. Another mechanism for increasing the functional β-cell mass is β-cell hypertrophy. Finally, functional β-cell mass can be increased through neogenesis or the process of generating new β cells from a progenitor. This idea remains controversial, especially in humans, and conflicting data exists as to whether or not it has a meaningful contribution to β-cell mass dynamics (186,187). Together, these mechanisms of increasing functional β-cell mass are at odds with forces such as apoptosis and dedifferentiation that reduce functional β-cell mass (Figure 1-21).

Traditionally it was believed that the loss of functional β-cell mass observed in diabetes was due to β-cell death, however, recent evidence suggests that loss of β-cell identity could have a more significant contribution than β-cell death (98,188,189). This information is impactful because it demonstrates that an adaptive response to metabolic stress is β-cell dedifferentiation or loss of β-cell maturity. Further, it is possible that the dedifferentiated state allows β cells to mount an adaptive response, such as increased proliferation. Indeed, it has long been postulated that β-cells may need to partially dedifferentiate in order to enter a proliferative state, but *in vitro* evidence does not support a differentiation to the extent of attaining mesenchymal characteristics (190). Different populations of β cells are more able to

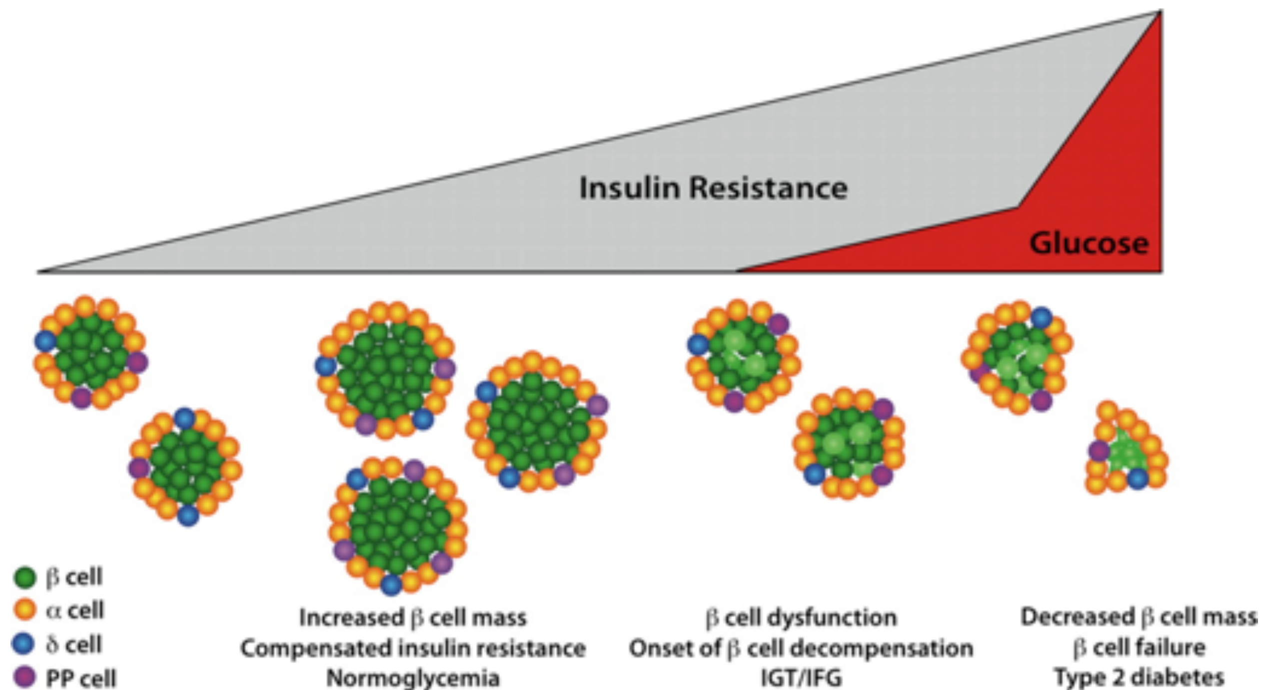

Figure 1-21: Dynamics of β-cell mass during the progression of insulin resistance to diabetes. In the setting of insulin resistance, the pancreatic islet insulin-secreting β-cells respond, in part, via a compensatory increase in β-cell mass, elevating plasma insulin levels to maintain normoglycemia. Changes in both β-cell proliferation and survival play important roles in this adaptive expansion of β-cell mass and in the reduction in mass that is associated with progressive β-cell dysfunction, eventually leading to T2D. These cellular processes are regulated by extracellular signals from a number of tissues, as described in detail in the text. IGT, Impaired glucose tolerance; IFG, impaired fasting glucose; PP, pancreatic polypeptide. Figure modified and reprinted with permission from Sachdeva and Stoffers, 2009 (191).

proliferate than other populations (192). Indeed, immature β cells lacking expression of the marker Flattop are nearly twice as proliferative as Flattop+ cells both postnatally and in response to pregnancy (192). One of the most notable things about β-cell dedifferentiation is that it appears to be a step-wise process. Indeed, there is a consistent and coordinated loss of regulatory transcription factors during β-cell dedifferentiation during T2D development. This loss is coordinated in the reverse of acquirement of β-cell maturity and function. In the setting of T2D, β cells first sequester MafA in the cytoplasm before losing expression completely. Shortly thereafter Pdx1 also becomes sequestered in the cytoplasm. The remaining cells are often Nkx6.1+ and may be insulin+, but they frequently begin to express glucagon and/or MafB suggesting a loss of β-cell identity (98,193). The increase in bihormonal cells then gives way to loss of insulin expression completely as the β cells become more dedifferentiated (193) and reviewed in (194). It is now appreciated that this process of dedifferentiation contributes to the loss of measurable β-cell mass in T2D (Figure 1-22). Additionally, stressed β cells may gain characteristics of other endocrine cell types further contributing reduced β-cell mass and/or a relative increase in α- or δ-cell mass (195).

Pancreatic Cancer

PDAC is by far the most common form of pancreatic cancer accounting for ~95% of cases. As noted earlier, PDAC has a tragically high mortality rate with only 8% of individuals diagnosed surviving past 5 years. One of the major contributing factors to this mortality rate is the late stage at which PDAC is generally diagnosed. Symptoms of early stage PDAC are generally mild and nondescript making diagnosis difficult until the disease is quite advanced

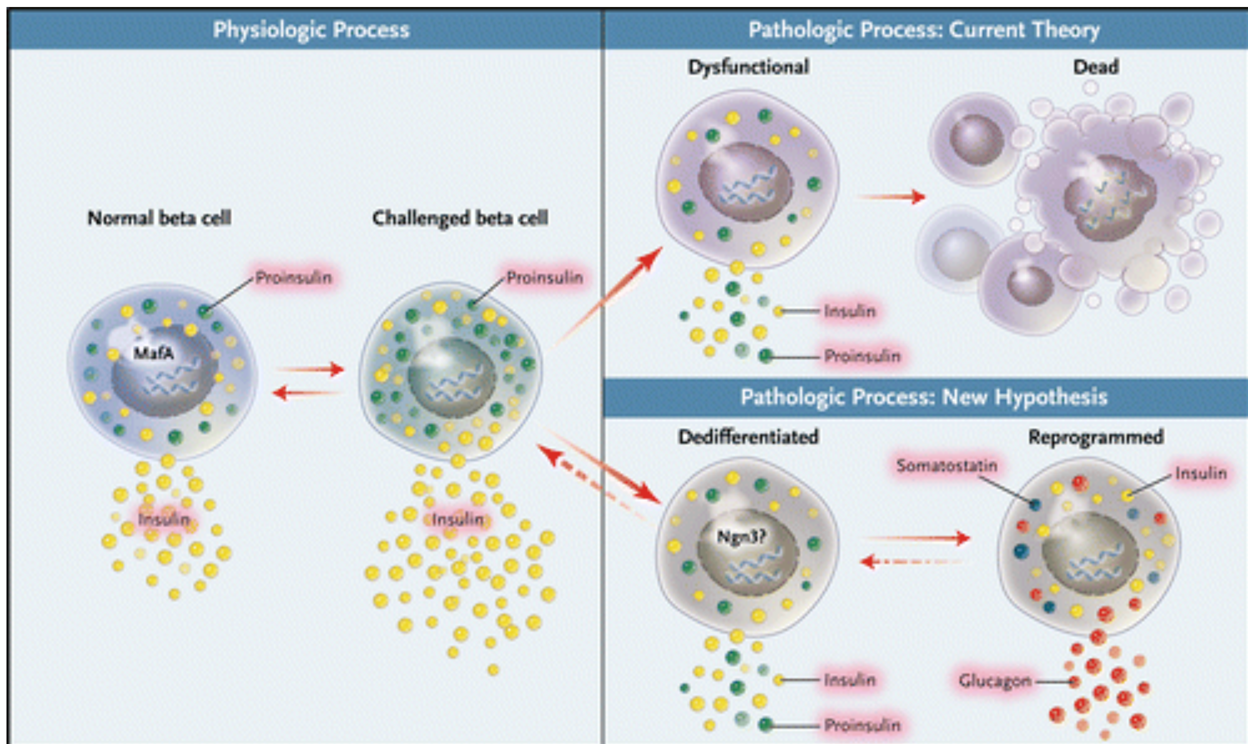

Figure 1-22: Redifferentiation — The Road to Future Therapy for Type 2 Diabetes? β-cell dedifferentiation, rather than death, may be the mechanism responsible for β-cell failure in T2D. When normal β cells (far left) are challenged by mild hyperglycemia or insulin resistance, they produce and secrete more insulin, thus maintaining euglycemia. Current thinking (upper right) suggests that oxidative stress, endoplasmic-reticulum stress, or both results in β-cell dysfunction, which, among other things, results in increased secretion of incompletely processed proinsulin. If unchecked, this process may trigger apoptosis. Talchai et al. (98) propose that stressed β cells may undergo dedifferentiation, decreasing expression of β-cell–specific genes, including transcription factors (such as MafA) and the enzyme that processes proinsulin; this may explain increased proinsulin secretion in T2D (lower right). Dedifferentiation may also involve gained expression of embryonic progenitor-cell markers such as Ngn3. Later on, these cells begin to express non–β-cell hormones such as somatostatin and glucagon. Figure modified and reprinted with permission from Dor and Glaser, 2013 (195).

and often metastatic. For this reason, it is also unclear if PDAC is a very slow disease developing over the course of years to decades, or if it is an extremely aggressive disease. It is possible that the poor survivability of PDAC is not due to its inherent aggressiveness, but rather that the presentation of symptoms only develops after a threshold of tumor growth and burden has been passed. Because early stage PDAC samples from humans are rare, we rely heavily on mouse models to try to understand the progression of precancerous lesions to PDAC.

Initiating mutations for PDAC are most often attributed to an activating mutation in the *Kras* gene that results in constitutive activity of the Kras GTPase giving it oncogenic characteristics. A single mutation in *Kras* is often inconsequential, but it can lead to genome instability and the accumulation of other mutations. Most frequently the second "hit" that occurs in PDAC oncogenesis is to a tumor suppressor such as *p53*, *Smad4*, or *Brca1*. As these mutations accumulate, it is believed that the pancreatic acinar cells progress through a series of well-defined metaplastic and neoplastic stages. The first stage in this progression, acinar-to-ductal metaplasia (ADM), does not require a mutation, but is rather a normal injury response of acinar cells whereby they partially dedifferentiate and become more like duct cells, both morphologically and at a gene/protein expression level (Figure 1-23). Importantly, ADM is reversible and the dedifferentiation aspect is thought to allow for recovery from whatever injury may have insulted the exocrine pancreas. A related, but not interdependent, lesion is pancreatic intra-epithelial neoplasia (PanIN). PanINs are true neoplastic conversions of cells rather than transient metaplasia, thus they are thought to often be independent of ADM (although ADM can progress to PanIN). PanINs can be staged by the level of transformation and neoplasia resulting in three classifications PanIN1, 2, and 3. Beyond PanIN3 is the

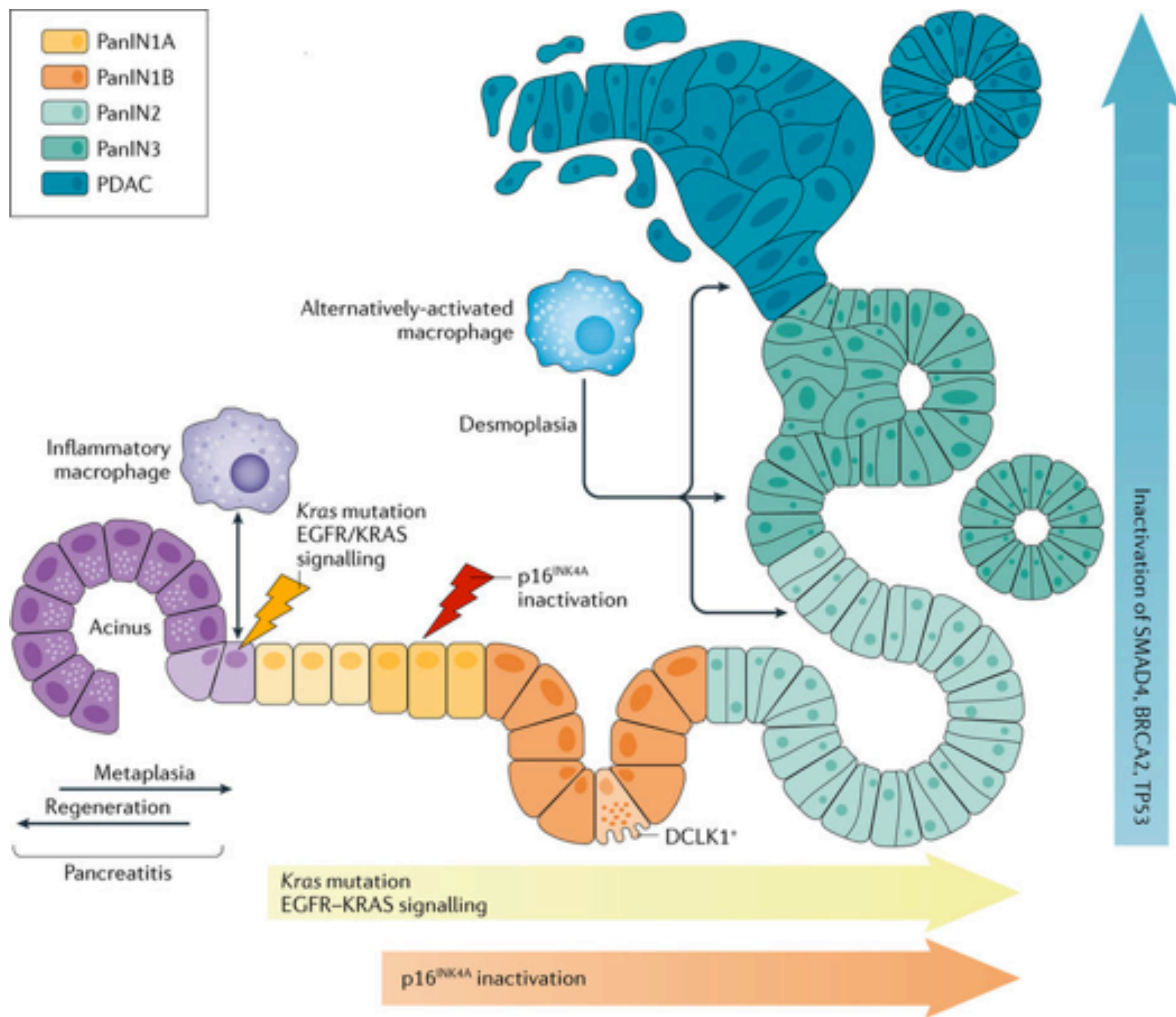

Figure 1-23: Oncogenic KRAS and inflammation as drivers of ADM and clonal expansion. Schematic showing how macrophage subtypes and genetic mutations contribute to ADM, clonal expansion and progression to pancreatic cancer. During pancreatitis ADM is a reversible process, but becomes irreversible when an oncogenic *Kras* mutation is present. The accumulation of KRAS activity as caused by oncogenic *Kras* mutations and epidermal growth factor receptor (EGFR)–wild-type KRAS signaling, as well as loss of senescence due to an additional inactivation of cyclin-dependent kinase inhibitor 2A (CDKN2A, also known as p16^{INK1A}), is needed for progression. Further progression to pancreatic intraepithelial neoplasia (PanIN)-2, carcinoma *in situ* (PanIN3) and PDAC occurs after acquisition of additional gene mutations in *Tp53* (p53), *Brca2* and *Smad4*. The progression to cancerous lesions occurs with an increase in desmoplasia. Cells positive for the serine/ threonine-protein kinase DCLK1 are of acinar origin, are formed mainly in low-grade PanIN lesions (PanIN1A, PanIN1B and PanIN2) and have cancer stem cell functions. Figure modified and reprinted with permission from Storz, 2017 (196).

adenocarcinoma of PDAC, although, definitive evidence of PanIN3 progressing to PDAC is not well established. Together, this information provides a collection of lesions in the pancreatic epithelium that are well-defined histologically and can be used to assess the severity of dysplasia (Figure 1-23).

Pancreatitis

Pancreatitis, in the simplest terms, is inflammation of the pancreas. It is, however, two distinct diseases that can have separate etiologies and outcomes. Regardless of the type of pancreatitis, there are some hallmarks of the disease that are consistent, including a substantial immune response with invasion of inflammatory cells, fibrosis (primarily collagen deposition), and possibly the development of ADM. Acute pancreatitis is defined by the rapid onset of the inflammatory state and severe abdominal discomfort. Most cases of acute pancreatitis are self-limiting, but those cases that are not require intervention which differs depending on the cause of the disease. The most common cause of acute pancreatitis is gallstones which block the pancreatic duct and result in activation of the pancreatic zymogens within the pancreas. In such cases the gallstones may need to be surgically removed. The second leading cause of acute pancreatitis is combined alcohol abuse and smoking for which there is no intervention at the time of disease presentation. While there are known genes mutations of which can lead to acute pancreatitis (e.g. *PRSS1*, *SPINK1*, *CFTR*), most of the remaining cases are idiopathic making them difficult to treat other than with palliative care (197).

The second form of pancreatitis is chronic pancreatitis which, as its name suggests, is defined by the long-term and progressive nature of the inflammation. Similar to acute

pancreatitis, alcohol abuse and smoking are significant contributors to risk of chronic pancreatitis development. Also, similar to acute pancreatitis, chronic pancreatitis can have genetic causes such as mutations in the genes listed above as well as a substantial contribution from uncharacterized mutations. It appears that a combination of environment and genetics determine whether the same stimulatory condition develops into acute or chronic pancreatitis (198). Pancreatitis in both forms serves as a potential, but not obligate, precursor for development of PanINs and PDAC (199).

Oc1 in Pancreatic Disease

Given the importance of Oc1 for the development of β cells and pancreatic ducts, it is possible that loss or mis-expression of Oc1 could predispose one to pancreatic disease. Oc1 dysfunction could contribute to defects in human pancreas development through its regulation of *Pdx1* expression (6,200). Loss of *Pdx1* expression results in pancreas agenesis in humans and mice, but some instances of human pancreatic hypoplasia or agenesis linked to impaired *Pdx1* expression show no alterations in the *Pdx1* coding region (200). In these cases, decreased *Pdx1* expression could potentially result from changes in the binding sites for or the activity of upstream regulatory factors such as Oc1.

Oc1 also regulates transcription factors and functional genes associated with diabetes, including genes encoding transcription factors associated with monogenic forms of diabetes known as Maturity Onset Diabetes of the Young (MODY). Oc1 directly regulates *Pdx1* (MODY 4), *Hnf4α* (MODY 1), and in the liver, *glucokinase* (MODY 2), and participates in a network regulating *Hnf1β* (MODY5) (15,145,146,201). Additionally, decreased or prematurely silenced

Oc1 expression in the endocrine lineage would be predicted to result in fewer differentiated endocrine cells, potentially predisposing one to diabetes later in life.

A stronger connection has been drawn between Oc1 and exocrine pancreas disease. In mice, inactivation of *Oc1* in the developing pancreatic epithelium results in ductal hyperplasia, ductal cysts, and periductal hemorrhaging (16). Further, this model has prominent ADM and is similar in many respects to human pancreatitis (16,202-204). Histological analysis has revealed that OC1 is up-regulated in human pancreatic acinar cells undergoing ADM, but OC1 expression is reduced in pre-cancerous PanIN lesions. Likewise, mouse models of ADM show a transient up-regulation of Oc1, but expression becomes reduced when the lesions progress to PanINs (Figure 1-24) (161,205). These results suggest a threshold level of Oc1 between normal acini and ducts with higher levels of Oc1 being required for the duct phenotype likely due to increased activation of duct-specific genes. Unexpectedly, the transient up-regulation of Oc1 in ADM occurs independently of the ductal transcription factor Sox9. Rather, Oc1 up-regulation in ADM is likely due in part to loss of micro-RNA-mediated Oc1 repression. Loss of micro-RNAs (through Dicer inactivation) in acini results in development of ADM, and this is dependent on Oc1 activity (206). The Jacquemin group has also shown that over-expression of Oc1 in acinar cells is sufficient to drive ADM onset (205). These results indicate that Oc1 (or its downstream effectors) is necessary for development of a ductal phenotype and possibly that different threshold levels of Oc1 regulate an acinar rather than duct phenotype (205). ADM is considered by many to be a precursor lesion for PanINs, which are themselves believed to be precursors to PDAC. As mentioned above, decreasing Oc1 expression correlates with increasing severity of PanINs in mice and humans. Indeed, OC1 is nearly undetectable in samples of human PDAC

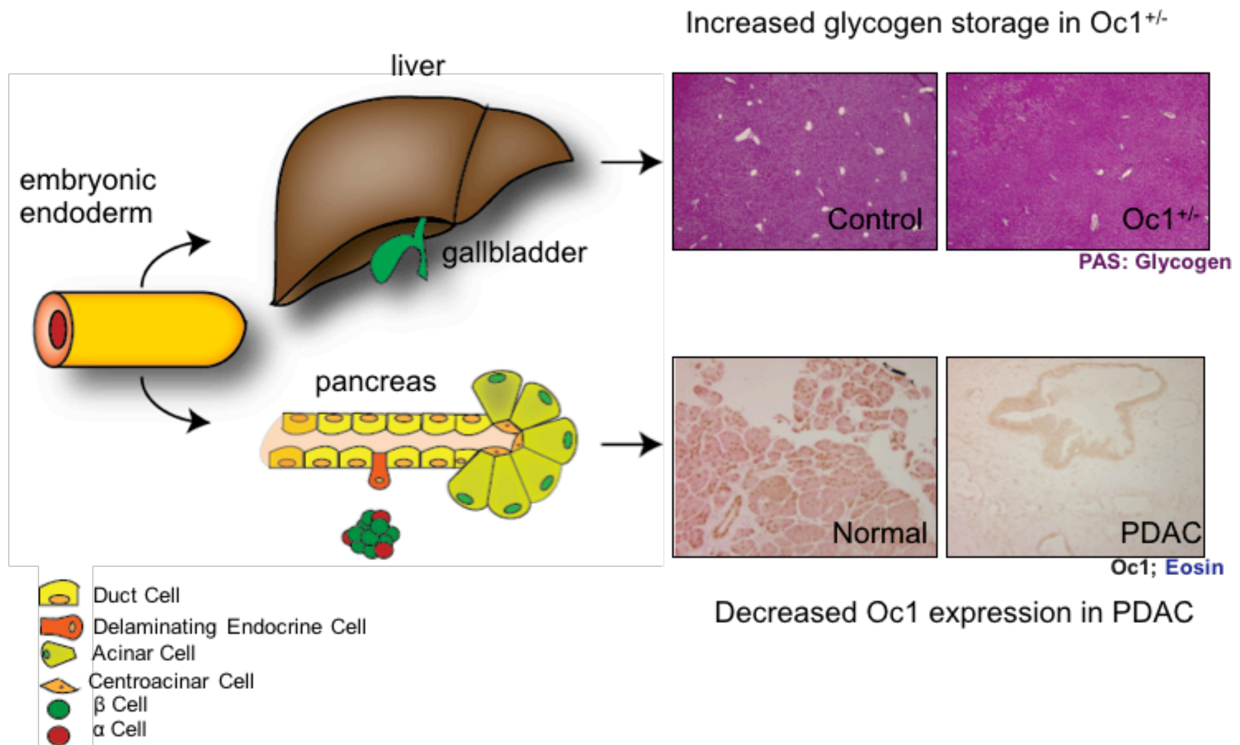

Figure 1-24: Implications of Oc1 loss in disease. The pancreas and liver, both endodermally-derived organs, are impacted by loss of Oc1 during development and disease. Above: Oc1 heterozygosity increases glycogen storage in the liver as shown by Periodic Acid Schiff staining in 3-week old mouse livers. Below: Oc1 is clearly expressed in the nuclei of normal, healthy ducts and acini of humans. Its expression is lost entirely from lesions of pancreatic ductal adenocarcinoma (PDAC). Figure modified and reprinted with permission from Kropp and Gannon, 2016 (151).

(161). These results are particularly interesting given that Oc1 has been shown to act through p53 to prevent epithelial-to-mesenchymal transition in lung cancer cells, setting a precedent for its role as a tumor-supressor (207)[74]. Together, these results demonstrate that Oc factors, especially Oc1, have a role in maintaining the differentiated state of the exocrine pancreas, and that loss of Oc1 leads to diseases of the exocrine pancreas.

Directed Differentiation

Of particular interest to the pancreas field is the directed differentiation of either embryonic or induced pluripotent stem cells to a β-cell fate. These protocols are designed to mimic the signaling that normally occurs during *in vivo* differentiation. With respect to directed differentiation of β cells, embryonic or induced pluripotent stem cells are manipulated in a step-wise fashion through the following stages using activators and inhibitors of different growth factor signaling pathways: definitive endoderm, posterior foregut, pancreatic progenitor, endocrine progenitor, β cell (208). Given that Oc1 regulation plays critical roles throughout this progression, it is surprising that it has not been utilized in protocols for *in vitro* differentiation of β cells. However, it has been used as a marker of effective differentiation down the posterior foregut pathway. Indeed, effective induction of definitive endoderm is often assessed by expression of *Oc1* (209). Signaling molecules including RA, activin A, FGF, and BMP are all capable of inducing an Oc1-expressing definitive endoderm and in many cases even more highly differentiated cell types (210-212).

Goals of the thesis

The regulation of pancreas development has been increasingly well defined in recent years, but many questions still remain about the role of pancreas regulatory factors in more nuanced contexts. Both Oc1 and Pdx1 are well studied for their individual roles, but less is known about how they function together to promote endocrine development and function. I have sought to determine the impact of reductions in both of these factors on β-cell specification, differentiation, maturation, and function. These studies have elucidated a novel cooperative function of Oc1 and Pdx1 in promoting competency for β-cell development and compensation. Additionally, I have investigated the role of Oc1 in development of the exocrine pancreas. While the role of Oc1 in duct development has previously been investigated, little was known about its role in development of acinar cells. I have sought to determine the necessity for Oc1 in acinar cell development and identify the direct and indirect transcriptional targets of Oc1 in that process.

CHAPTER II

MATERIALS AND METHODS

Methods for Chapters III and IV

Mutant and transgenic mice

Pdx1^{XSLacZ} (*Pdx1*^{LacZ}) animals are described in (9). *Oc1* floxed mice are described in (16). The *Protamine-Cre (Prm-Cre)* transgene is described in (213). All mice were on a mixed genetic background. Mice were maintained on a 12-hour light/dark cycle and provided food and water *ad libitum* (except where indicated). All experiments involving mice 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 2-1.

High-fat diet

8-week-old male mice were placed on high-fat diet (HFD; 60% kcal from fat; BioServ F3282, Frenchtown, NJ) for 5 weeks.

Tissue dissection, preparation, and histology

Digestive organs were fixed for 4 hours in 4% paraformaldehyde (PFA) at room temperature, dehydrated, cleared in xylenes, and embedded in paraffin. Livers were fixed 24 hours in 4% PFA at 4°C, dehydrated, cleared in xylenes and embedded in paraffin. Paraffin embedded tissues

Table 2-1: Genotyping primers

Allele	Forward Primer	Reverse Primer
Oc1 Floxed	GTCGTCGACCTCTCTCCTGTCTCCCTC AGTATCC	ATAAGCGGCCGCCCTCCCTCTCTCTTTCCATC
Oc1 Deletion	GTCGTCGACCTCTCTCCTGTCTCCCTC AGTATCC	ATAAGCGGCCGCGCCTGCAGGTTTGGAATCTGTG
Cre	ACCTGAAGATGTTCGCGATTATCT	ACCGTCAGTACGTGAGATATCTT
LacZ	AATCGTCTGACCGATCC	ACATCCAGAGGCACTTCACC

were cut at 5 µm, deparaffinized in xylenes and rehydrated in water for all downstream analyses. Periodic Acid Schiff staining was performed following the manufacturer's protocol (Sigma-Aldrich). In brief, slides were incubated 5' in Periodic Acid solution, rinsed in water, incubated 15' in Schiff's Reagent, rinsed in water, counterstained with Hematoxylin Solution, (Gill No.3), dehydrated and mounted with a xylene-based mounting medium. Fluorescent and bright field images were captured using an Olympus BX41 microscope, the Aperio ScanScope microscope and slide scanner (Vista, CA), or Nikon 600. Digital images were captured and quantified using MagnaFire software (Optronics Engineering, Goleta, CA), or ImageScope software of the Aperio software suite (Vista, CA) for insulin, glucagon, Ki67 and Nkx6.1.

β-cell mass and immunofluorescence analysis

For β-cell mass, sections ~250 µm apart (5-10 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. At least 1-2% of the entire pancreas was imaged. Slides were imaged using MetaMorph or a macro built in Genie (Aperio System, Vista, CA). For all immunofluorescence analyses, 2-3 (embryo and neonate) or 5-7 (adult) sections per animal ~200 µm apart were analyzed. For the mixed islet phenotype, non-mantle α-cells were classified as those more than 2 cell diameters from the edge of the islet. Primary antibodies are listed in Table 2-2. Species-specific secondary antibodies were conjugated to Cy2, Cy3 or Cy5

Table 2-2: Primary antibodies used for Chapters III-IV

Antibody	**Vendor**	**Dilution**
Guinea Pig anti-Insulin	Dako	1:500
Guinea Pig anti-Insulin	Millipore	1:400
Mouse anti-Glucagon	Millipore	1:500
Guinea Pig anti-Glucagon	Millipore	1:400
Rabbit anti-Ki67	Abcam	1:500
Goat anti-Glut2	Santa Cruz Biotechnologies	1:250
Rabbit anti Nkx6.1	Novus Biologicals	1:500
Goat anti-Pdx1	Beta Cell Biology Consortium	1:10,000
Mouse anti-Neurog3	Beta Cell Biology Consortium	1:1000
Rabbit anti-Pax6	Covance	1:1000
Rabbit anti-MafA	Bethyl	1:400
Rabbit anti-MafB	Bethyl	1:800
Mouse anti-E-Cadherin	Santa Cruz Biotechnologies	1:400
Mouse anti-Synaptophysin	Millipore	1:500
Mouse anti-Proinsulin	Developmental Studies Hybridoma Bank	1:100
Rabbit anti-Urocortin3	Phoenix Pharmaceuticals	1:1000
Mouse anti-Ghrelin	Santa Cruz Biotechnologies	1:250

and diluted 1:500. Labeling with the following antibodies required heat-mediated antigen retrieval with citric acid buffer: α-Insulin (Dako), mouse α-Glucagon, α-ghrelin, α-Ki67, α-Glut2, α-Nkx6.1, α-Pdx1, α-Urocortin3, α-Proinsulin. α-Pax6. Labeling with the α- Neurog3 antibody required citric acid antigen retrieval and amplification with Perkin Elmer tyramide tissue amplification kit. Labeling with the following antibodies required heat-mediated antigen retrieval with TEG (10 mM Tris; 0.5 mM EGTA): α-MafA, α-MafB, α-Synaptophysin.

Islet isolation for RNA acquisition

P1 and P14

Isolation of islets from newborn or neonatal mice required collagenase digestion of whole dissected pancreas at 37°C and hand-picking of islets away from exocrine tissue. For P14 islets, hand-picking took place following a histopaque-1077 (Sigma-Aldrich) gradient.

At P1, picked islets were placed immediately in 500 µl Trizol reagent (ThermoFisher), lysed by vortexing, and RNA was isolated using the RNeasy Micro kit (Qiagen) according to manufacturer's instructions. 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 instruction (Clontech).

At P14, islets from more than one animal were pooled (if necessary) to reach 40 islets per sample. Islets were placed immediately in 500 µl Trizol reagent (ThermoFisher), lysed by

vortexing and RNA was isolated using the RNeasy Micro kit (Qiagen) following the manufacturer's instructions. RNA concentration and integrity were assessed using a Qubit® Fluorometer (ThermoFisher) and the Qubit® RNA HS Assay Kit.

P28

Isolation of islets from 4-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 500 µl Trizol reagent (ThermoFisher), lysed by homogenizing with a Tissuemiser (Fisher Scientific), and RNA was isolated using the RNeasy Mini kit (Qiagen) following the manufacturer's instructions. RNA concentration and integrity were assessed using a ND-1000 Spectrophotometer (NanoDrop) and the 2100 Electrophoresis Bioanalyzer (Agilent) at VANTAGE. cDNA was prepared from 50-350 ng islet RNA using the Superscript III First-Strand synthesis system (Invitrogen).

Gene expression analysis

Quantitative Real-Time PCR

Real-time PCR reactions were carried out in technical triplicates with iQ SYBR Green supermix (Bio-Rad) according to the manufacturer's instructions on a CFX Real-Time PCR Detection system (Bio-Rad) in the Vanderbilt Molecular and Cellular Biology Resource Core. Primers used for quantitative reverse-transcriptase PCR are listed in Table 2-3. Gene expression from P14 islets was analyzed with a custom-designed codeset from nanoString. 50-100 ng of total RNA

Table 2-3: qRT-PCR primers

Gene/Region	Forward Primer	Reverse primer
Ins1 3' UTR	GCTTGTGATAAAACACCAGGAA	CAGTGGCATTTACACGGTTG
Ins1 3' coding sequence	AGCATCTGCTCCCTCTACCA	GGTGGGCCTTAGTTGCAGTA
Ins1 5' UTR	GTAACCCCCAGCCCTTAGTG	CTGCTTGCTGATGGTCTCTG
Ins2 3' coding sequence	CAGAAGCGTGGCATTGTAGA	GCTGGTAGAGGGAGCAGATG
Ins2 5' UTR	GCCCTAAGTGATCCGCTACA	CTGGAAGATAGGCTTCCTGCT
Gcg Exon 1	ACTTTGTGGCTGGATTGCTT	TGATCTGGGGTTCTCCTCTG
Gcg 3' coding sequence	GGCTGATTCAAACCAAGATCA	GGCACGAGATGTTGTGAAGA
Gcg middle coding sequence	ATTCACCAGCGACTACAGCA	TGACGTTTGGCAATGTTGTT
Sst	ACCCCAGACTCCGTCAGTTT	CCAGGGCATCATTCTCTGTC

was used in each reaction. Differential expression and pathway analysis were performed using the nSolver software from nanoString (214).

TaqMan Low Density array (TLDA)

RNA was isolated as described above and 150-300 ng cDNA was prepared using the SuperScript III First Strand Synthesis System Kit according to manufacturer's instructions (Invitrogen). Genes were analyzed using TaqMan Universal PCR Mastermix (with UNG, Applied Biosystems) on custom-designed TLDA cards. 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.

Pancreatic insulin/glucagon content

Pancreatic hormones were extracted from whole pancreas using an acid-alcohol extraction. Insulin and glucagon content were measured by the Vanderbilt Hormone Assay and Analytical Services Core as described in (16,215).

Serum insulin and glucagon measurements

At P14, whole blood was collected and serum isolated by coagulating blood on ice for 30' before centrifugation at 1,800xG. Serum insulin levels were measured with the Alpco Mouse Ultrasensitive Insulin ELISA following the manufacturer's recommended protocol. Serum glucagon levels were measured using the Crystal Chem Mouse Glucagon ELISA following the manufacturer's recommended protocol. In adults, whole blood was collected in EDTA-coated

microvettes (Sarstedt) prior to centrifugation at 13,000 RPM at room temperature. Insulin was measured by the Vanderbilt Hormone Assay and Analytical Services Core as described in (216).

***In vivo* and *in vitro* analysis of glucose homeostasis**

Intra-peritoneal glucose tolerance tests (IPGTT) were performed as in (217). Static incubations were performed as follows: Islets were isolated, purified, and cultured overnight in RPMI 1640, 1% pen/strep, 10% FBS, 5.6 mM glucose. The next day, islets were washed through a plate of DMEM with 0.1 % BSA, 5.6 mM Glucose. Islets were size-matched to approximately 20 islet equivalents (IEQ) for each sample into 12-well untreated plates containing 5.6 mM glucose, 16.7 mM glucose or 5.6 mM glucose plus 20 mM arginine. Islets were cultured for 70 minutes (10 minutes "warm-up", and 60 minutes for secretion), after which the plates were placed on ice for 10 minutes. Islets were collected into 1.5 mL tubes, spun down and hormones extracted using acid ethanol for 24 hours. Media was collected into 1.5 mL tubes. Media and extracts were measured for insulin using RIA (Millipore RI 13-K). Islet perifusion analyses were performed on islets isolated from 3 month old male mice as in (157).

***Ex vivo* Proliferation Assay**

Islets were isolated from 8-week-old male mice. Islet culture and analysis of proliferation was assessed as in (218).

Statistics

All data are presented as the mean ± SD. Data was tested for unequal variance with a Brown-Forsyth test. Analyses performed for parametric data were two-tailed students t-test, One- or Two-way ANOVA (where appropriate) with Tukey correction for multiple comparisons. Analyses performed for nonparametric data were Mann-Whitney U-test and Kruskal-Wallace tests. using GraphPad Prism version 6.

Methods for Chapter V

Mutant and transgenic mice

Oc1 floxed mice are described in (16). The *Pdx1-Cre* transgene is described in (219). $Oc1^{\Delta panc}$ mice had the genotype *Oc1*$^{Fl/Fl}$; *Pdx1*-Cre^{Tuv}. The Cre-dependent enhanced yellow fluorescent protein (EYFP) reporter allele was generated as described previously (220), where homologous recombination was used to introduce *EYFP* into the *ROSA26* (R26R) locus. The *Elastase-Cre*^{ERT2} was a gift from Steve Koneiczny (Purdue) and has not been previously published. $Oc1^{\Delta acini}$ mice had the genotype *Oc1*$^{Fl/Fl}$; *Elas-Cre*^{ERT2}; *R26R*$^{LSL\text{-}eYFP/+}$. All mice were on a mixed genetic background. Mice were maintained on a 12-hour light/dark cycle and provided food and water *ad libitum* (except where indicated). All experiments involving mice 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. Genotyping primers for the *Oc1-flox* allele and *Cre* are listed in Table 2-1 and the primers for the *eYFP* allele are listed in Table 2-4.

Table 2-4: Genotyping primers for Chapter V

Allele	Forward Primer	Reverse Primer
Rosa26 WT	TCCGAATTCAGTGACTACAGATG	CTAGCCACCATGGCTTGAGT
Rosa26$^{LSL\text{-}eYFP}$	TCCGAATTCAGTGACTACAGATG	ATGTCTTTCCCCAGCACAGT

Tissue dissection, preparation, and histology

Digestive organs were fixed for 4 or 24 hours in 4% PFA at room temperature, dehydrated, cleared in xylenes, and embedded in paraffin. Paraffin embedded tissues were cut at 5 μm, deparaffinzed in xylenes and rehydrated in water for all downstream analyses. Fluorescent and bright field images were captured using an Olympus BX41 microscope, the Aperio ScanScope microscope and slide scanner (Vista, CA), or Nikon 600. Digital images were captured and quantified using MagnaFire software (Optronics Engineering, Goleta, CA), ImageScope software of the Aperio software suite (Vista, CA) for eYFP and amylase. For hematoxylin and eosin staining, slides were incubated 3' in hematoxylin, washed 15' in running tap water, rinsed once in acid alcohol, rinsed 2x in water, and counterstained with eosin. Slides were then dehydrated and mounted with a xylene-based mounting medium. For Sirius Red/Fast Green staining, slides were incubated 1 hour in the staining solution (1mg/ml Direct Red 80 [Sigma], 1mg/ml Fast Green [Sigma] in 3% Picric Acid solution), washed 2x5' in acidified water (1% glacial acetic acid solution), dehydrated, and mounted with a xylene-based mounting medium.

Immmunohistochemistry and immunofluorescence analyses

For embryonic and postnatal analyses, tissue sections ~150 μm apart (2-4 per animal) were analyzed. Primary antibodies are listed in Table 2-5. For immunohistochemistry analyses, a peroxidase-conjugated secondary antibody was used prior to visualization with a DAB Peroxidase Substrate Kit (Vector Laboratories) or a biotinylated secondary antibody with amplification by an ABC Standard Peroxidase Kit (Vector Laboratories). For immunofluorescence analyses, species-specific secondary antibodies were conjugated to Cy2,

Table 2-5: Primary antibodies used for Chapter V

Antibody	Vendor	Dilution
Rabbit anti-GFP	Novus Biologicals	1:500
Rat anti-CK19 (Troma III)	Developmental Studies Hybridoma Bank	1:500
Goat anti-Amylase	Santa Cruz Biotechnologies	1:500
Mouse anti-Synaptophysin	Sigma	1:500
Rabbit anti-Ki67	Abcam	1:500
Rabbit anti-Mist1	Gift from Steve Koneiczny	1:250

Cy3 or Cy5 and diluted 1:500. Labeling with the α-GFP antibody required heat-mediated antigen retrieval in a basic (Tris, pH 10.0 or TEG, pH 9.0) solution. Labeling with the α-Synaptophysin antibody required heat-mediated antigen retrieval with TEG, pH 9.0 solution.
Labeling with the α-CK19 antibody required incubation with 0.2 mg/ml Proteinase K. Labeling with the α-Mist1 antibody required heat-mediated antigen retrieval with a citric acid (pH 6.0) solution. The signal was amplified with a biotinylated secondary antibody and the Peroxidase Standard Kit (Vector).

Gene expression analysis

Tissue processing for RNA acquisition

At e15.5, whole pancreata were dissected from embryos, placed immediately in 500 μl Trizol reagent (ThermoFisher), and lysed by vortexing. At e18.5 and P2, pancreata were dissociated with a collagenase digestion and exocrine-enriched samples (islets excluded) were collected for RNA extraction in 500 μl Trizol reagent (ThermoFisher). Total RNA was generated from 3 animals per genotype. RNA was isolated using the RNeasy Micro kit (Qiagen) according to manufacturer's instructions. 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.

RNA Sequencing

Libraries were generated for each sample by the Vanderbilt University VANTAGE Core. Libraries were pair-end sequenced to 100 bp on an Illumina hiSeq2000. EdgeR or CuffDIff was used to

determine differentially expressed genes using an FDR cutoff of 0.05 and log_2-fold change of 2. We used the comparison enrichment analysis using Ingenuity Pathway Analysis (IPA) from Ingenuity Systems (Qiagen, MD), which allows for the visualization of canonical pathways and biological functions across multiple genotypes. (http://www.ingenuity.com/products/ipa).

Chromatin immunoprecipitation

McDonald Protocol

For each biological replicate, 2 P21 pancreata were combined and homogenized with mortar and pestle in liquid nitrogen. Samples were fixed 10 minutes in 1% formaldehyde at 37°C and reactions were quenched by adding glycine to a final concentration of 0.125 M. Chromatin was sheared with a Diagenode Bioruptor for a total of 45 minutes to an average length of 300 bp. ChIP was performed with 200 μg of DNA and 10 μg of Oc1 antibody (Rabbit α-Hnf6, Santa Cruz Biotechnology sc-13050) and Protein A Dynabeads (Invitrogen). DNA was purified from each reaction with phenol-chloroform extraction.

Stoffers Protocol

For each biological replicate, 3-5 e18.5 pancreata were combined and minced with fine scissors. Samples were fixed 10 minutes in 1.11% formaldehyde at room temperature and reactions quenched by adding glycine to a final concentration of 0.125 M. Samples were homogenized by hand with plastic pestles. Chromatin was sheared with a Diagenode Bioruptor for a total of 22.5 minutes to an average length of 300bp. ChIP was performed with 200 μg of DNA and 10 μg of Oc1 antibody (Rabbit α-Hnf6, Santa Cruz Biotechnology sc-13050) and Protein A/G plus agarose

beads (Santa Cruz Biotechnology). DNA was purified from each reaction with the MinElute PCR Purification kit (Qiagen).

DNA Sequencing and analysis

Libraries were generated by Hudson Alpha Institute for Biotechnology. Libraries were pair-end sequenced at 50 bp on an Illumina HiSeq 2000 platform. Sequences were aligned to the mm10 genome and peaks were called using MACS2 software with an FDR of 0.05

Quantitative PCR

Enrichment for Oc1 targets was assessed by quantitative PCR. Reactions were carried out in technical triplicates with iQ SYBR Green supermix (Bio-Rad) according to the manufacturer's instructions on a CFX Real-Time PCR Detection system (Bio-Rad) in the Vanderbilt Molecular and Cellular Biology Resource Core. Primers used for quantitative reverse-transcriptase PCR are listed in Table 2-6. 125 pg of DNA was used in each reaction.

Statistics

All data are presented as the mean ± SD. Data was tested for unequal variance with a Brown-Forsyth test. Analyses performed for parametric data were two-tailed students t-test, One- or Two-way ANOVA (where appropriate) with Tukey correction for multiple comparisons. Analyses performed for nonparametric data were Mann-Whitney U-test and Kruskal-Wallace tests. using GraphPad Prism version 6.

Table 2-6: Primers used for ChIP-PCR

Human	Forward Primer	Reverse Primer
HNF4α	CCAGAGTGCAGGACTAGGA	GGTGGGTGGATACGTTAAAGAG
FoxA2	GACACACTGTTAGCCTTGAGATA	CGCGCTGCCAAACATAAC
SLC2a2	GGCAGCTCAGCATATCTCAT	CCAGAGGCAATCACATTTCAAG
G6PC2	CACCCTGAACATGTTTGCATC	CAACCCAGCCCTGATCTTT
HNF1α	GCCAGGGAGTCAGAGAGA	GATCAGGACCTTGGACAGAAA
HNF1β	GAGGACCCACAAACGATCC	CAGAGGATCTGGTCTCAGTCT
Negative region	CTCTGTCCTAAAGCGGAGTG	CTAGACTTTGACAGCTGGTTACT
Mouse		
Hnf1β	AAGCACCCACTGCTCTT	GATTACAGGTGGTTGTGAGC
Pdx1	GGAGATAGCATCGAGTCCCT	ACCCGTGCACCTAATCTCTA
Negative region (Oc1)	GGTGGCACAGTGACGCA	CATCTAGCCAGACTCCAGGAA
Negative region (H3K27ac)	GCCATTGATAGCATAGGGCTTA	TAGGGTGAGTCTCAGTGATCTT

CHAPTER III

THRESHOLD-DEPENDENT COOPERATION BETWEEN PDX1 AND OC1 IS NECESSARY FOR SPECIFICATION AND DIFFERENTIATION OF PANCREATIC β CELLS

Adapted from Henley *et al.,* "Threshold-dependent cooperativity of Pdx1 and Oc1 in pancreatic progenitors establishes competency for endocrine differentiation and β cell function", *Cell Reports*, 2016

Introduction

Knowledge gained from developmental biology has been instrumental in deriving glucose-responsive, insulin-secreting pancreatic β cells from embryonic stem (ES) cells or induced pluripotent stem (iPS) cells to generate a cell-based therapy for the treatment of diabetes (208,221,222). Of particular interest are signaling molecules and transcriptional regulators that direct the β-cell fate or facilitate the generation of fully functional β cells. Many elegant single gene inactivation studies revealed critical roles for specific transcription factors in different stages of pancreas development and endocrine differentiation. However, very few studies analyzed the functional consequences of combinatorial genetic manipulations of structurally un-related pancreas transcription factors during development (14,60,223). In this series of experiments, I collaborated with Dr. Doris Stoffers laboratory at the University of Pennsylvania to analyze the functional cooperativity of the Pdx1 and Oc1 transcription factors in endocrine specification and differentiation. We identified a requirement for a combined

threshold of activity of these two factors in setting up a genetic program for endocrine differentiation and β-cell function.

Pdx1 and *Oc1* are co-expressed in MPCs in the early pancreatic bud and later in the undifferentiated, bipotential duct/endocrine cell pool located within the "trunk" domain of the pancreatic epithelium. Pdx1 and Oc1 each activate *Neurog3* expression and evidence from in vitro studies suggests that a physical interaction between these two factors promotes endocrine specification (154). Pdx1 occupies an evolutionarily conserved *Neurog3* enhancer at e13.5 and, in reporter assays, Pdx1 transactivation via this enhancer was significantly enhanced by Oc1. Mice homozygous for a *Pdx1* allele with a premature C-terminal truncation ($Pdx1^{\Delta C/\Delta C}$) display a global reduction in endocrine lineages and decreased numbers of $Neurog3^{+}$ progenitors at e13.5. The Pdx1-Oc1 interaction is greatly attenuated, though not abolished, with the *Pdx1* C-terminal truncation (154).

We hypothesized that the Pdx1-Oc1 interaction is critical at the multi- or bipotent stages to promote the specification of pancreatic endocrine progenitors by regulating *Neurog3* and other developmentally important genes. To assess the significance of the Pdx1-Oc1 interaction *in vivo*, we generated animals globally heterozygous for either or both genes. To date, no developmental phenotype for either single heterozygous animal had been reported. At e13.5, I found that double heterozygote pancreata contained normal numbers of glucagon+ and Neurog3+ cells, suggesting that the first wave of endocrine differentiation is unaffected. By e15.5 at the height of the secondary wave of differentiation, the numbers of Neurog3+ endocrine progenitors and insulin+ and glucagon+ cells were reduced. Whole transcriptome analysis at e15.5 revealed a dramatic and unique impact of *Pdx1-Oc1* heterozygosity on the

endocrine compartment. Later stages of endocrine differentiation and function, well after the normal down-regulation of *Oc1* in the endocrine lineage, were also defective in double heterozygotes. Thus, Pdx1 and Oc1 cooperate to promote endocrine specification and subsequent β-cell maturation, most likely by establishing a state of competency in progenitors that allows for later steps in endocrine differentiation.

Results

Combined *Pdx1* and *Oc1* heterozygosity has a broad effect on the transcriptional network regulating endocrine development

To determine the effect of combined global heterozygosity for *Pdx1* and *Oc1* on global pancreatic gene expression during development, we analyzed the transcriptome of pancreata from control (CON), *Pdx1*$^{lacZ/+}$ (in which one allele of *Pdx1* contains a lacZ cassette in place of the homeodomain rendering it a null allele (9)), *Oc1*$^{+/-}$, and *Pdx1*$^{lacZ/+}$;*Oc1*$^{+/-}$ (hereafter: double heterozygous, DH) animals. Pancreata were collected at e15.5 since all pancreatic lineages are present at this stage, and the greatest number of Neurog3+ cells can be detected (43). RNA-Sequencing (RNA-Seq) was performed and the results analyzed at the University of Pennsylvania. A total of 2304 genes were differentially expressed in at least one of the three experimental genotypes (Figure 3-1A). Expression of 102 genes was altered in *Pdx1*$^{lacZ/+}$ pancreata (Figure 3-1B). *Oc1*$^{+/-}$ pancreata showed the greatest number of gene expression changes, with more than 2000 genes affected. In contrast to *Pdx1* single-heterozygotes (SH), β-cell genes such as *Ins1, Ins2,* and *Iapp* were increased in *Oc1*$^{+/-}$ SH, consistent with a role for Oc1 in suppressing β-cell differentiation (157). The transcriptome of Pdx1-Oc1 double heterozygotes

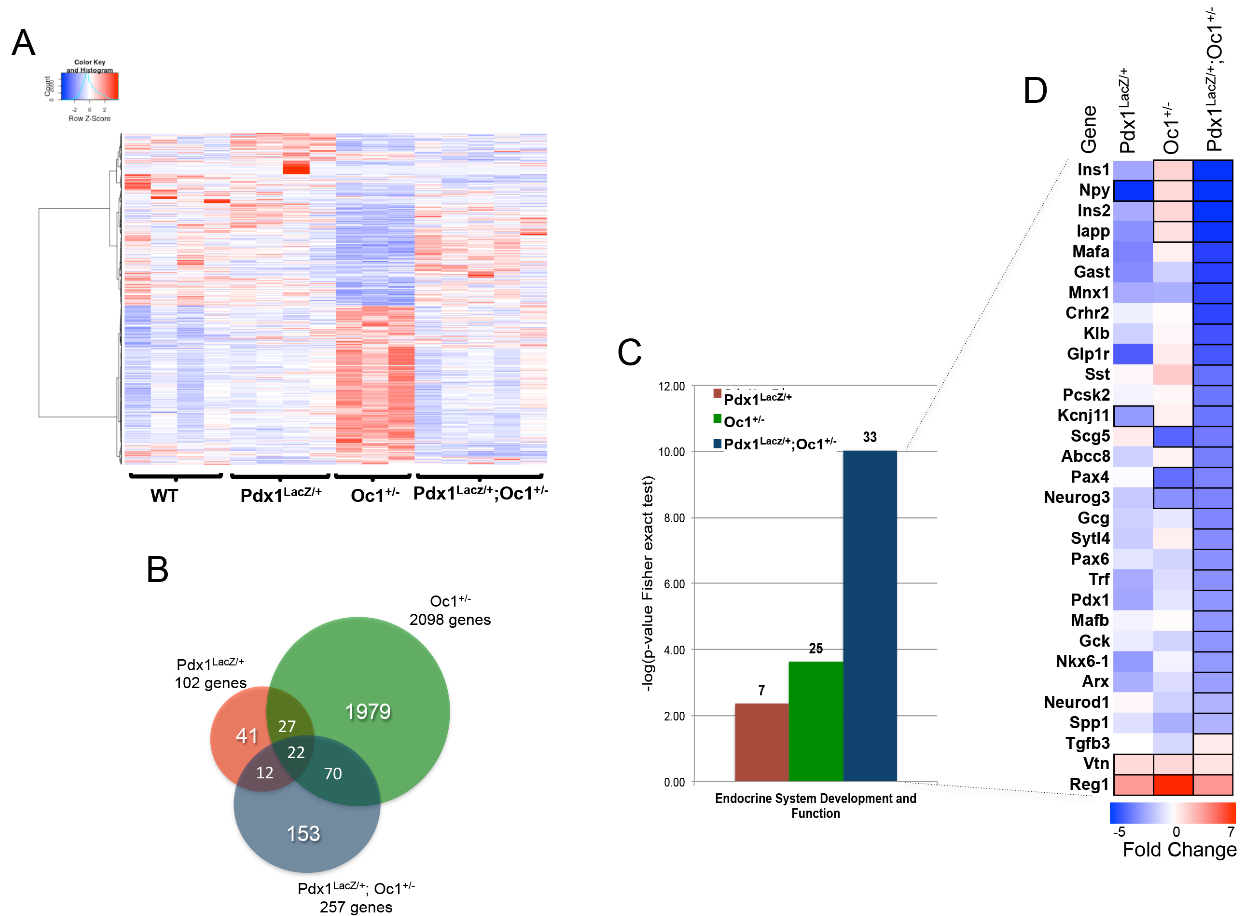

Figure 3-1: Combined heterozygous reduction in *Pdx1* and *Oc1* gene dosage has a broad impact on the transcriptional network of endocrine pancreas progenitors. (A) Hierarchical clustering of 2331 differentially expressed genes in individual pancreata at e15.5 from *Pdx1$^{LacZ/+}$*, *Oc1$^{+/-}$* and *Pdx1$^{LacZ/+}$;Oc1$^{+/-}$* mice; (B) Venn diagram depicting the number of altered genes in the *Pdx1$^{LacZ/+}$*, *Oc1$^{+/-}$* and *Pdx1$^{LacZ/+}$;Oc1$^{+/-}$* mice compared to Control; (C) Endocrine system development and function gene ontology category in each genotype, according to negative log of p-value from Fisher exact test. The numbers above each column represent the number of genes enriched in each category; (D) Heat map of endocrine development and function genes. Up- or down-regulated genes reaching statistical significance 9false discovery rate less than 0.1 and fold change higher than 0.5 versus Control) are highlighted with bold black borders. Figure modified and reprinted with permission from Henley *et al*, 2016 (224).

showed a pattern of gene dysregulation distinct from either single heterozygote transcriptome. Of the 257 genes affected in DH, 153 genes such as *MafA, MafB, NeuroD1, Nkx6.1,* were specifically altered in DH compared to CON (Figure 3-1B), providing strong support for functional cooperation between these two transcription factors to regulate a distinctive genetic program. Furthermore, the shift of gene expression patterns of *Oc1*$^{+/-}$ SH versus DH or *Pdx1*$^{lacZ/+}$ SH versus DH (Figure 3-1A) suggests the Pdx1-Oc1 interaction acts cooperatively or antagonistically at the level of broad categories of genes. Interestingly, there were 27 genes with altered expression in either SH but not DH. These genes tended to be associated with the exocrine lineage including *Amy2b*, *Serpina6*, *Serpinf2*, and *Ear6/7*. It is possible that Oc1 and Pdx1 have independent effects on this lineage, but somehow double heterozygosity mitigates the effect of either factor alone.

To assess systematic changes in expression of genes involved in canonical signaling pathways, disease and biological function categories, and molecular networks of genes altered in the three experimental genotypes, our collaborator, Dr. Diana Stanescu (University of Pennsylvania) performed comparison-enrichment analysis using the Ingenuity Pathway Analysis (IPA). The top gene ontology categories ascribed to genes altered in *Oc1*$^{+/-}$ or in *Pdx1*$^{lacZ/+}$ SH pancreata were "cancer", "embryonic development" and "cellular development" or "gene expression", respectively (Appendix Table A-1. The genes altered in DH pancreata clustered primarily in the "endocrine system development and function", "carbohydrate metabolism", "endocrine system disorders" categories (Figure 3-1C, Appendix Table A-1). The "endocrine system development and function" category was enriched for genes associated with "quantity of endocrine cells" and "quantity of beta and alpha islet cells" (Appendix Table A-2), indicating a

possible impact on endocrine progenitors and endocrine differentiation. Genes in these categories clustered less strongly in *Pdx1*$^{lacZ/+}$ or *Oc1*$^{+/-}$ SH because the vast majority of genes were specifically altered in the DH dataset and not in SH pancreata (Figure 3-1C, D and Appendix Table A-2).

The largest effects of double heterozygosity were on pancreatic endocrine-hormone expression. *Insulin* (both *Ins1 and Ins2*) was most affected in the DH pancreata, being significantly reduced ~3.5 fold compared to both CON and SH animals (Figure 3-1D). mRNA levels of other islet hormones (*Gcg*, *Iapp* and *Sst*) were also decreased in the DH animals by 30-50% compared to CON or SH pancreata, suggesting an overall synergistic action of Pdx1 and Oc1 on the entire endocrine compartment. Within the gene set significantly altered in DH pancreata, we also observed an impressive array of transcription factors crucial for development of the endocrine pancreas. The decreased expression of *MafA, MafB, Pax4, Pax6, Mnx1* (*Motor neuron and pancreas homeobox 1*)*, Nkx2.2,* and *Nkx6.1* suggested that the simultaneous *Pdx1* and *Oc1* reduction acts at multiple levels of endocrine differentiation and not just at the level of *Neurog3* activation. Expression of *Neurog3* and *Pax4* was decreased in *Oc1* SH and DH*,* while *neuropeptide Y* (*NPY*) was decreased similarly in the *Pdx1* SH and DH compared with CON, indicating that some genes show sensitivity to reductions in either *Pdx1* or *Oc1* gene dosage alone.

In DH pancreata, we also observed large decreases in expression of genes encoding proteins involved in multiple aspects of glucose-stimulated insulin secretion– glucose metabolism (*Gck*, *G6pc2*), K_{ATP} channel components (*Abcc8* and *Kcnj11*), vesicle trafficking (*Scg5* and *Sytl4*), G protein coupled receptors (*Glp1r* and *Ffar1*), and transmembrane proteins (*Klb*)

(Figure 3-1D). These findings are likely related to the decreased number of differentiated endocrine cells in DH at this developmental age (see below). Although the majority of changes were associated with down-regulation of gene expression, some genes were up-regulated in all three mutant genotypes, including vitronectin (*Vtn*), which is increased in delaminating endocrine-committed cells (225) and inhibits insulin production and secretion (226), and regenerating islet-derived 1 (*Reg1*), which has been associated with islet regeneration (227,228).

Embryonic endocrine progenitor specification and endocrine maturation during the secondary transition are impaired by double *Pdx1-Oc1* heterozygosity

The RNA-Seq data at e15.5 suggested that endocrine lineage commitment and differentiation were impaired in DH pancreata. Specifically, expression of *Neurog3,* the critical endocrine specification transcription factor, and *Pax6,* a pan-endocrine-lineage transcription factor downstream of Neurog3*,* were down-regulated. Pancreas development is asynchronous and multiple developmental stages can be observed at a single time point (51). To obtain cellular resolution of gene expression changes, we quantified the number of cells expressing either Neurog3 or Pax6 at e15.5 using immunolabeling. DH pancreata had approximately 50% fewer Neurog3+ endocrine progenitors than CON pancreata (Figure 3-2A, C). These data supported our hypothesis that combined Pdx1 and Oc1 deficiency leads to reduced endocrine lineage specification. Despite a reduction in *Neurog3* mRNA expression in *Oc1* SH (Figure 3-1D), the number of Neurog3+ cells in *Oc1* SH was not statistically significantly affected compared to CON (Figure 3-2C), suggesting a decrease in *Neurog3* expression per cell in *Oc1* SH. Although the

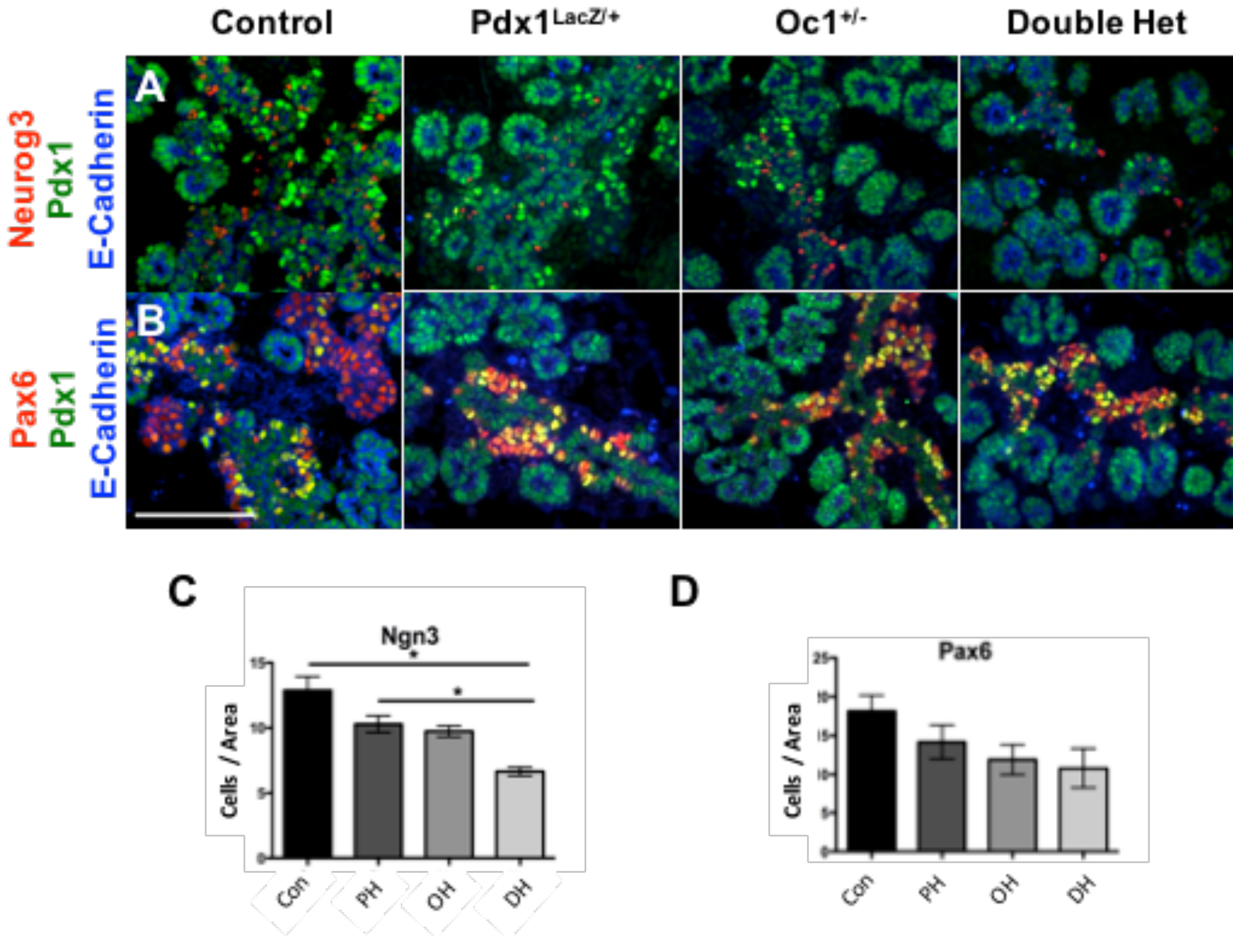

Figure 3-2: Reduced number *Neurog3*-expressing endocrine progenitors in DH mice at e15.5. Control, *Pdx1*$^{LacZ/+}$, *Oc1*$^{+/-}$ and DH pancreata were immunolabeled for (A) Neurog3 or (B) Pax6 (in red), Pdx1 (green) and E-cadherin (blue). (C,D) Quantification for Neurog3 and Pax6. A and B are at 20X magnification. p-value for all marked comparisons was <0.05 by One-Way Anova with Tukey correction. Figure modified and reprinted with permission from Henley *et al*, 2016 (224).

reduction in Pax6+ endocrine precursor cells did not achieve statistical significance (Figure 3-2B, D), the ratio of Neurog3+ cells to Pax6+ cells at e15.5, was similar among genotypes (Figure 3-3), supporting an overall decrease in the number of both specified and committed endocrine cells. In contrast, we found that e13.5 Neurog3+ progenitor numbers were not affected in SH or DH pancreata (Figure 3-4). Pax6+ and glucagon+ cells were also normal at this stage (Figure 3-4; too few insulin+ cells were detected to quantify). Thus, the impact of decreased *Pdx1-Oc1* dosage appears to be restricted to the second wave of endocrine differentiation that gives rise to cells within the mature islets of Langerhans (51). Additionally, I did not observe any alteration in the total epithelial area of the pancreas at e15.5 suggesting that reduced *Pdx1-Oc1* levels specifically impacted the endocrine compartment and not the development of exocrine cells of the pancreas (Figure 3-5).

During normal pancreas development, *Neurog3* expression initiates within a subset of bipotential trunk epithelial cells (229) and becomes elevated in cells destined to undergo commitment to the endocrine lineage. It is thought that the Neurog3^{hi} cells give rise to hormone-expressing cells after delaminating from the ductal epithelium (230). Closer examination of DH pancreata at e15.5 revealed that, in contrast to CON pancreata, fewer Neurog3+ cells could be found within the pancreatic trunk epithelium. A surprising proportion of the Neurog3+ cells present were instead located adjacent to the epithelium in DH compared with the other three genotypes; these extra-truncal Neurog3+ cells seemed to express high levels of Neurog3 (Figure 3-6A, B white arrows compared to yellow arrows).

To characterize the impact of Pdx1-Oc1 cooperativity on the endocrine compartment during the second wave of differentiation, I examined markers of differentiated endocrine

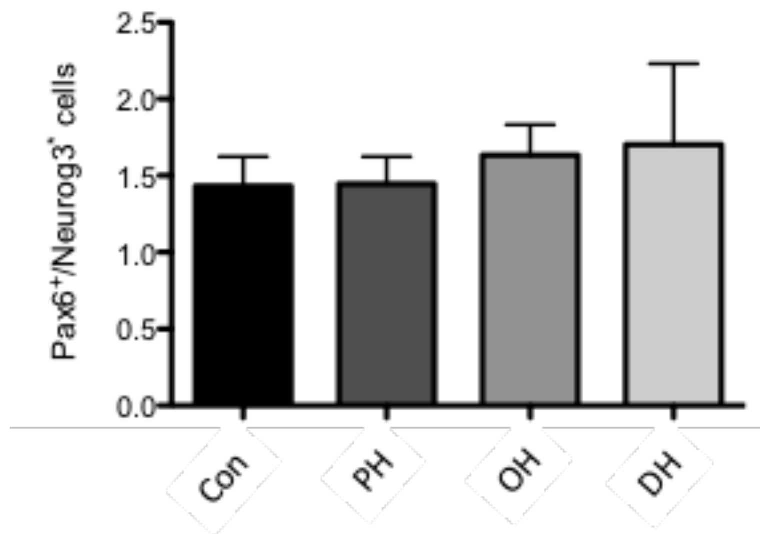

Figure 3-3: Ratio of Pax6+:Neurog3+ cells. There is no difference in the ratio of Pax6+ cells to Neurog3+ cells in any genotype. Figure modified and reprinted with permission from Henley *et al*, 2016 (224).

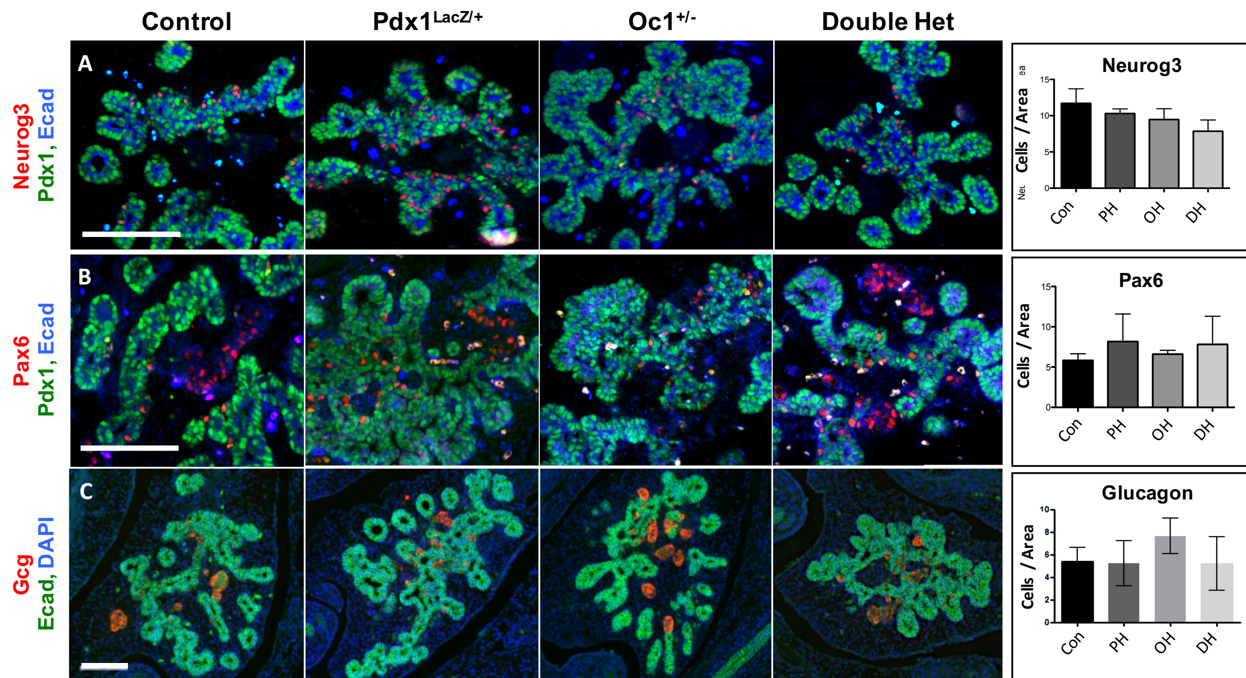

Figure 3-4: No change in endocrine specification or differentiation at e13.5. e13.5 Control, *Pdx1*$^{LacZ/+}$, *Oc1*$^{+/-}$ and DH pancreata were immunolabeled for (A) Neurog3, (B) Pax6 (in red), Pdx1 (green), and E-Cadherin (blue). (C) e13.5 pancreata were immunolabeled for Gcg (red), E-Cadherin (green) and DAPI (blue). Quantification of each is to the right. There were no changes in the number of Neurog3+, Pax6+, or Gcg+ cells at e13.5. Scale bars: 100 μm. Figure modified and reprinted with permission from Henley *et al*, 2016 (224).

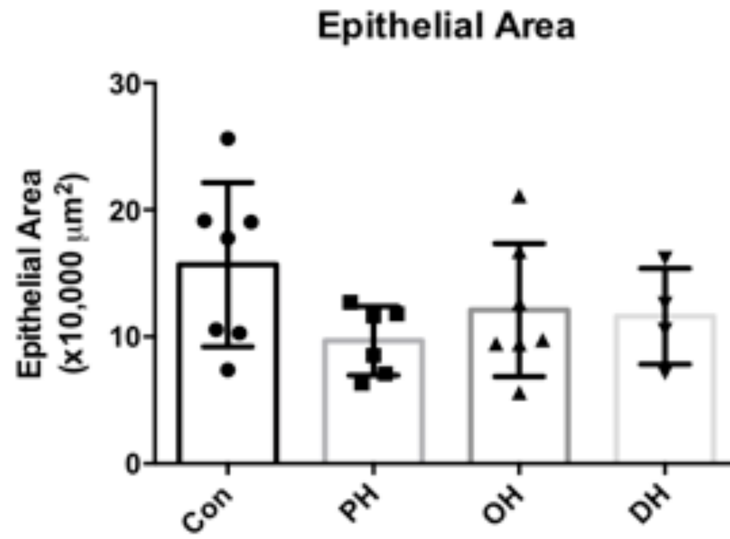

Figure 3-5: No difference in e15.5 epithelial area. The epithelial area e15.5 pancreata was measured and no difference was found in any genotype.

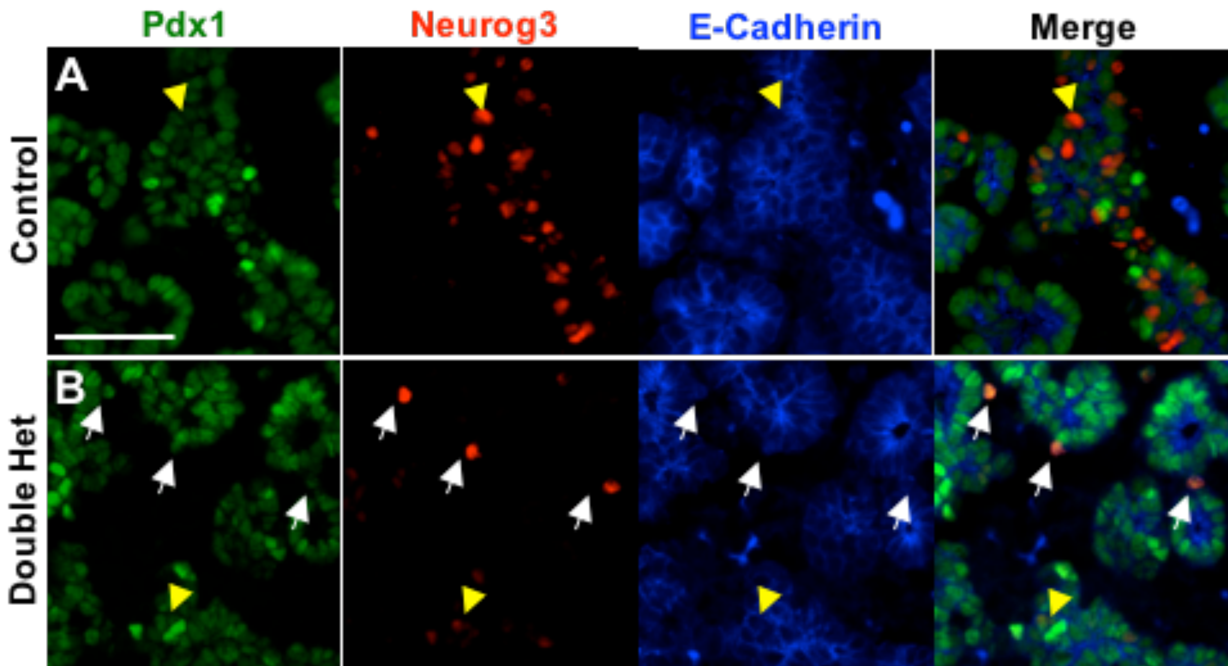

Figure 3-6: Altered location of Neurog3 endocrine progenitors in e15.5 DH pancreata. (A,B) Control and DH pancreata were labeled for Pdx1 (green), Neurog3, Red, and E-Cadherin (blue). White arrows: delaminated Neurog3^{+} progenitors; yellow arrowheads: Neurog3^{+} progenitors within developing trunk. Images captured at 40X magnification. Figure modified and reprinted with permission from Henley *et al*, 2016 (224).

lineages at e15.5, including the hormones insulin and glucagon. DH pancreata showed significant decreases in the numbers of both insulin+ and glucagon+ cells (Figure 3-7A, B). Only DH pancreata showed a reduction in glucagon+ cell number (Figure 3-7B), whereas similar decreases in the number of insulin+ cells were observed in *Pdx1* SH and DH (Figure 3-7A). While previous studies have shown decreased β-cell function and impaired glucose homeostasis in *Pdx1* SH post-weaning (170,171,231), this is the first report of a defect in the β-cell lineage in *Pdx1* SH during embryonic development. Defective β-cell development could contribute to the susceptibility to adult onset diabetes observed in mice and humans with heterozygous *PDX1* mutations (171,174,176,232,233). Taken together, my data reveal a reduction in the numbers of emerging hormone-expressing cells at e15.5 in mice with combined reduction in Pdx1 and Oc1 gene dosage.

In view of the transcriptomic impact of Pdx1 and Oc1 reduction on markers of α- and β-cell maturation, I next assessed the number of cells expressing the "large Maf" transcription factors, MafA and MafB. *MafB* is activated soon after endocrine progenitor delamination in both glucagon+ and insulin+ cells, and is down-regulated postnatally in insulin+ cells in mice. *MafA* expression initiates later, specifically in insulin+ cells and is maintained in these cells. Despite measurable decreases in *MafA* and *MafB* transcripts (Figure 3-1D), I observed no differences among genotypes in the total number of MafA+ or MafB+ cells at e15.5 (Figure 3-8A, B). Normal cell numbers in the face of reduced transcripts suggests that *MafA* and *MafB* expression per cell is decreased. When I compared the numbers of MafA/B+ cells to the number of hormone-expressing cells, I found a significant increase in the number of Maf+/hormone- cells in DH pancreata (Figure 3-9). These Maf+ cells may derive from Neurog3+

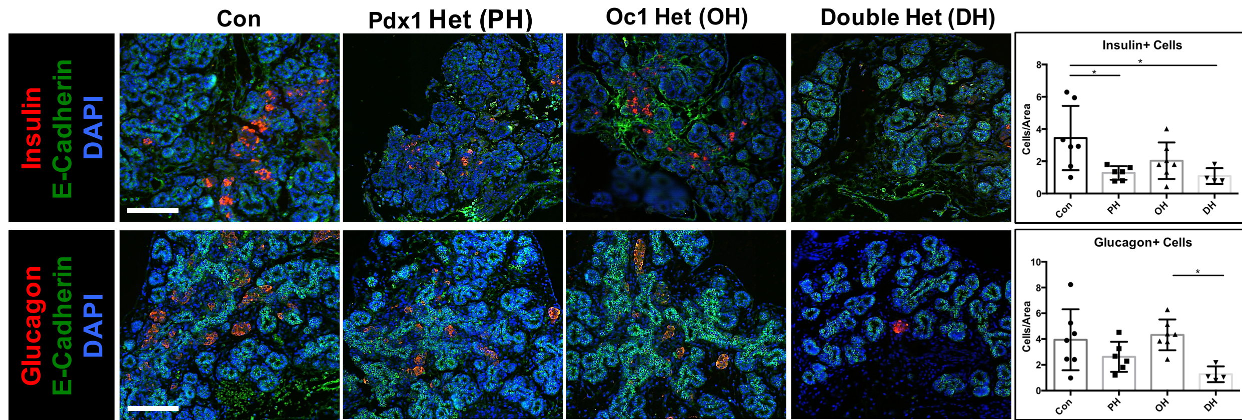

Figure 3-7: Combined heterozygous reduction in *Pdx1* and *Oc1* leads to reductions in hormone-positive cells at e15.5. Control, *Pdx1*$^{LacZ/+}$, *Oc1*$^{+/-}$ and DH pancreata were immunolabeled for insulin (A), glucagon (B), in red, E-Cadherin (green), and DAPI (blue). Images are at 20X magnification. Scale bar represents 100 µm. p-value for all marked comparisons was <0.05 by One-Way Anova with Tukey correction. *p<0.05. Figure modified and reprinted with permission from Henley *et al*, 2016 (224).

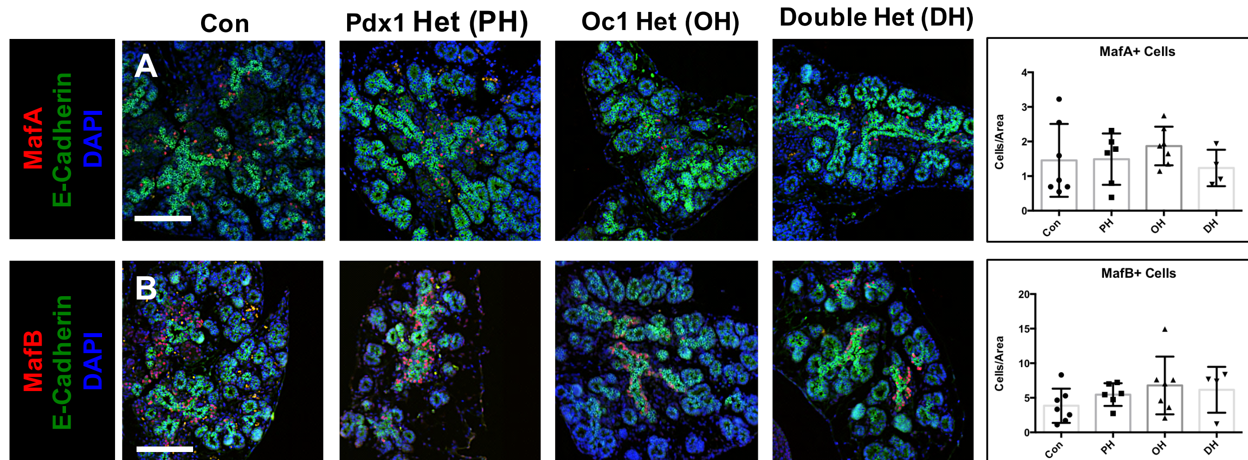

Figure 3-8: No change in MafA+ of MafB+ cells at e15.5. Control, *Pdx1*$^{LacZ/+}$, *Oc1*$^{+/-}$ and DH pancreata were immunolabeled for MafA (A), MafB (B), in red, E-Cadherin (green), and DAPI (blue). Images are at 20X magnification. Scale bar represents 100µm. Figure modified and reprinted with permission from Henley *et al*, 2016 (224).

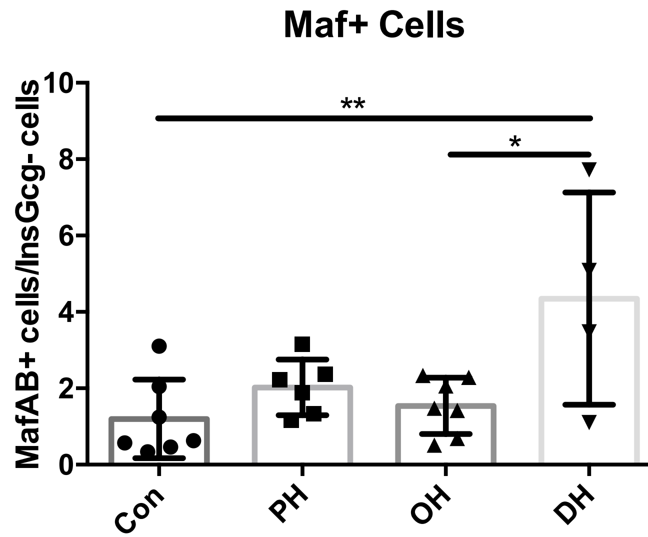

Figure 3-9: Increased ratio of large Maf-expressing/insulin or glucagon-negative cells in DH at e15.5. *: p<0.05; ** p<0.01 by One-Way ANOVA with Tukey correction. Figure modified and reprinted with permission from Henley *et al*, 2016 (224).

cells generated prior to e13.5 that fail to further gain mature hormone expression. Interestingly, the number of cells expressing the β-cell transcription factor Nkx6.1 was not different between Control and DH pancreata (Figure 3-10); however, there was a decrease in DH pancreata when compared to SH animals. As with the expression of MafA and MafB, the reduction in *Nkx6.1* mRNA is likely due to a reduced amount of expression on a per-cell basis rather than a reduction in the total number of Nkx6.1+ cells. Taken together these data show that simultaneous decrease in *Pdx1-Oc1* dosage preferentially affects the endocrine progenitor program during the second wave of endocrine differentiation.

Impaired terminal differentiation and function of hormone-positive cells with double *Pdx1-Oc1* heterozygosity

To determine whether the developmental defects in DH persist after birth, we examined early postnatal stages physiologically and morphologically. Immediately at birth, prior to feeding, there were no significant differences in body weight or blood glucose levels (Figure 3-11A, B). However, with the start of feeding at P1 DH animals failed to increase in body weight compared to the other genotypes (Figure 3-12A). At P1 DH pups exhibited elevated *ad lib* blood glucose compared to CON and *Oc1*$^{+/-}$ animals (Figure 3-12B). Another graduate student in the lab therefore analyzed whether DH neonates had reduced α- or β-cell mass, consistent with our observations of decreased insulin+ and glucagon+ cells at e15.5. While there was a trend toward reduced α- and β-cell mass at P1 in *Pdx1* SH and DH (Figure 3-12C, D), this was not statistically significant. However, I found that there was a dramatic and significant decrease in total pancreatic insulin protein content in DH when compared with CON or either SH (Figure 3-

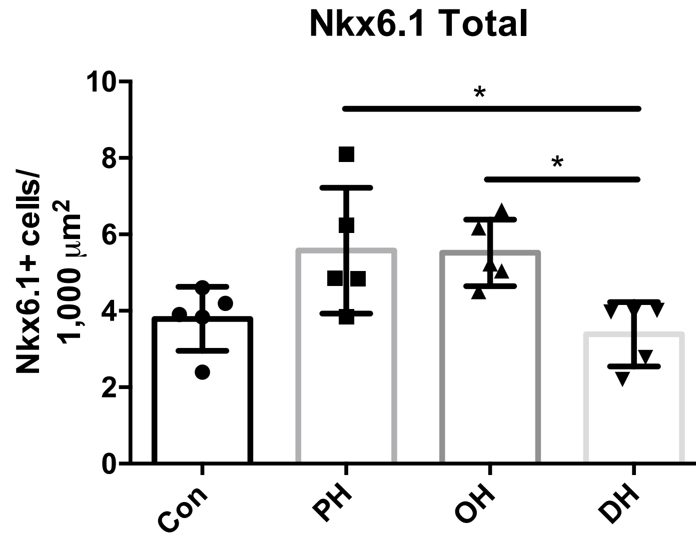

Figure 3-10: Nkx6.1+ cells in e15.5 pancreata. There is no difference in the number of Nkx6.1+ cells between the pancreata of e15.5 Con and DH mice, but there is a significant reduction in the number of Nkx6.1+ cells in DH pancreata compared to PH and OH mice. *: $p<0.05$ by One-Way ANOVA with Tukey correction. Figure modified and reprinted with permission from Henley *et al*, 2016 (224).

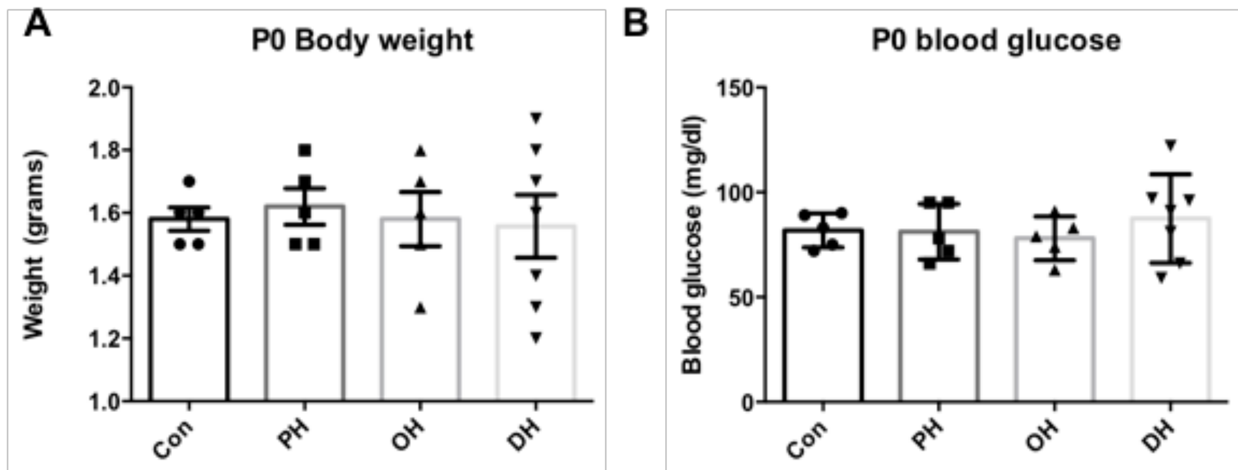

Figure 3-11: Body weight and blood glucose are similar between genotypes at P0. Body weight (A) and blood glucose (B) were measured at birth prior to feeding. Figure modified and reprinted with permission from Henley *et al*, 2016 (224).

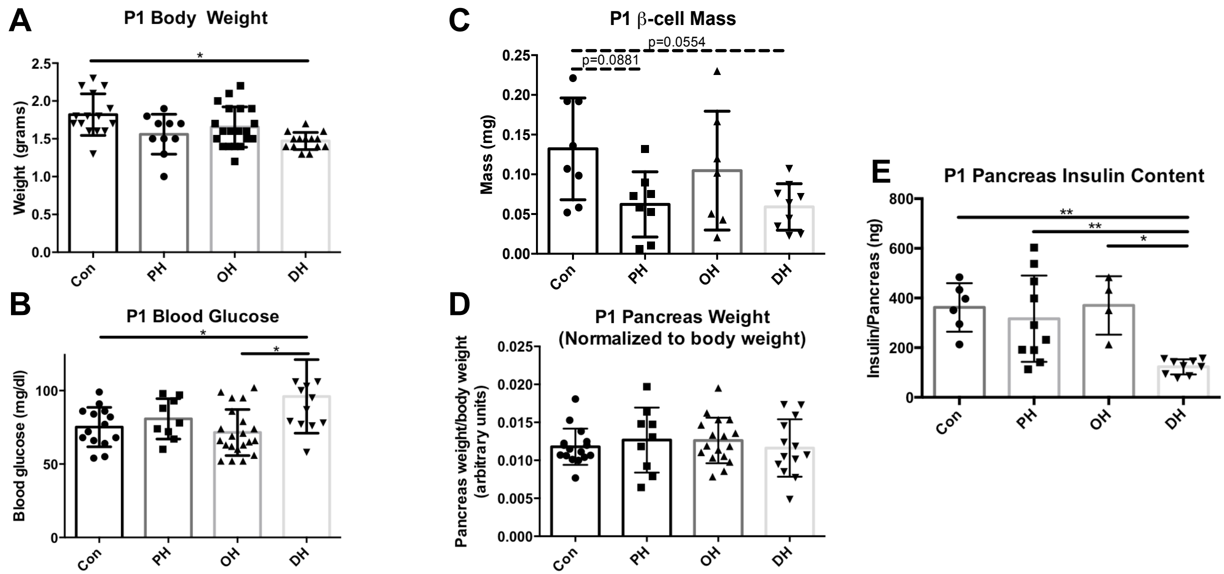

Figure 3-12: Defects in glucose homeostasis in DH mice at P1. Body weight (A) and ad lib feeding blood glucose measurements (B). (C) β-cell mass, (D) pancreas mass, and (E) Total pancreatic insulin content. *p <0.05. **p<0.01 by One-Way ANOVA with Tukey correction. Figure modified and reprinted with permission from Henley *et al*, 2016 (224).

12E), indicating a functional β-cell defect in DH mice that persists after birth in a cell population in which Pdx1 and Oc1 no longer co-localize.

A postnatal functional defect in DH islets was supported by my analysis of islet gene expression at P1 (Figure 3-13). Using TaqMan Low-Density Array (TLDA) I found that DH neonatal islets had undetectable levels of *insulin* and *glucagon* mRNA; expression of these hormones was unaffected in islets from SH (Figure 3-13A). To determine if the lack of detection of these transcripts in the TLDA analysis was due to premature truncated transcription, I performed quantitative RT-PCR using primers that spanned the *insulin* and *glucagon* transcripts. These analyses confirmed the decrease in *insulin* and *glucagon* in DH islets at P1 with no evidence of truncated transcription (Figure 3-14). *Sst* mRNA expression was not changed in any genotype at P1.

My TLDA analyses also revealed that expression of several key islet transcription factor genes (e.g. *Isl1*, *Nkx6.1*, *MafA*, *Pax6*) known to regulate either *insulin* or *glucagon* gene expression was substantially increased specifically in DH at P1. These results suggest an attempt at compensation to overcome the decreased expression of hormone genes, but this altered expression could be secondary to stress of islet isolation at P1. In contrast, expression of *Pax4,* a critical β-cell differentiation factor (54), was undetectable (Figure 3-13B), consistent with the reduction in insulin+ cells and the decrease in *Pax4* expression in DH at e15.5 detected by RNA-Seq. Similarly, expression of genes involved in glucose sensing and hormone-granule exocytosis was also increased at P1 (e.g. *Gck*, *Snap25*, *Vamp2*) (Figure 3-13C). The BMP inhibitor *Sostdc1* was increased in DH P1 islets (Figure 3-13C), possibly contributing to the impaired islet function (217), as autocrine BMP activity was shown to be important for GSIS (234). I also observed

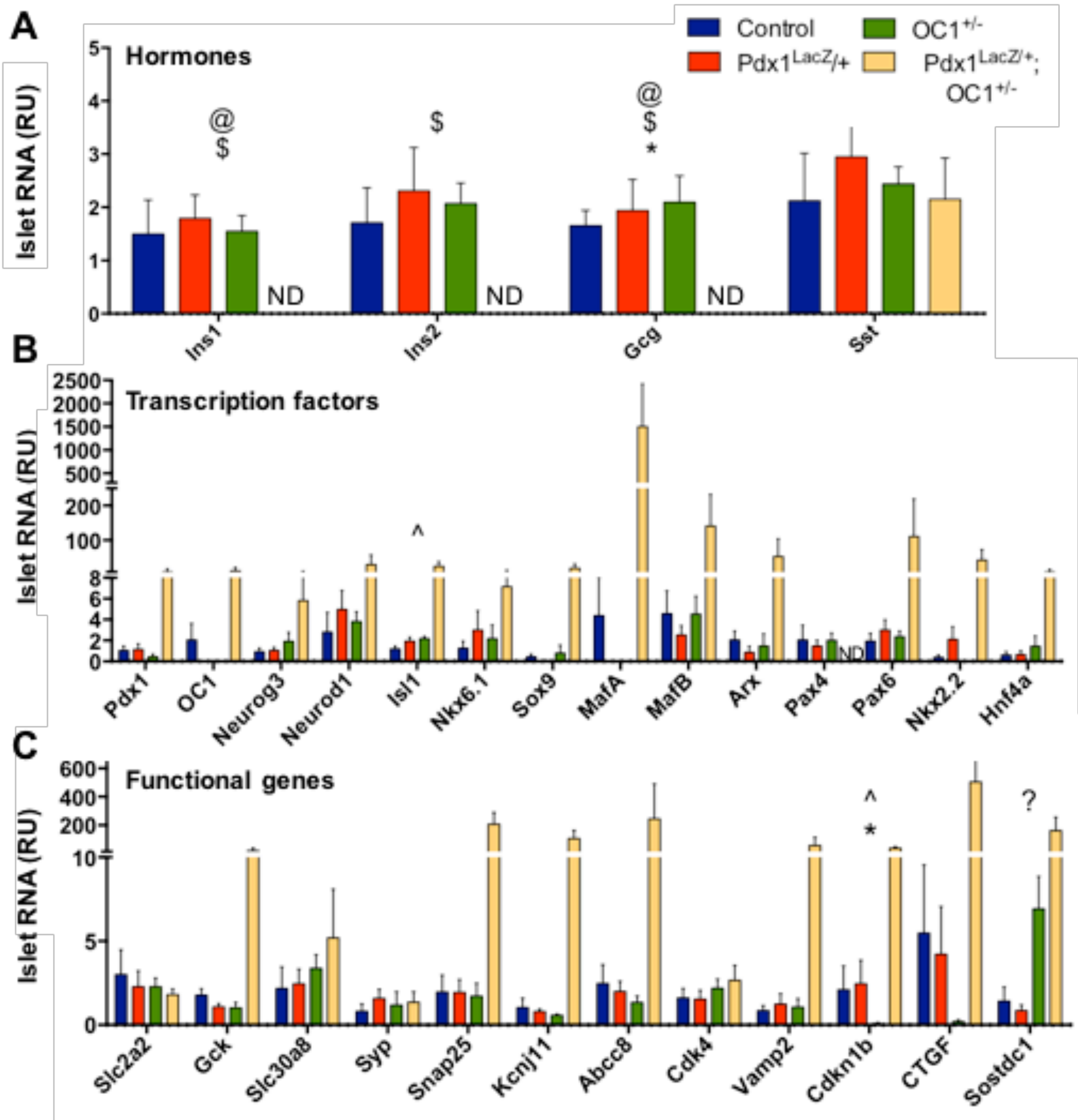

Figure 3-13: Dramatic alterations in gene expression of P1 DH islets. Control, *Pdx1*$^{LacZ/+}$, *Oc1*$^{+/-}$ and *Pdx1*$^{LacZ/+}$;*Oc1*$^{+/-}$ islets were analyzed for gene expression of hormones (A), endocrine-associated transcription factors (B), and secretory functional genes (C). p-value for marked comparisons was <0.05 by Kruskal-Wallace Test followed by two-tailed Student's T-Test. *: Control v. *Pdx1*$^{LacZ/+}$;*Oc1*$^{+/-}$; #: Control v. *Pdx1*$^{LacZ/+}$; ^: Control v. *Oc1*$^{+/-}$; @: *Pdx1*$^{LacZ/+}$ v. *Pdx1*$^{LacZ/+}$;*Oc1*$^{+/-}$; $: *Oc1*$^{+/-}$ v. *Pdx1*$^{LacZ/+}$;*Oc1*$^{+/-}$; ?: *Pdx1*$^{LacZ/+}$ v. *Oc1*$^{+/-}$. Figure modified and reprinted with permission from Henley *et al*, 2016 (224).

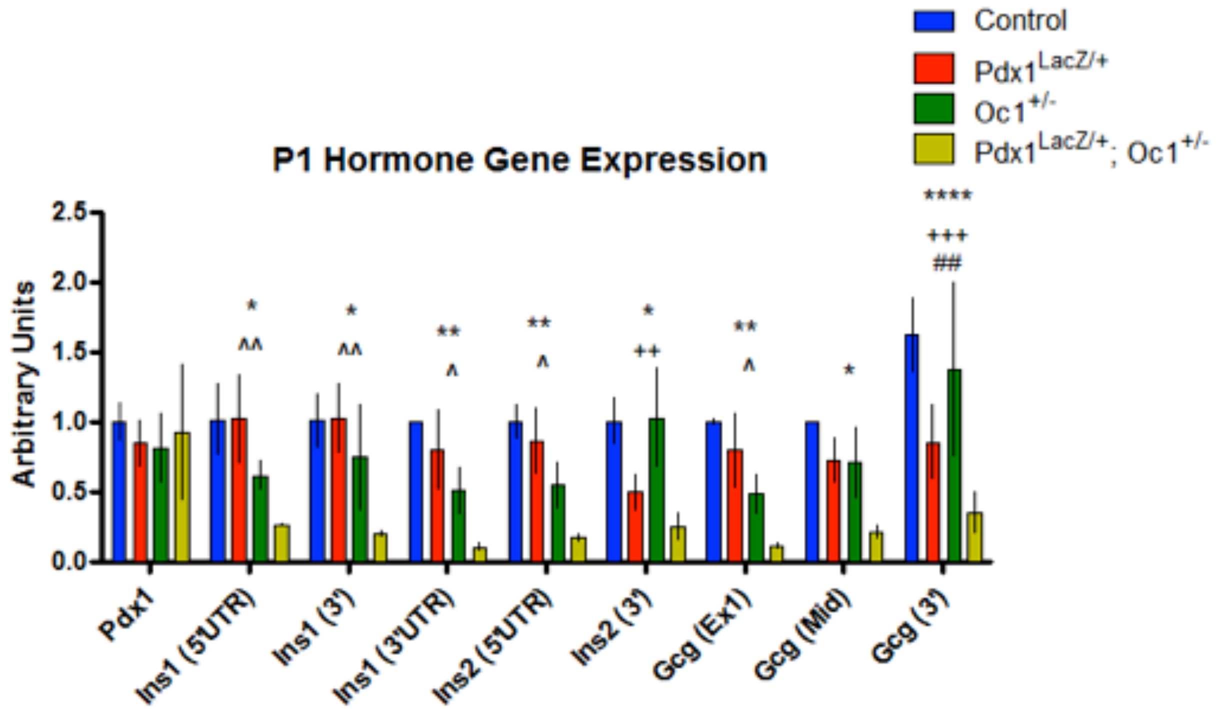

Figure 3-14: Expression of hormones in P1 whole pancreata. Expression of Ins1, Ins2, and Gcg is reduced in DH as measured by qRT-PCR. Single mark: $p<0.05$; Two marks: $p<0.01$; Three marks: $p<0.001$; Four marks: $p<0.0001$ by One-Way ANOVA with Tukey correction for multiple comparisions. Control v. $Pdx1^{LacZ/+;}$ $Oc1^{+/-}$; ^: $Pdx1^{LacZ/+}$ v. $Pdx1^{LacZ/+}$; $Oc1^{+/-}$; +: $Oc1^{+/-}$ v. $Pdx1^{LacZ/+}$. Figure modified and reprinted with permission from Henley *et al*, 2016 (224).

increased expression of *Ctgf*, a β-cell-derived growth factor that is critical for embryonic β-cell proliferation and capable of inducing proliferation of embryonic α- and β-cells (235-237) (Figure 3-13C). The dramatic increase in *Ctgf* expression in DH suggested increased α- and/or β-cell proliferation as a mechanism for restoring α- and β-cell mass by birth. Indeed, we detected a significant increase in α- and β-cell proliferation specifically in DH pancreata at e18.5 (Figure 3-15). While *Ctgf* expression remained elevated at P1, the increased α- and β-cell proliferation was restricted to late gestation as there was no significant difference in α- or β-cell proliferation immediately after birth at P0 (Figure 3-16). A second possible explanation for the partial recovery of α- and β-cell mass at P1 was increased specification of endocrine cells late in gestation. This mechanism could work in conjunction with the increased proliferation to increase α- and β-cell mass. However, I did not detect any evidence of increased specification at e18.5 as measured by the number of Neurog3+ cells (Figure 3-17).

In order to better understand how and when this increased α- and β-cell proliferation contributed to the partial recovery of α- and β-cell mass at P1, I examined multiple measures of the α- and β-cell compartments at e18.5. Unsurprisingly, the increased proliferation at this time point did not yet translate into to an increase in α- or β-cell area, which was also reduced at e18.5 (Figure 3-18A, B), similarly to what I observed at e15.5. The reduction in β-cell area at e18.5 was due to both decreased β-cell number and decreased β-cell size (Figure 3-18C, D). In contrast, there was no difference in α-cell size at e18.5. The reduction in α-cell area appears to be due to a modest reduction in total α-cell number (Figure 3-18E, F). Together, these data indicate that the reduction in differentiation of both α- and β-cells observed during the second wave of endocrine differentiation results in persistent reductions in those cell types at e18.5.

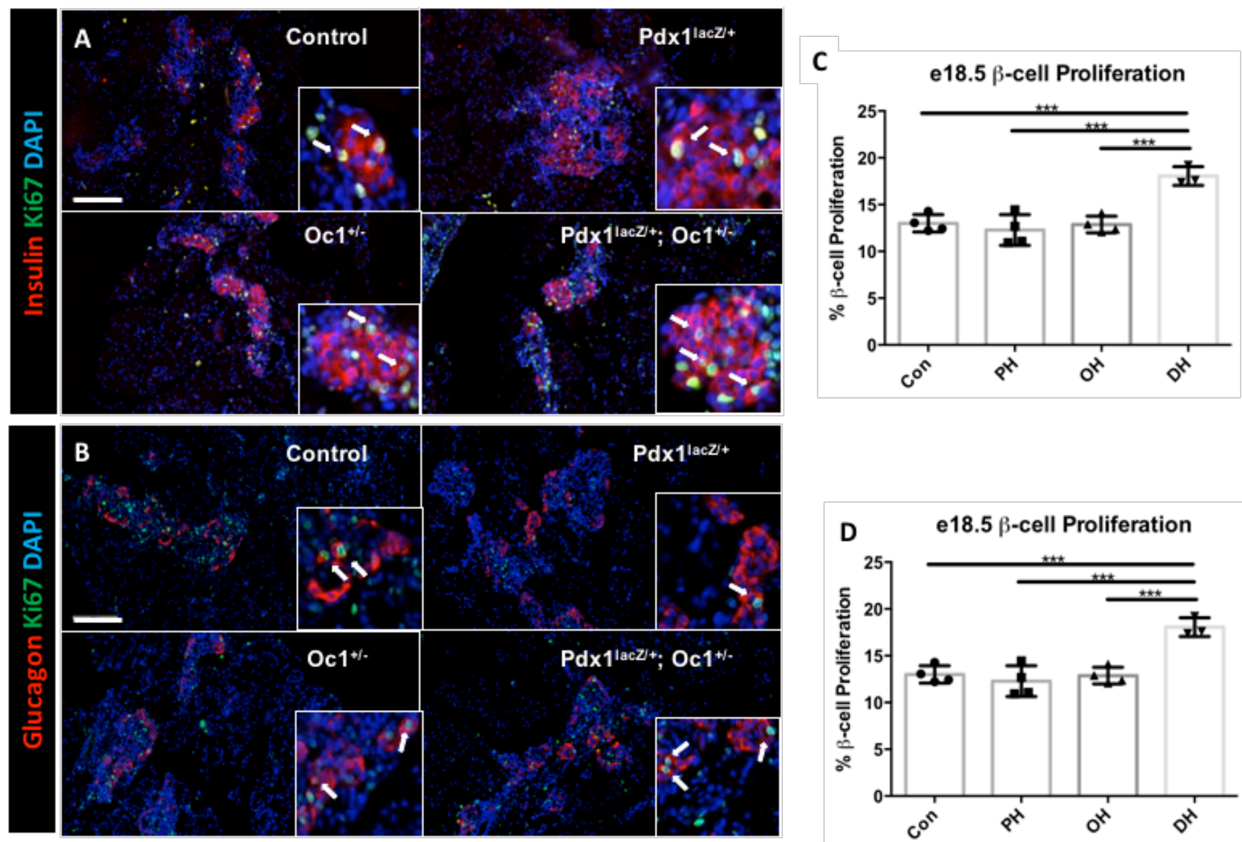

Figure 3-15: Increased α- and β-Cell Proliferation at e18.5 in DH Pancreata. (A and B) Representative images of proliferating β cells (A; red: Glut2 and green: Ki67) and a cells (B; red: glucagon and green: Ki67) at e18.5. α-cell proliferation at e18.5. The arrows point to proliferating hormone+ cells. (C and D) Quantification of β-cell proliferation (C), and quantification of α-cell proliferation (D). Scale bar represents 100 µm. *p < 0.05; ***p < 0.001 by One-Way ANOVA with Tukey Correction for multiple comparisons. Figure modified and reprinted with permission from Henley *et al*, 2016 (224).

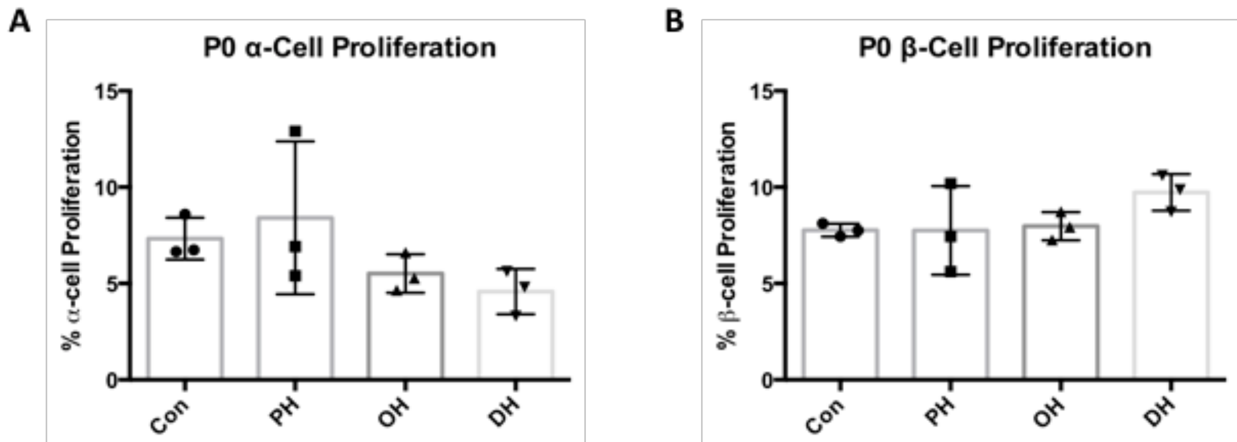

Figure 3-16: No change in P0 α- and β-Cell Proliferation. (A) α-cell proliferation and (B) β-cell proliferation are unchanged in any group at P0.

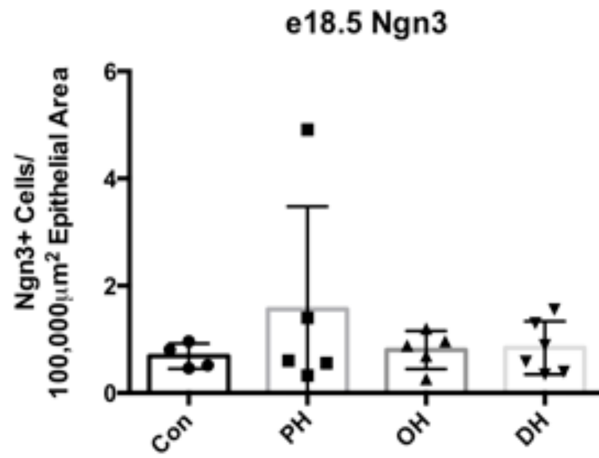

Figure 3-17: No increase in Neurog3+ cells at e18.5.

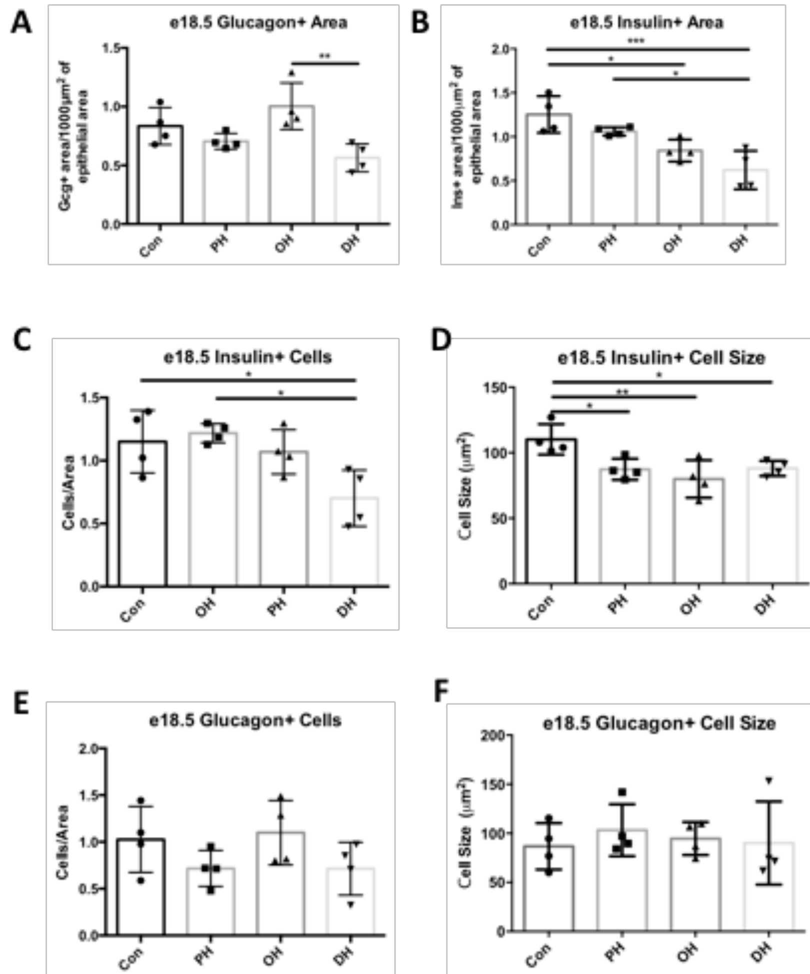

Figure 3-18: Reduced glucagon+ and insulin+ area in e18.5 DH pancreata. (A) Glucagon+ area is reduced in DH compared to OH. (B) Insulin+ area is reduced in DH compared to Con and PH as well as OH compared to Con. Insulin+ cell number (C) and size (D) are reduced between multiple groups. Glucagon+ cell number (E) and size (F) are unchanged. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ by One-Way ANOVA with Tukey correction for multiple comparisons.

Increased α- and β-cell proliferation at e18.5 results in a partial recovery of α- and β-cell area by P1. In spite of the reduction in hormone+ cells at e18.5, the number of Pax6+ cells committed to the endocrine lineage was unchanged in any group (Figure 3-19).

Restoration of normal glucose homeostasis and islet gene expression in DH animals at weaning

A former graduate student in our lab examined the DH phenotype at weaning (three weeks of age) when β cells become fully mature (62,169). At this time point, she found that DH animals had normal body weight (Figure 3-20A), and, with the exception of two mice, restoration of normal fasting and *ad lib* blood glucose levels (Figure 3-20B,C); however, *Pdx1* SH had elevated *ad lib* blood glucose (Figure 3-20B), consistent with the adult phenotype presented in the literature (78,170,171,238). When challenged with glucose during an intraperitoneal glucose tolerance test (IP-GTT) at three weeks of age, there were no significant differences in glucose clearance among the genotypes although Pdx1 heterozygous (PH) mice trended toward impaired glucose tolerance (Figure 3-20D) in agreement with the literature (170,171).

Surprisingly, DH blood glucose levels trended more closely toward controls than PH throughout the IP-GTT. I hypothesized that the moderately improved glucose tolerance in DH mice was due to the liver acting as a glucose sink because of altered glycogen storage as a consequence of Oc1 heterozygosity (Oc1 regulates genes important for glycogenolysis and gluconeogenesis). Oc1 null mice have an inability to mobilize liver glycogen and die of hypoglycemia within one week after birth (239-241). This idea was supported by evidence from

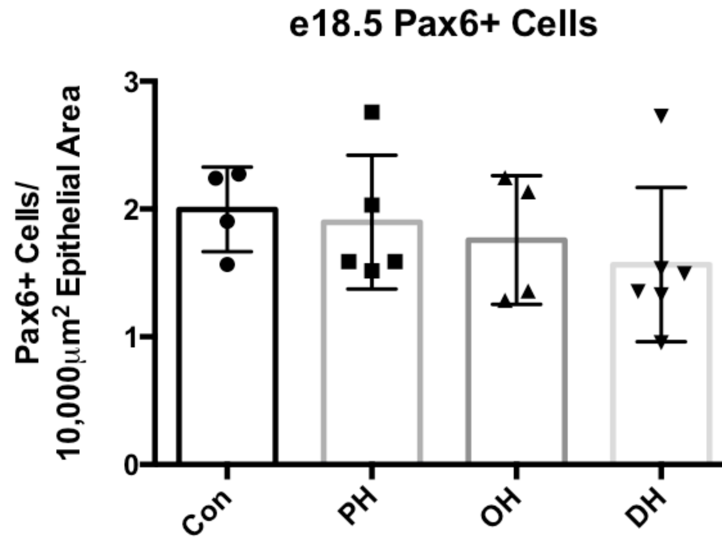

Figure 3-19: No change in Pax6+ cells in any group at e18.5.

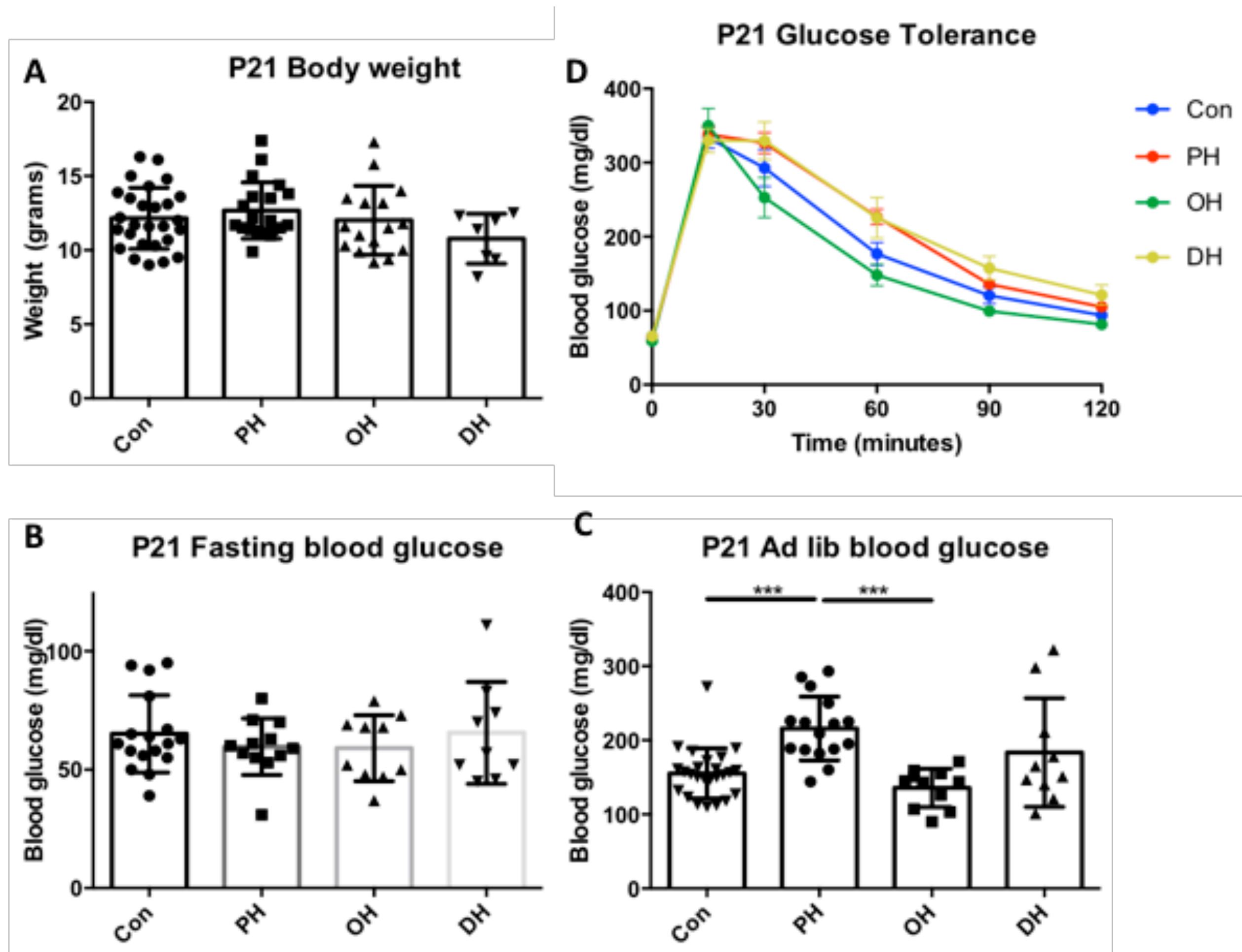

Figure 3-20: Defects in DH glucose homeostasis resolve by weaning. (A) P21 Body weight, (B) fasting blood glucose, (C) Ad lib blood glucose measurements, and (D) IPGTT at P21. ***: $p<0.001$ by One-Way ANOVA with Tukey correction for multiple comparisons. Figure modified and reprinted with permission from Henley *et al*, 2016 (224)

Period Acid Shiff staining on liver tissue sections where I observed that DH livers contained noticeably more glycogen than PH livers (Figure 3-21).

Since the physiological phenotype of DH animals appeared to resolve by weaning, I next examined islet gene expression at four weeks of age. Although expression of *insulin* could be detected in DH islets at this age (unlike at P1), it was still significantly reduced compared with Oc1 SH (Figure 3-22A). At this time point, expression of *Sst* was also reduced in DH islets. Expression levels of islet transcription factors that were elevated in DH at P1 were normalized at four weeks of age, with the exception of *MafA*, which was significantly decreased (Figure 3-22B). Likewise, expression of genes involved in glucose sensing and insulin secretion was also restored to normal levels in DH islets at four weeks (Figure 3-22C). *Ctgf* expression remained slightly elevated while *Sostdc1* levels were normal (Figure 3-22C).

Discussion

Pdx1 and *Oc1* are co-expressed in MPCs at very early stages of pancreas development and previous work from the Stoffers lab suggested that these two factors cooperate to regulate transcription of the endocrine progenitor transcription factor *Neurog3*. We hypothesized that a threshold of cooperative *Pdx1* and *Oc1* activity is required for realization of the endocrine program from MPCs. Transcriptome analyses of DH pancreas at e15.5 supported this hypothesis, revealing a highly compromised islet differentiation program with reduced expression of several key endocrine lineage transcription factors, including *Neurog3* and *Pax6*, as well as multiple genes involved in mature β-cell function. Complementary morphological and physiological studies point to distinct effects of combined *Pdx1 Oc1* reduction in endocrine

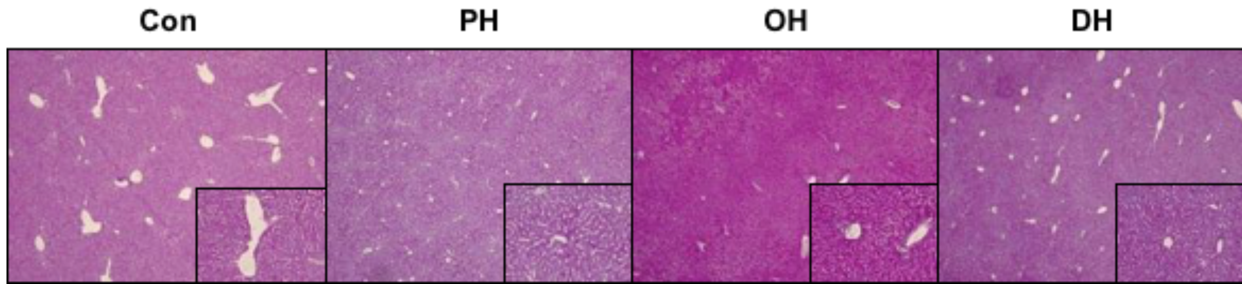

Figure 3-21: Liver glycogen deposition at P21. Period Acid Schiff staining of livers from CON, Pdx1 and Oc1 SH and DH. Compared to CON, Pdx1 SH have less glycogen deposition; Oc1 single heterozygotes have increased glycogen deposition. DH have more glycogen deposition than Pdx1 SH. Images taken at 4x; Insets at 20x. Figure modified and reprinted with permission from Henley *et al*, 2016 (224)

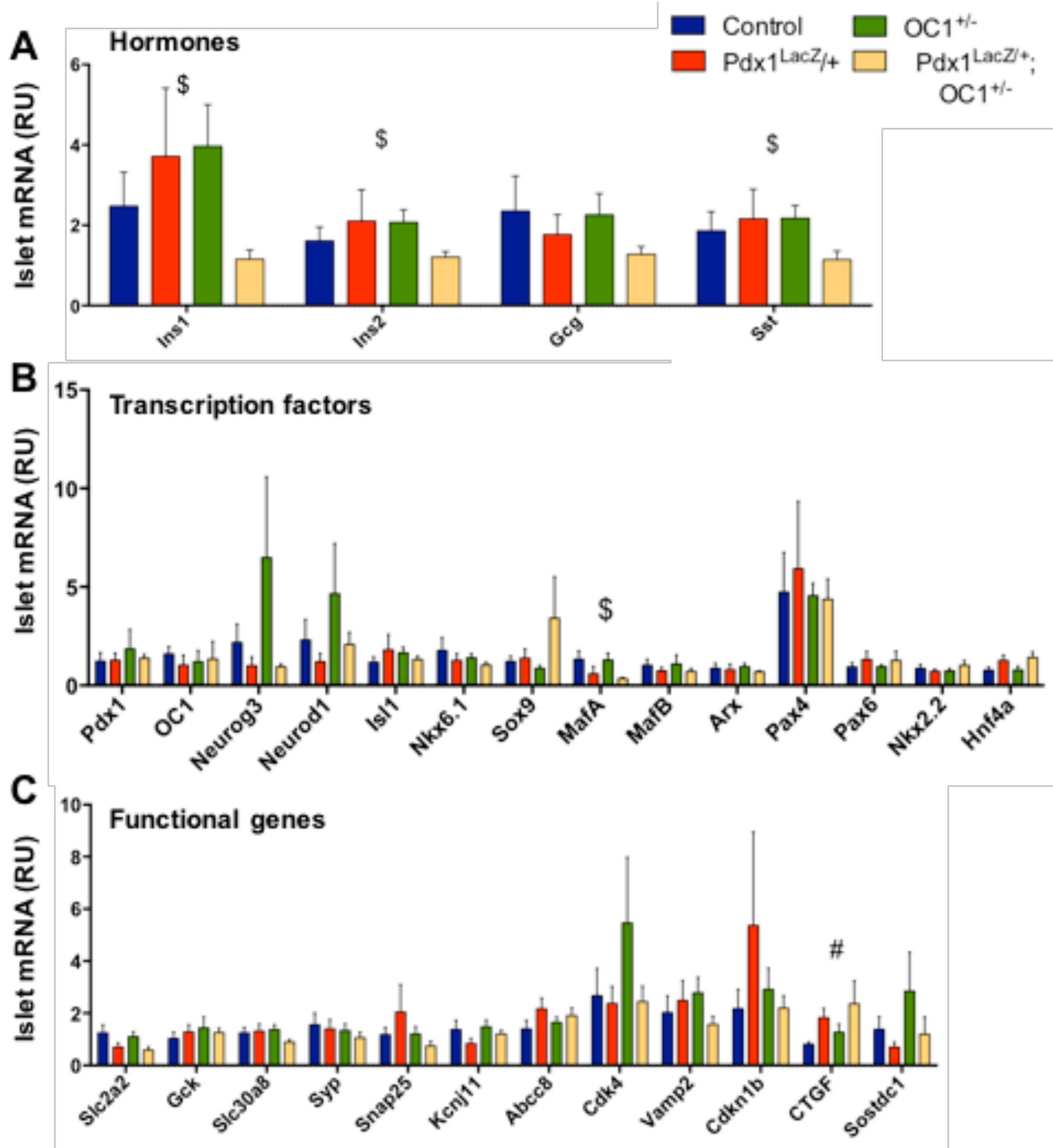

Figure 3-22: Early Reductions in Pdx1 and Oc1 Lead to Persistent Alterations in Islet Gene Expression at Weaning. (A–C) Con, $Pdx1^{LacZ/+}$, $Oc1^{+/-}$, and DH islets were analyzed for gene expression of hormones (A), endocrine-associated transcription factors (B), and secretory functional genes (C) at P28. p value for marked comparisons were < 0.05 by Kruskal- Wallace Test followed by two-tailed Student's t-test (#, CON versus $Pdx1^{LacZ/+;}$ $, $Oc1^{+/-}$ versus $Pdx1^{LacZ/+;}Oc1^{+/-}$). Figure modified and reprinted with permission from Henley *et al*, 2016 (224).

progenitor specification and maturation with long-term effects on gene expression and function.

These findings highlight a specific role for combined *Pdx1* and *Oc1* activity in establishing the endocrine progenitor program during the second wave of endocrine differentiation. Decreased dosage of *Pdx1-Oc1* impacted Neurog3+ cell numbers and subsequent endocrine differentiation at e15.5 but not e13.5. Since the duration of the Neurog3+ state is short (estimated to be ~12 hours; (19)), the reduced numbers of Neurog3+ cells at e15.5 suggest that endocrine progenitors are more sensitive to *Pdx1-Oc1* dosage after e13.5. The normal number of glucagon+ and Pax6+ cells at e13.5, which derive from even earlier Neurog3+ progenitors (46), supports this timeline of events. The relative increase in the proportion of delaminated cells expressing high Neurog3 levels in DH pancreata suggests that Pdx1 and Oc1 synergize to regulate the timing of transition from a Neurog3^{lo} to Neurog3^{hi} cell. The significance of duration of the Neurog3^{lo} state is currently unclear, but may affect subsequent steps in endocrine maturation (19,50).

The morphologic and transcriptomic analyses at e15.5 further suggest that *Pdx1-Oc1* reduction leads to defective maturation of α and β-cell lineages. This could occur either directly by decreased dosage of *Pdx1* and *Oc1*, and/or indirectly due to decreased expression of maturational and other endocrine cell markers per cell. The persistence of gene expression defects and elevated blood glucose at P1 indicates that the endocrine maturation program is not being completed successfully by birth in DH embryos.

Our results suggest that the combined activity of two structurally unrelated transcription factors within a progenitor-cell population (MPCs) affects subsequent differentiated cell

populations (α and β cells) in which the two factors are not co-expressed. Expression of *Pdx1* and *Oc1* initially overlaps in MPCs and in bipotential duct/endocrine progenitors in the pancreatic epithelial "trunk", but they diverge with *Oc1* silenced and *Pdx1* maintained almost exclusively in the pro-β cell lineage, as endocrine cells become specified. It is possible that the cooperative activity of *Pdx1* and *Oc1* in MPCs and endocrine progenitors primes the cells for subsequent steps of the differentiation program. This notion that transcription factors can have temporally separated effects on cell behavior is not novel, as deletion of *Hnf4α* in the embryonic liver affects gene expression in differentiated hepatocytes long after its expression is down-regulated (242). Similarly, the FoxD3 transcription factor, which is expressed in adult, quiescent β cells is required for maternal β-cell proliferation during pregnancy despite being dispensable under normal conditions (243). Our preferred model is that Pdx1 and Oc1 cooperate at an early stage in multi- or bi-potent progenitors to establish a state of poised gene activation via chromatin modifications in regulatory regions of both instructional and functional classes of islet endocrine genes. This competency state is realized in later stages of differentiation. The timing of the Pdx1-Oc1 interaction is critical as inactivation of one *Oc1* allele in *Pdx1* heterozygotes later in development (using a later-acting pancreas-specific Cre driver line) did not impact endocrine function (experiments performed by Dr. Kathryn Henley while a student in the Gannon lab, not shown).

The gene expression of DH islets at P1 suggests potential mechanisms for the recovery of normal numbers of insulin-expressing cells by birth, but also for the persistent defect in β-cell function. The Oc1 target *Ctgf* is essential for embryonic β-cell proliferation; increased *Ctgf* expression in embryonic β cells induces both α- and β-cell proliferation, resulting in increased α-

and β-cell mass at birth (236). The dramatically increased *Ctgf* expression in DH islets likely promoted the increased α- and β-cell proliferation, thereby increasing α- and β-cell mass by P1. It is also possible that decreased insulin expression itself contributes to increased β-cell proliferation during late gestation. As shown by Duvillie et al, mice with targeted disruption of the both insulin genes in early development exhibited increased β-cell proliferation at e18.5, similar to the DH animals (244). There is an ongoing reduction in *insulin* and *glucagon* expression at P1, which likely contributes to impaired glucose homeostasis. In addition, the increase in *Sostdc1* could impair β cell function. Sostdc1 is a BMP inhibitor, and autocrine BMP signaling was suggested to enhance insulin secretion and glucose homeostasis (234). Indeed, inactivation of *Sostdc1* enhances glucose-stimulated insulin secretion and glucose homeostasis (217). We were surprised to observe that the majority of DH animals show restored glycemic control by weaning. *Sostdc1* expression was no longer elevated and most islet transcription factors, including *Pax4*, had returned to normal expression levels. However, decreases in *insulin* and *glucagon* expression persisted, now along with reduced *MafA* expression. Loss of MafA is associated with impaired β cell function in adult mice (61,245). Thus, it is likely that islets from DH animals still have reduced functionality. Future studies will address the ramifications of Pdx1-Oc1 early developmental defects in endocrine programming on susceptibility to adult β cell dysfunction provoked by stressful conditions such as high fat diet.

Taken together, our results suggest that Pdx1 and Oc1 cooperate within primary pancreatic MPCs to promote endocrine specification and to establish a permissive intracellular environment for later steps of endocrine differentiation and maturation. Together, these two transcription factors initiate a network of gene expression beyond simple activation of the

endocrine progenitor determinant, *Neurog3*. Current directed differentiation protocols are based heavily on Pdx1 and Neurog3 induction. The concerted action of Oc1 and Pdx1 is critical for the future timely maturation of endocrine cells and that their cooperative role in ES or iPS cell differentiation toward functional β cells should be considered.

CHAPTER IV

PDX1 AND OC1 COOPERATIVITY IS NECESSARY FOR POSTNATAL β-CELL MATURATION AND ADAPTABILITY

Adapted from Kropp *et al*., "Cooperative function of Pdx1 and Oc1 in multipotent pancreatic progenitors impacts postnatal islet maturation and adaptability", *American Journal of Physiology: Endocrinology & Metabolism*, In press

Introduction

T2D affects nearly 10% of the adult population in the USA. In spite of the depth and breadth of research into the origins of T2D and β-cell dysfunction, the distinguishing factors that predispose individuals to developing T2D remain unclear. Of particular importance are the transcription factors that regulate β-cell development and function since establishment and maintenance of functional β-cell mass is essential for maintaining healthy glucose homeostasis. β-cell function is established in the postnatal period through the process of β-cell maturation. In particular, P9-P14 is appreciated as a critical window of mature β-cell characteristics, especially regulation of GSIS. Multiple groups have demonstrated that immature β-cells secrete insulin more constitutively, even at low (<5.6 mM) glucose conditions, whereas mature β-cells have very low insulin secretion at low glucose (81-84). This functional change is accompanied by changes in gene expression including a switch from MafB to MafA expression (in mice), decreased expression of "disallowed" genes involved in glucose metabolism (*hexokinase 1/2*, *lactate dehydrogenase A*, *aldolase B*, etc…), and increased expression of factors such as Ucn3

which participate in feedback loops to suppress prolonged insulin secretion (61,62,81,82,90,91,246). The attainment of this mature state is vital for the establishment of functional β-cell mass and adult glucose homeostasis.

Autopsy studies reveal a wide range of β-cell mass in the human population and that obese non-diabetic individuals have greater β-cell mass compared to lean individuals or obese individuals with T2D. It is possible that the increased β-cell mass observed in pancreata from obese non-diabetic individuals is due to a compensatory response to the insulin resistance and increased insulin demand associated with obesity (247). Multiple possibilities exist for the decreased β-cell mass in pancreata from obese individuals with T2D (248). One explanation is variations in developmental processes in which some individuals having reduced β-cell mass at birth, leading to increased T2D susceptibility with age and weight gain. In this circumstance, a lower β-cell mass could be sufficient to maintain normoglycemia in lean conditions, but would be insufficient to maintain glucose homeostasis in the face of increased metabolic stress (e.g. increased glucose metabolism and insulin secretion) associated with obesity and insulin resistance. Another possibility is that it is not the β-cell mass that one starts with that determines susceptibility to T2D, but rather the ability of one's β cells to compensate with increased insulin output and/or increased β-cell proliferation in response to the increased stress of obesity and/or insulin resistance. A third possibility is that the increased stress on β cells due to obesity and insulin resistance induces β-cell exhaustion, dedifferentiation, and/or death subsequently causing a reduction in functional β-cell mass (Figure 4-1). Baseline β-cell mass at birth, the ability to undergo β-cell mass expansion, and susceptibility to β-cell apoptosis can all vary depending on genetic makeup, and these possibilities could collectively contribute

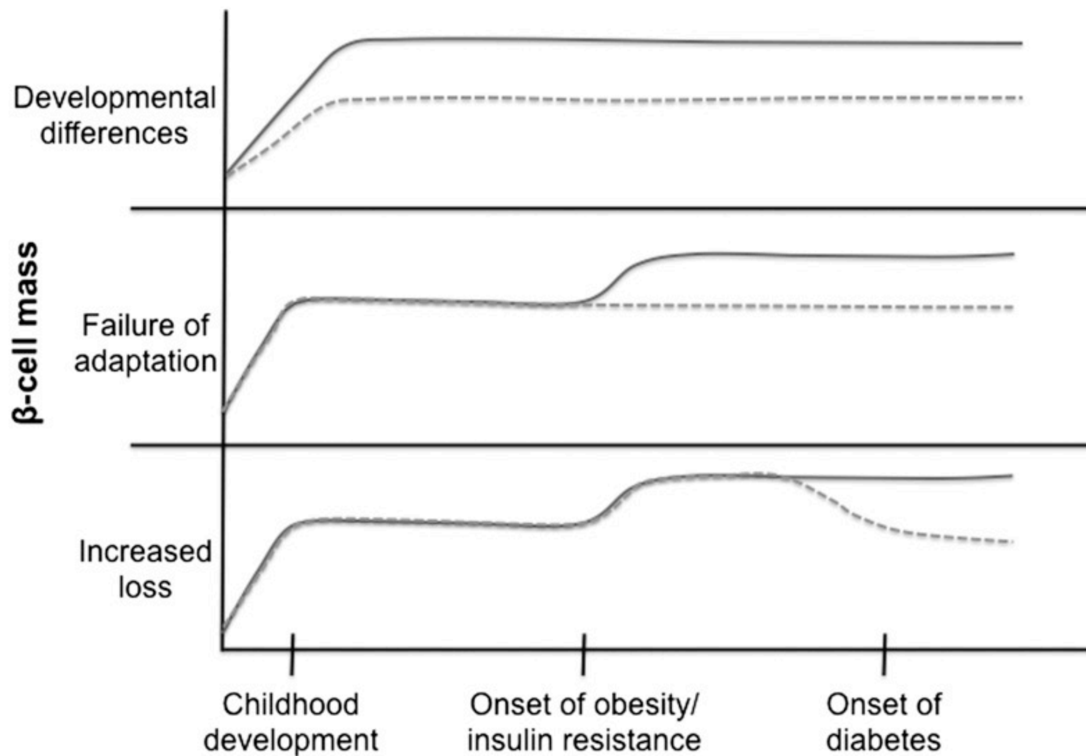

Figure 4-1: Three possible timelines resulting in diminished β-cell mass in type 2 diabetes at the time of autopsy. In the *top panel*, a failure to achieve adequate β-cell mass during development or early childhood expansion results in decreased β-cell mass that persists throughout life, increasing susceptibility to type 2 diabetes. In the *middle panel*, a failure to expand β-cell mass in adult life in response to obesity and insulin resistance results in failure to produce adequate insulin and the development of type 2 diabetes. In the *bottom panel*, compensatory expansion occurs, but then there is increased loss of β-cells that results in lower β-cell mass when measured at autopsy. The *dotted lines* represent the timeline in an individual with increased susceptibility to diabetes. Figure modified and reprinted with permission from Linnemann *et al*, 2014 (249).

to T2D risk. We previously showed that combined reduction in two critical pancreatic transcription factors, Pdx1 and Oc1, leads to reduced endocrine cell specification during embryonic development. Here, I report that the function of these two factors during embryonic development also affects postnatal β-cell maturation and adaptability to stress.

Previous work showed that Pdx1 and Oc1 act cooperatively to promote expression of *Neurog3* (154), so I hypothesized that these two transcription factors act within the same genetic pathway to promote endocrine specification and differentiation (224). In a classic genetic test using double heterozygosity, I aimed to determine if simultaneous reductions in each factor would have a greater impact on endocrine development than single heterozygosity for either factor alone. As predicted, I observed significant alterations in endocrine gene expression at e15.5 and subsequent reductions in endocrine specification and differentiation. Further, I observed a functional defect in β cells of DH evidenced by significantly blunted insulin production and subsequent hyperglycemia at P1. Although the blood glucose phenotype resolved by weaning in these mice, they continued to have reduced expression of *MafA,* a transcription factor involved in β-cell maturation and function (62,64,70), suggesting a potential defect in these processes.

I thus predicted that, postnatally, DH would demonstrate impairments in or delayed expression of genes important for mature β-cell function and would have altered GSIS as adults. Further, I predicted that the underlying defects in β cells of DH would impair their ability to adapt to stressors such as high-fat diet and insulin resistance. Here, I report that islets of postnatal DH indeed have altered morphology and substantial defects in expression of genes involved in β-cell identity, maturation, and function. Additionally, β cells from DH have an

inherent defect in adaptive proliferation that cannot be attributed to single heterozygosity of either *Pdx1* or *Oc1*.

Results

Pdx1 **and** ***Oc1*** **single or double heterozygosity results in reduced β-cell mass at postnatal day 7**

We previously reported that global heterozygosity for *Pdx1* and *Oc1* resulted in significant defects in the development of the pancreatic endocrine compartment (224). At P1, DH mice had reduced body weight, elevated blood glucose, and substantial defects in *insulin* mRNA and protein expression. Since DH mice had normal body weight and blood glucose by weaning (P21), I examined intermediate time points to determine when these phenotypes resolved. I first examined control, SH and DH mice at P7, an age at which β cells are still immature. DH mice no longer presented with body weight or *ad lib* blood glucose impairments suggesting an improvement from P1 (Figure 4-2A, B). In spite of normal weight gain and glucose homeostasis, both *Pdx1* heterozygous (PH) and DH mice had slight but significant decreases in pancreas mass (Figure 4-2C). Pancreas mass was normal in both groups at P1 (Figure 3-12D), so this result suggests an impairment in growth of the pancreas in the early postnatal window. The β-cell compartment was disproportionately affected by either *Pdx1* or *Oc1* heterozygosity compared to the entire pancreas since PH, OH, and DH mice all presented with significantly reduced β-cell mass compared to controls (Figure 4-2D). The most likely explanation for this finding would be reduced β-cell proliferation, but only DH mice presented with reduced β-cell proliferation compared to controls (Figure 4-2E). It is possible that β-cell

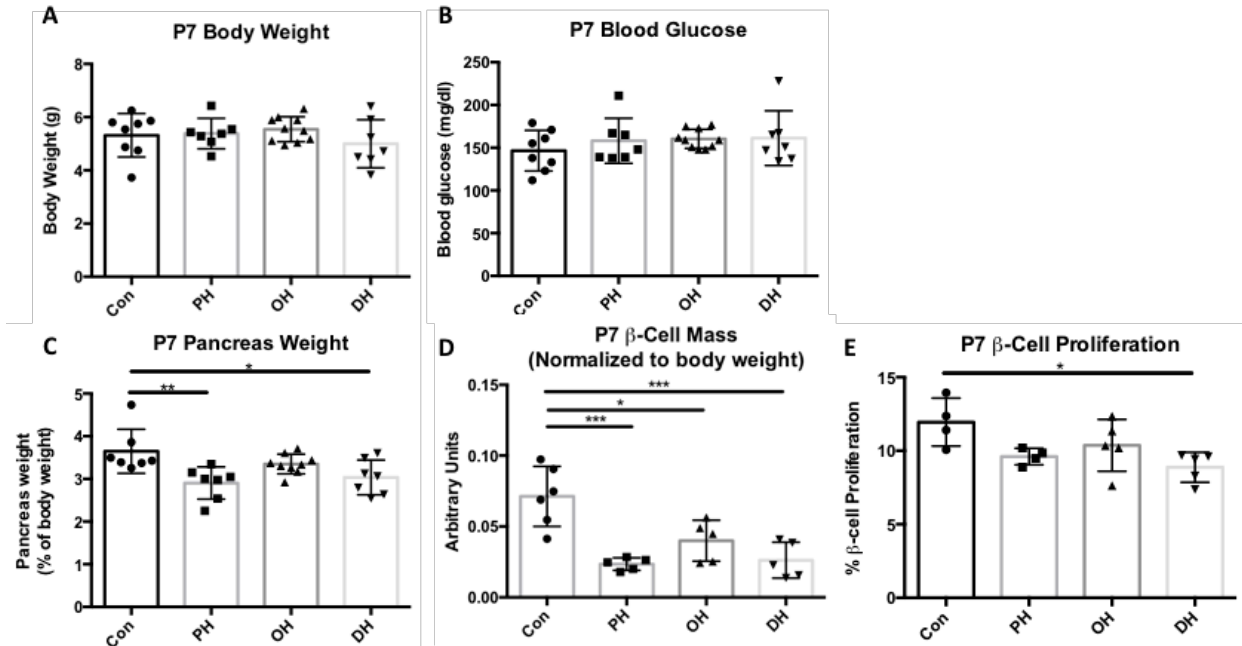

Figure 4-2: P7 SH and DH mice have complex pancreas-specific defects. Body weight (A) and blood glucose (B) is unchanged in any group at P7. (C) Pancreas weight is reduced in PH and DH mice compared to controls. (D) SH and DH mice have reduced β-cell mass compared to Con. (E) DH mice have reduced β-cell proliferation compared to Con. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ by One-Way ANOVA with Tukey correction for multiple comparisons.

proliferation in the SH groups was reduced between P1 and P7, but that decrease was no longer present at the time of analysis.

I next analyzed whether the decreased β-cell mass was correlated with decreased pancreatic insulin content at P7. I had previously observed that exclusively DH mice had decreased insulin production at P1 in spite of a trend toward reduced β-cell mass in both PH and DH mice, so I predicted that DH mice would continue to have a specific impairment in pancreatic insulin content. While DH mice did have reduced insulin content compared to OH, PH had an equal reduction also compared to OH (Figure 4-3A). PH mice also had reduced pancreatic glucagon content compared to OH mice (Figure 4-3B), but none of these changes in hormone content resulted in significant changes in serum insulin or glucagon (Figure 4-3C, D). The normal levels of serum insulin and glucagon likely accounts for the normal blood glucose observed in each group. Together, these data demonstrate that whole-body physiology normalizes in the window from P1 to P7, but islet defects remain in DH and are beginning to develop in PH.

Pdx1 **and** ***Oc1*** **double heterozygotes show normal growth at postnatal day 14**

While the whole-body phenotype of DH resolves by P7, I hypothesized that the persistent defects in insulin production at P1 (224) and P7 and reduced *MafA* expression at weaning (224) suggested a problem in β-cell development that impaired the ability of DH β cells to functionally mature. As noted in the Chapter I, a critical window for β-cell maturation exists between P9 and P14, thus, I chose to analyze DH mice at P14 to determine if there was evidence of a β-cell maturation defect. Similar to what I observed at P7, body weight was

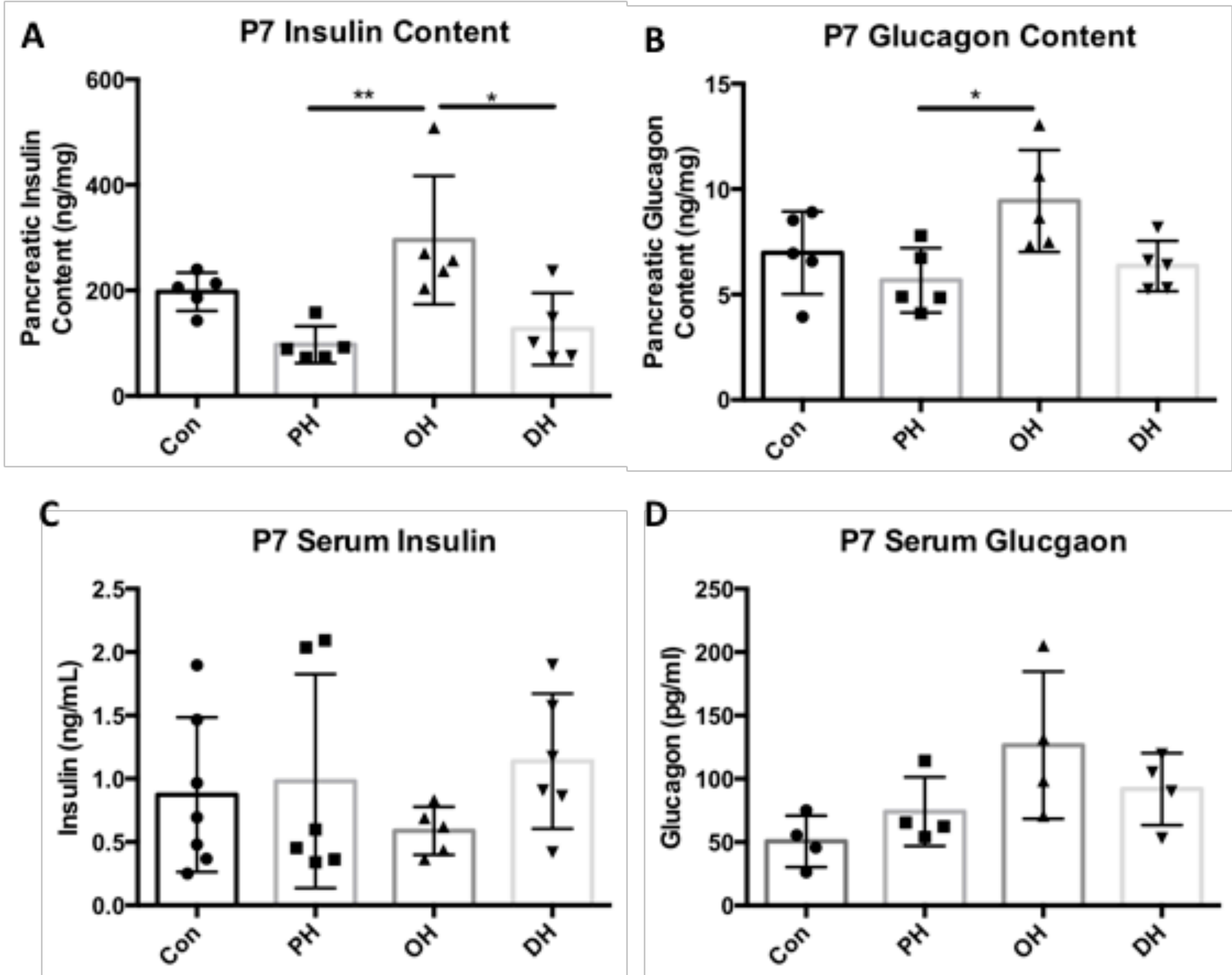

Figure 4-3: Changes in hormone content do not affect serum hormone levels at P7. (A) Pancreatic insulin content is reduced in PH and DH mice compared to OH. (B) PH mice have reduced pancreatic glucagon content compared to OH. No group has any change in serum insulin (C) or glucagon (D). *: $p<0.05$, **: $p<0.01$ by One-Way ANOVA with Tukey correction for multiple comparisons.

normal in all groups at P14 (Figure 4-4A) suggesting that there were no gross defects in whole body metabolism. Unlike at P7, both PH and DH mice had normal pancreas mass, and, surprisingly, there were no significant differences in β-cell mass in any group (Figure 4-4B, C). The recovery in β-cell mass was not due to increased β-cell proliferation, size, or number (Figure 4-5) although there was a trend toward reduced β-cell number in DH mice. These data suggest that pancreas, and β-cell, expansion normalizes by P14 but by an unknown mechanism.

Pdx1-Oc1 double heterozygosity results in lower blood glucose and reduced circulating insulin

P1 and P7 DH mice had decreased insulin production and P1 DH mice had elevated blood glucose (224). By contrast, I observed an unexpected reduction in *ad lib* blood glucose levels in DH mice at P14 compared to littermate controls and SH (Figure 4-6A). I had predicted that if proper levels of Pdx1 and Oc1 in MPCs were important for postnatal islet maturation, then insulin production would be low in DH animals just as it had been at P1 and P7. As anticipated, DH mice had reduced total pancreatic insulin content at P14 as did the PH (Figure 4-6B). Mature β cells normally secrete very low levels of insulin at basal glucose and have a strong insulin secretory response to elevated glucose; immature β cells secrete insulin even at low glucose concentrations and show limited responsiveness to glucose elevation (81). Serum insulin during *ad lib* feeding was not elevated in DH mice and was in fact marginally lower than in PH animals (Figure 4-6C), despite both genotypes having similar reductions in pancreatic insulin content. However, serum insulin was not measured after a fast and thus, we may have missed the time point for elevated insulin secretion in DH pups. I hypothesized that β cells from DH mice are immature and inappropriately secrete insulin at basal glucose conditions, thus

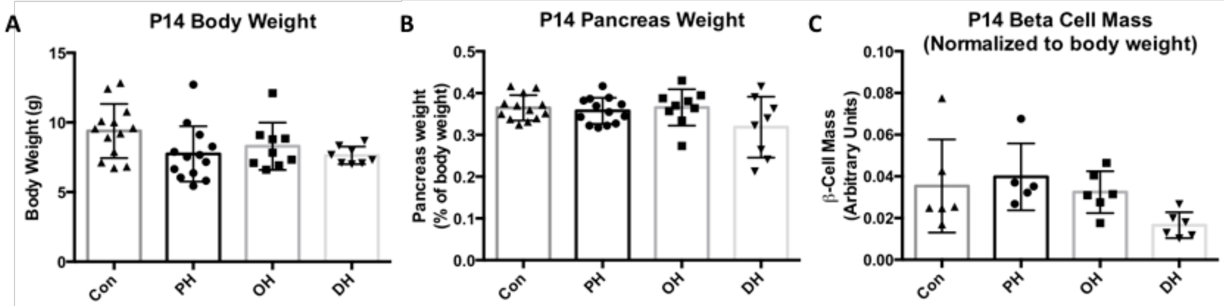

Figure 4-4: Double heterozygosity has no impact on postnatal body, pancreas, or β-cell mass. At postnatal day (P)14, neither single heterozygous nor DH mice have any difference in (A) β-cell mass (n=5-6), (B) body mass (n=8-13), or (C) pancreas mass (n=8-13). β-cell mass is normalized to body mass. Figure modified from Kropp *et al*, 2017, in press.

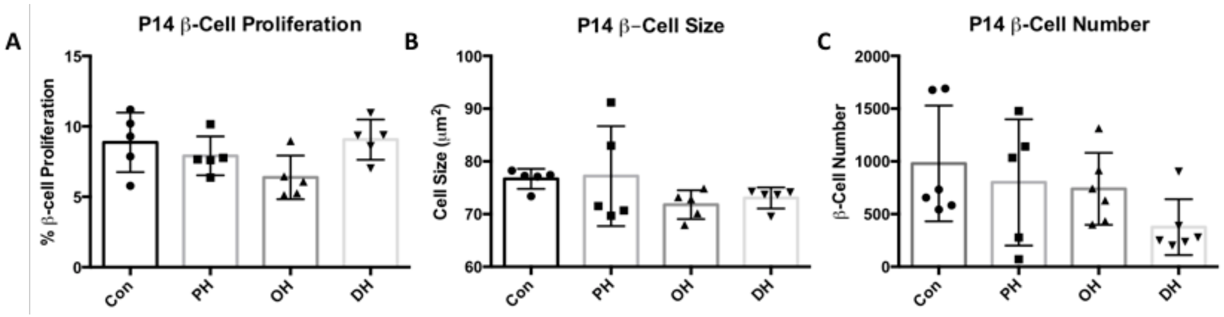

Figure 4-5: P14 DH mice have no difference in β-cell physiology. β-cell proliferation (A), size (B), and number (C) are unchanged in any group at P14.

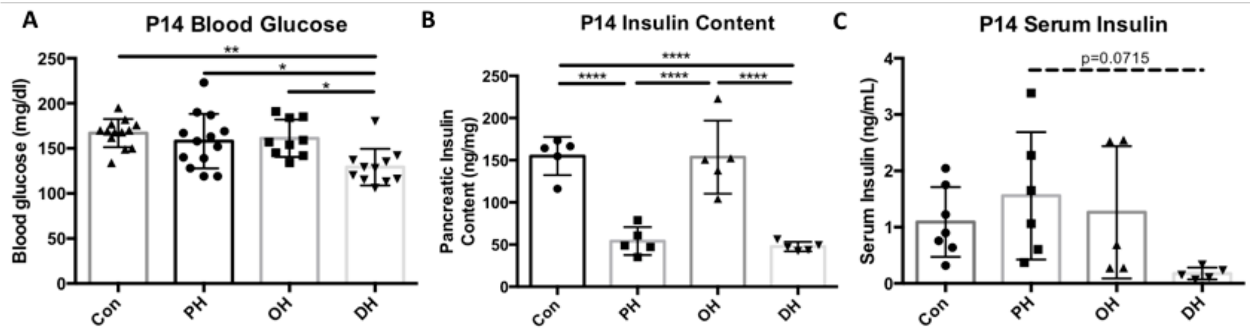

Figure 4-6: Postnatal double heterozygotes have unique insulin and blood glucose alterations. (A) P14 *ad lib* blood glucose is reduced in DH mice compared to littermate controls (n=9-13). (B) DH also have reduced pancreatic insulin content (n=5). (C) Serum insulin levels are highly variable in all genotypes except DH, which have consistently low serum insulin (n=5-7). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<.0001$ by One-Way ANOVA with Tukey correction for multiple comparisons. Figure modified from Kropp *et al*, 2017, in press.

leading to the observed lower blood glucose levels. Static incubation of islets isolated from P14 mice at both basal (5.6 mM) and high (16.7 mM) glucose revealed no difference in basal insulin secretion or GSIS (Figure 4-7A). However, a static incubation is an end-point assay and defects in either the first or second phase of insulin secretion could be missed. An islet perifusion assay would reveal such a defect, but the limited number of islets in P14 mice precluded such an assay. DH islets also had no difference in insulin secretion when stimulated with 20 mM L-arginine, an amino acid stimulant of depolarization that bypasses the glucose metabolism step of GSIS (Figure 4-7B). However, PH islets had an elevated insulin-secretion response to 20 mM L-arginine compared to control and Oc1 heterozygous (OH) islets (Figure 4-7B). This increased amino acid-stimulated insulin secretion could help explain the difference in serum insulin between PH and DH mice even though they have equally reduced pancreatic insulin content.

I previously observed increased liver glycogen content in OH and DH mice at P21 due to Oc1 heterozygosity, in agreement with the role of Oc1 in regulation of genes involved in hepatic glycogen breakdown (201,239,240). Thus, another possible explanation for the reduced blood glucose levels in DH at P14 was an increase in liver glycogen. However, there were no gross differences in liver glycogen at P14 (Figure 4-8A) indicating that the increase in liver glycogen in DH animals occurs sometime between P14 and P21. A third potential explanation for the reduced blood glucose levels in DH at P14 is decreased circulating glucagon. No changes in serum glucagon levels or pancreatic glucagon content were observed (Figures 4-8B, C). These results also indicated that the defects in hormone production and secretion in P14 DH mice appear to be unique to β cells since α-cell function (with respect to glucagon production) was unaltered. Finally, I hypothesized that a defect in islet vascularization could account for the

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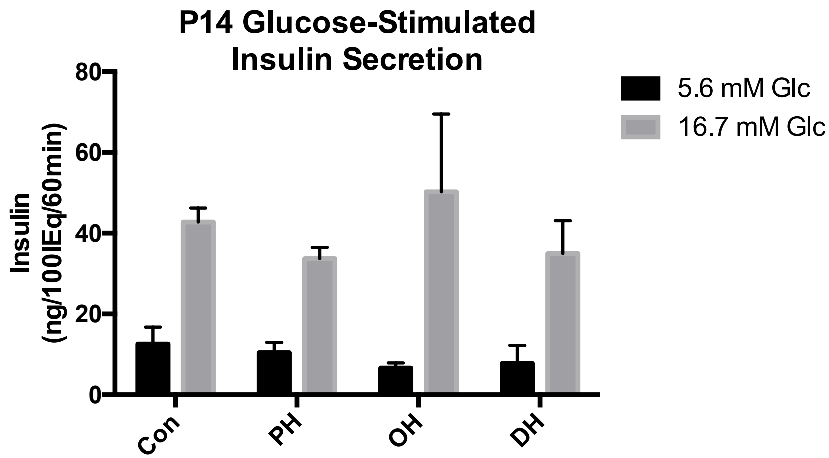

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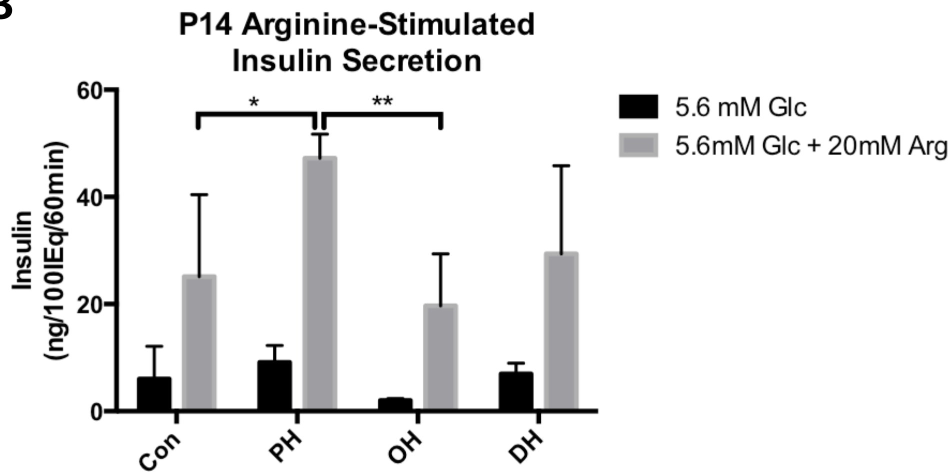


Figure 4-7: P14 DH islets display normal insulin secretion in static incubation assays. (A) Glucose-stimulated insulin secretion and (B) L-arginine-stimulated glucose secretion assayed by static incubation are unchanged in DH islets, but PH islets have increased L-argnine-stimulated insulin secretion (n=3). *: $p<0.05$, **: $p<0.01$ by One-Way ANOVA with Tukey correction for multiple comparisons. Figure modified from Kropp *et al*, 2017, in press.

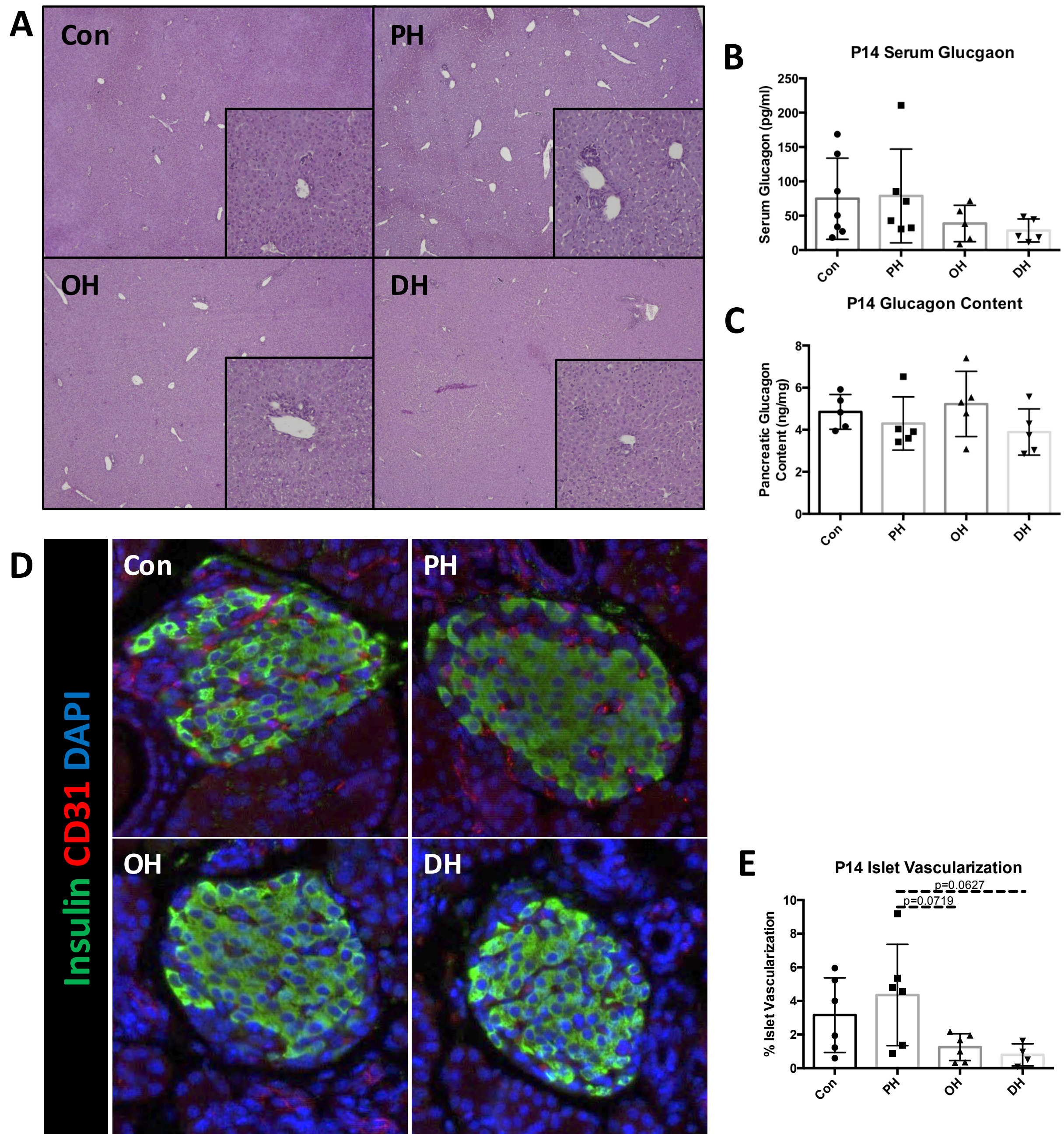


Figure 4-8: Limited differences in confounding reasons for reduced serum insulin. (A) Liver glycogen content, as assessed by Periodic Acid Schiff staining (purple), is unchanged in any group (n=5). Images acquired at 4x magnification; insets at 20x magnification. (B) Single and double heterozygosity has no impact on serum glucagon levels or (C) whole pancreatic glucagon content. (D) Representative images of islet vascularization measured with insulin (green), CD31 (red), and DAPI (blue). (E) Quantification of D. p-value from PH-DH (0.0627) and PH-OH (0.0719). Figure modified from Kropp *et al*, 2017, in press.

discrepancy between serum insulin levels *in vivo* and unstimulated insulin secretion *ex vivo*. While no statistically significant differences in islet vascularization were observed, there was an appreciable trend toward reduced vascularization in both OH and DH islets when compared to PH islets (Figure 4-8D, E). It is possible that the slightly reduced islet vascularization in DH and increased amino-acid stimulated insulin secretion in PH account for the difference observed in serum insulin.

Developmental haploinsufficiency of Pdx1 and Oc1 has a lasting impact on postnatal islet gene expression

The reduced pancreatic insulin content in PH and DH mice at P14, as well as the unique decrease in serum insulin in DH mice, suggested that defects in β-cell maturation could be occurring at the molecular level. Using the altered gene expression from our previously published RNA-Seq analysis as a guide (224), we utilized a custom nanoString Codeset array to assay a set of genes important for β-cell function to determine how double heterozygosity impacted these genes. The DH mice uniquely clustered together in an unbiased clustering analysis (Figure 4-9), whereas gene expression changes between the single heterozygotes and Control groups were insufficient to distinguish those groups. This result is suggestive of an absence of substantial changes in expression of these genes at this age in either PH or OH mice.

We previously reported that *insulin* and *glucagon* transcripts were significantly reduced in DH mice at P1 (224). At P14, we did not observe any significant differences in expression of any of the islet hormones in DH mice (Figure 4-10A). PH mice had significantly increased levels of *ghrelin* at this age, which has not previously been described. Among the transcription factors

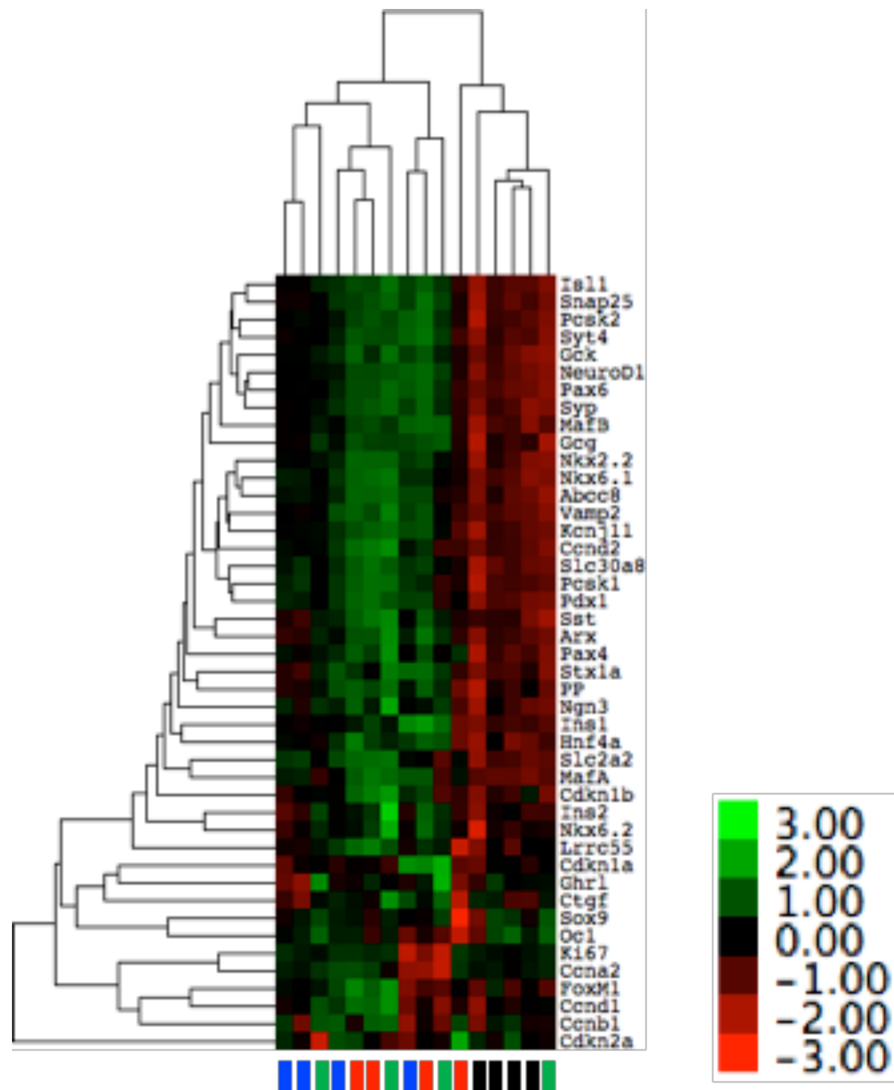


Figure 4-9: DH islets have mostly unique gene expression changes. Expression of select endocrine development and functional genes in P14 islets represented in a heatmap. Green indicates increased gene expression; red indicates reduced gene expression. Controls (blue boxes), PH (green boxes), OH (red boxes), DH (black boxes). Figure modified from Kropp *et al*, 2017, in press.

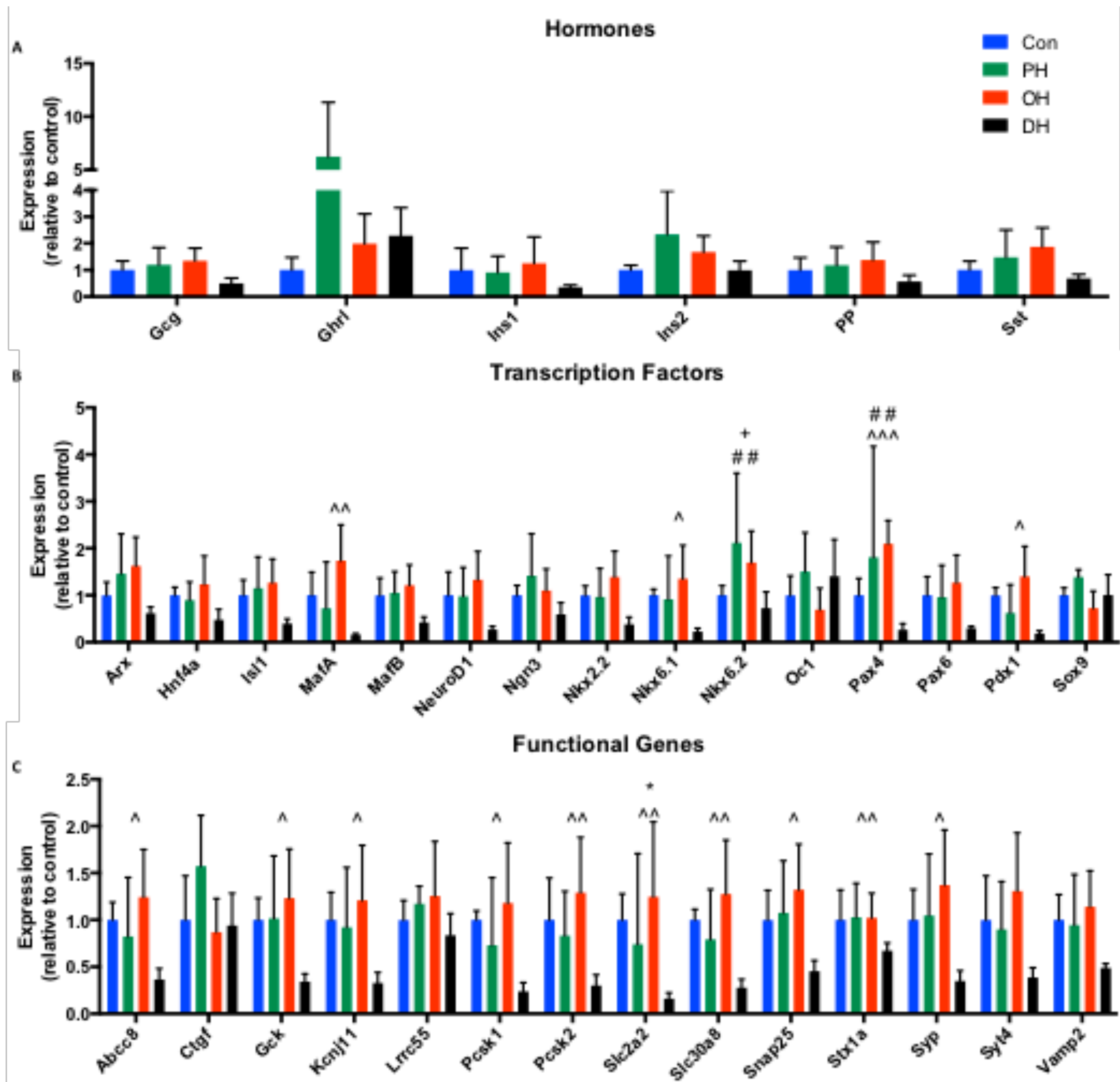


Figure 4-10: Gene expression analysis of P14 islets. Relative expression levels of (A) hormones, (B) regulatory transcription factors, and (C) islet functional genes (n=4). One symbol: p≤0.05; two symbols: p≤0.01; three symbols: p≤0.001; four symbols p≤0.0001. +: Con v. PH; @: OH v. PH; #: DH v. PH; ^: OH v. DH; *: Con v. DH by Two-Way ANOVA with Tukey correction for multiple comparisons or Kruskal-Wallis with Dunn's correction for multiple comparisons. Figure modified from Kropp *et al*, 2017, in press.

that are necessary for β-cell maturation, mRNA levels of *MafA*, *Nkx6.1*, and *Pax4* were reduced in DH mice (Figure 4-10B). Such reductions suggest that the β cells of DH mice have an underlying defect in gene expression resulting in delayed maturation. Impaired gene expression was not limited to regulatory transcription factors. A general trend of reduced expression was observed for genes necessary for glucose sensing (*Slc2a2*, *Gck*), insulin processing (*Pcsk1/2*, *Slc30a8*), and insulin secretion (*Abcc8*, *Kcnj11*) (Figure 4-10C). Importantly, all of these alterations in gene expression are specific to DH mice.

To understand how the changes in gene expression in DH islets at P14 are manifested at a cellular level, we analyzed protein expression patterns for a select number of the factors. Figure 4-11A shows that the intensity of Nkx6.1, a transcription factor important in establishing and maintaining β-cell identity, was reduced in insulin-positive cells in DH mice, suggesting lower levels of Nkx6.1 protein per cell. Further, the number of insulin-positive cells expressing Nkx6.1 was significantly reduced only in DH mice (Figure 4-11B). Together, these results indicate that Pdx1-Oc1 double heterozygosity reduces the number of properly differentiated β cells and that those cells that do express Nkx6.1 appear to do so at a reduced level. We also observed reduced expression of *MafA* in P14 DH islets (Figure 4-10B). Immunofluorescence analysis of MafA did not reveal a change in the number of cells expressing MafA protein (Figure 4-11C), suggesting a decreased level of expression per cell rather than a decreased number of cells, as observed with Nkx6.1. Transcript levels of the primary glucose transporter in the β cell, Glut2 (*Slc2a2*), were also reduced in DH islets (Figure 4-10C). Although somewhat variable, it was readily apparent that DH mice had weaker Glut2 protein expression and that the Glut2 protein in DH β cells showed reduced localization at the plasma membrane compared to single

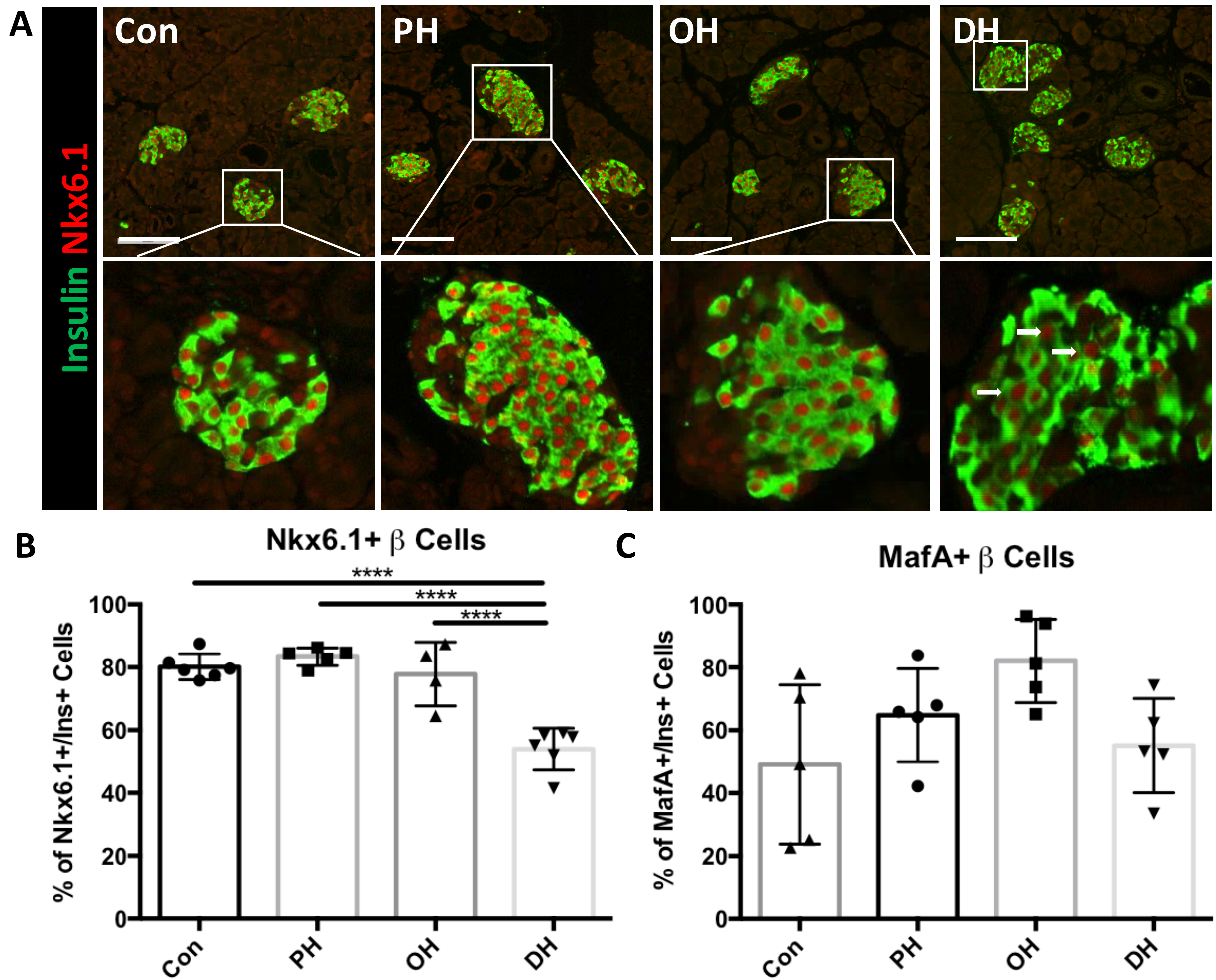


Figure 4-11: Reduced Nkx6.1 expression in P14 DH islets. (A) Representative images of islets with insulin (green) and Nkx6.1 (red) labeled. Arrows pointing to weakly Nkx6.1+ β-cells in DH islets. (B) Quantification of A. (C) Quantification of MafA+ β-cells. Scale bar represents 100 μm. ****: $p<0.0001$ by One-Way ANOVA with Tukey correction for multiple comparisons. Figure modified from Kropp *et al*, 2017, in press.

heterozygote and control animals (Figure 4-12). Gene expression analysis also revealed decreased expression of the pro-hormone convertases *Pcsk1* and *Pcsk2* (Figure 4-10C), which are the primary enzymes responsible for cleaving the proinsulin peptide to mature insulin. Immunofluorescence analysis of proinsulin revealed diffuse localization throughout β cells rather than localization to the Golgi as would be expected (Figure 4-13). This finding is consistent with *Pcsk1/2* knockout animals and highly suggestive of impaired insulin processing (250,251). Together, these data suggest an impaired maturation state in DH β cells that could lead to defects in specific phases of GSIS.

Pdx1-Oc1 double heterozygotes have altered islet morphology

An essential characteristic of mature mouse islets is the presence of a β cell core surrounded by a mantle consisting predominantly of α cells. This typical islet morphology is initiated during development, but becomes fixed in the early postnatal period. This morphology is essential for β cell-to-β cell communication via gap junctions and the coordinated secretion of insulin (252). I analyzed the distribution of α cells in the islet at P14 to determine if Pdx1-Oc1 double heterozygosity impacted the sorting of endocrine cells, resulting in mixed islets. Non-mantle α cells (cells more than 2 cell-diameters from the edge of the islet) were counted in all groups. While PH mice did have some mixed islets, DH mice showed a consistent tendency toward islets having an altered morphology (Figure 4-14A, quantified in 4-14B). Not only did DH mice have an increased percentage of islets with a mixed phenotype, but also an increased proportion of α cells that were non-mantle in DH mice (Figure 4-14C). This was in spite of no

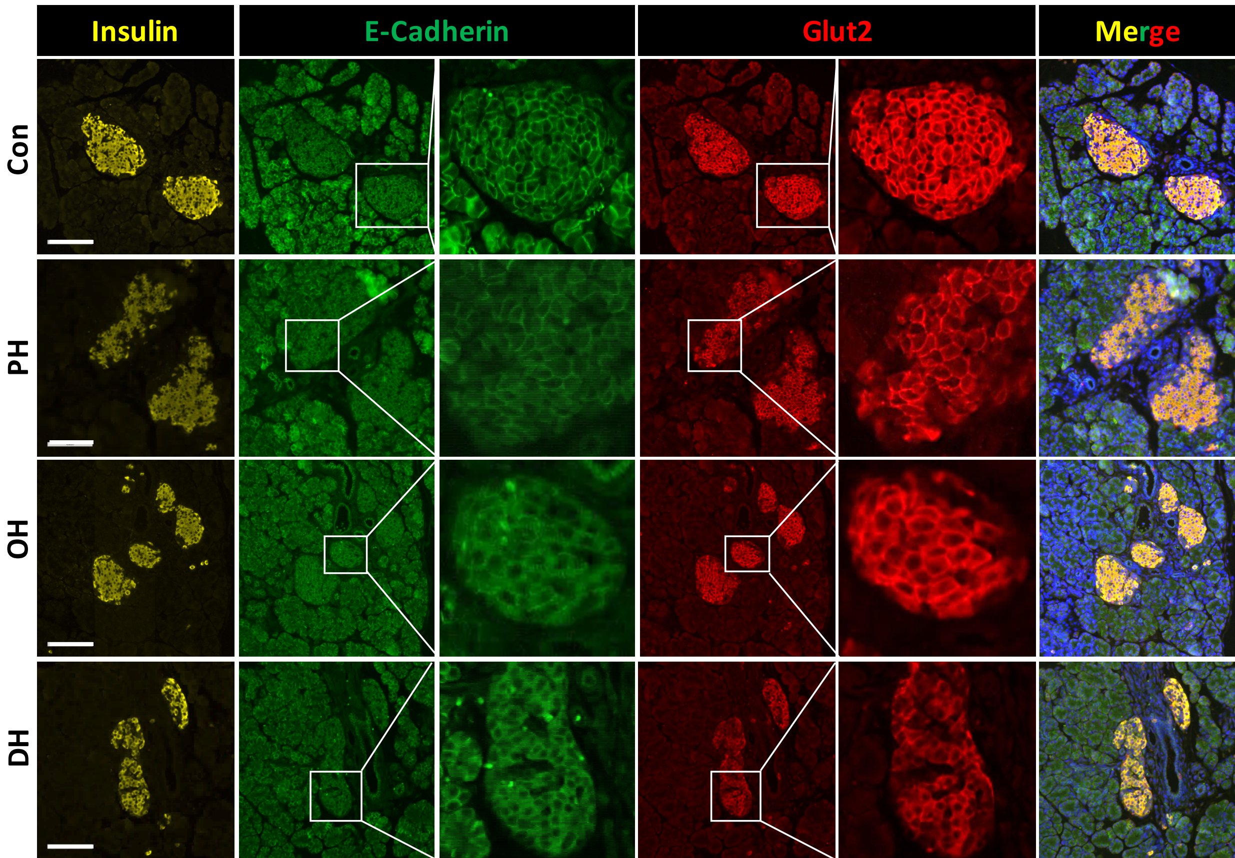


Figure 4-12: Glut2 expression is weak and diffuse in DH β-cells. Representative images of immunofluorescence for insulin (yellow), E-Cadherin (green), Glut2 (red) and DAPI (blue). E-cadherin is used as a membrane marker. Glut2 does not localize to the membrane in DH β cells. Scale bar represents 100 μm. Figure modified from Kropp *et al*, 2017, in press.

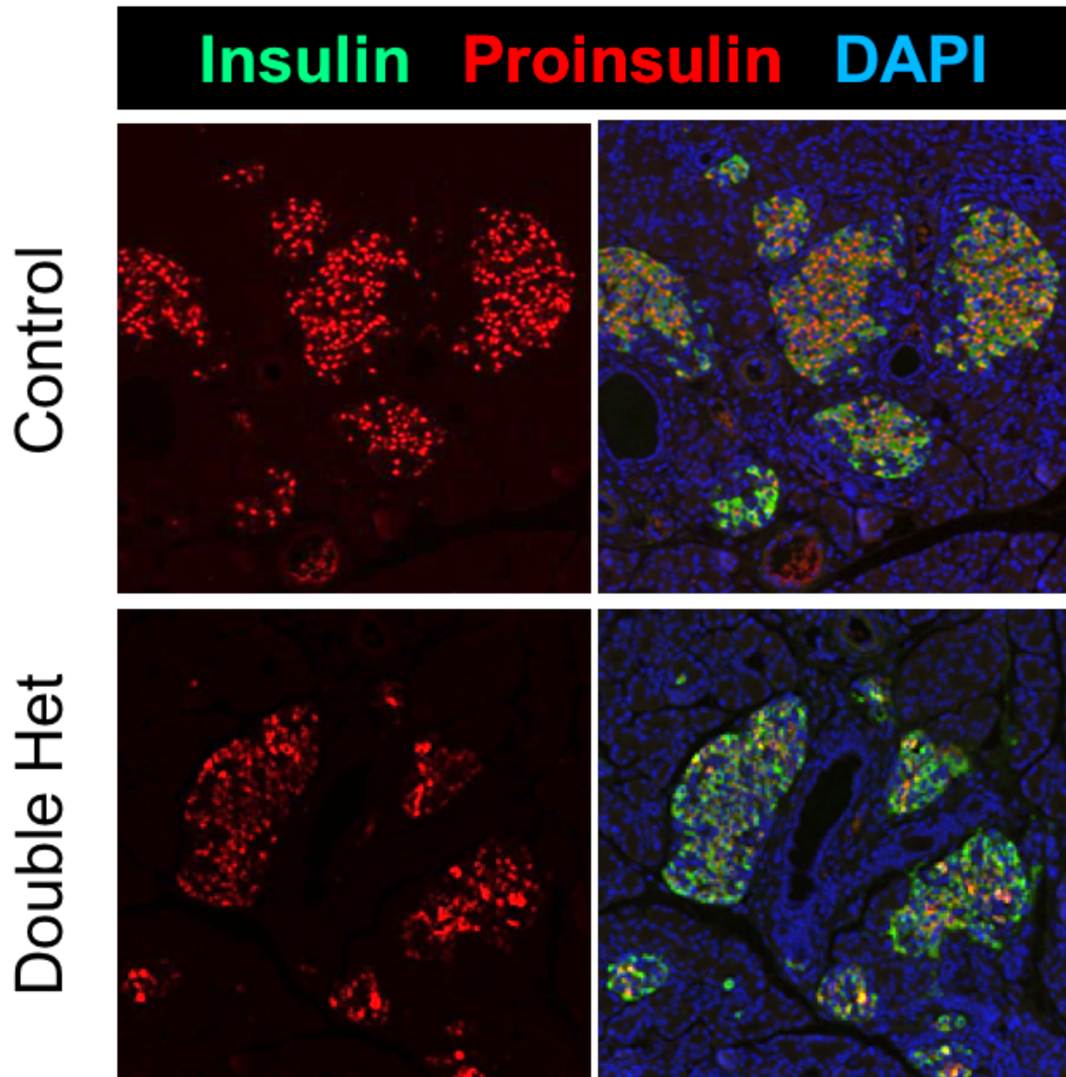
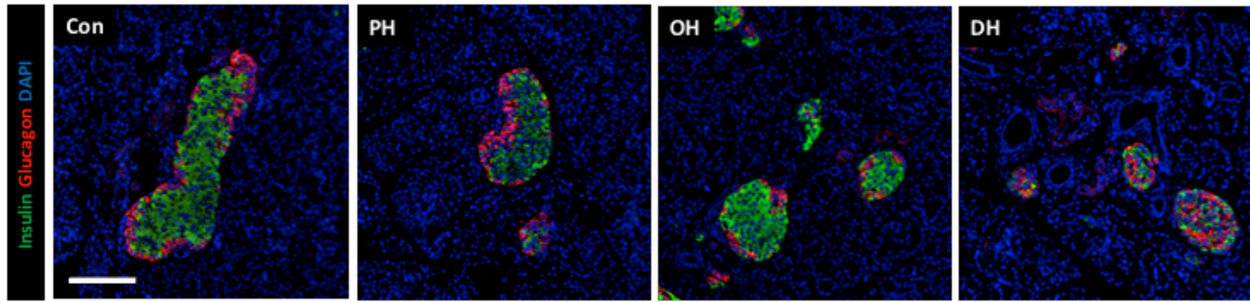


Figure 4- 13: Aberrant proinsulin localization in P14 PH β cells. Above: Control β cells have foci of proinsulin in the Golgi. DH β cells have diffuse and mislocalized expression of proinsulin.



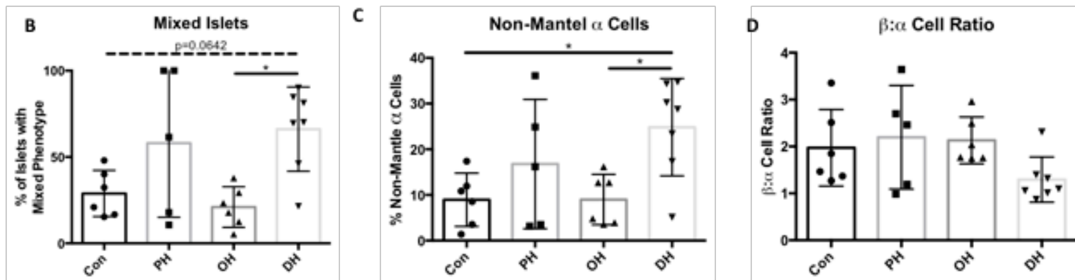


Figure 4-14: Islets of double heterozygotes show altered islet architecture. (A) P14 DH islets lack a distinct β-cell core and α-cell mantle (Insulin, green; Glucagon, red; DAPI, blue). Quantification of (B) the percentage of islets with a mixed phenotype and (C) the percentage of non-mantle α cells in each genotype at P14, without a change in β:α cell ratio (D) (n=5-7). Scale bar represents 100 μm. Figure modified from Kropp *et al*, 2017, in press.

change in the α:β cell ratio (Figure 4-14D). Together, these data suggest that Pdx1-Oc1 double heterozygosity impairs the ability of islet cells to organize into functional units.

I additionally wanted to assess the maturation state of DH β cells by examining expression of Ucn3, a peptide hormone expressed exclusively in mature mouse β cells. Recent work from multiple groups has demonstrated that Ucn3 expression reaches the level of adult β cells by P10, and that Ucn3+ β cells are glucose responsive. Immunofluorescence analysis demonstrated that DH β cells had noticeably lower and inconsistent Ucn3 expression (Figure 4-15). This finding further supports the idea that P14 DH β cells have an impairment in their maturation state and is consistent with the reduced Nkx6.1 and Glut2 expression.

In adult mice, impaired β-cell function in Pdx1-Oc1 double heterozygotes is offset by increased liver glycogen storage

We had previously observed that DH mice had normal glucose tolerance at weaning likely due to increased liver glycogen storage (224). However, mRNA expression of the transcription factor *MafA* remained reduced in DH, suggesting that there could be inherent β-cell immaturity and/or impaired function. To determine if persistent underlying defects in β-cell maturation and function result in a reemergence of defects in glucose tolerance in DH mice as they age, I analyzed male mice at 12 weeks. There were no differences in body weight, pancreas weight or β-cell mass at this age (Figure 4-16A-C). As multiple groups have observed, adult PH mice were glucose intolerant after a 16-hour fast(Figure 4-16D) (170,171,238). In contrast, 12-week old DH mice had normal glucose tolerance (Figure 4-16D), similar to what was observed at weaning. This finding is especially interesting considering the observation that

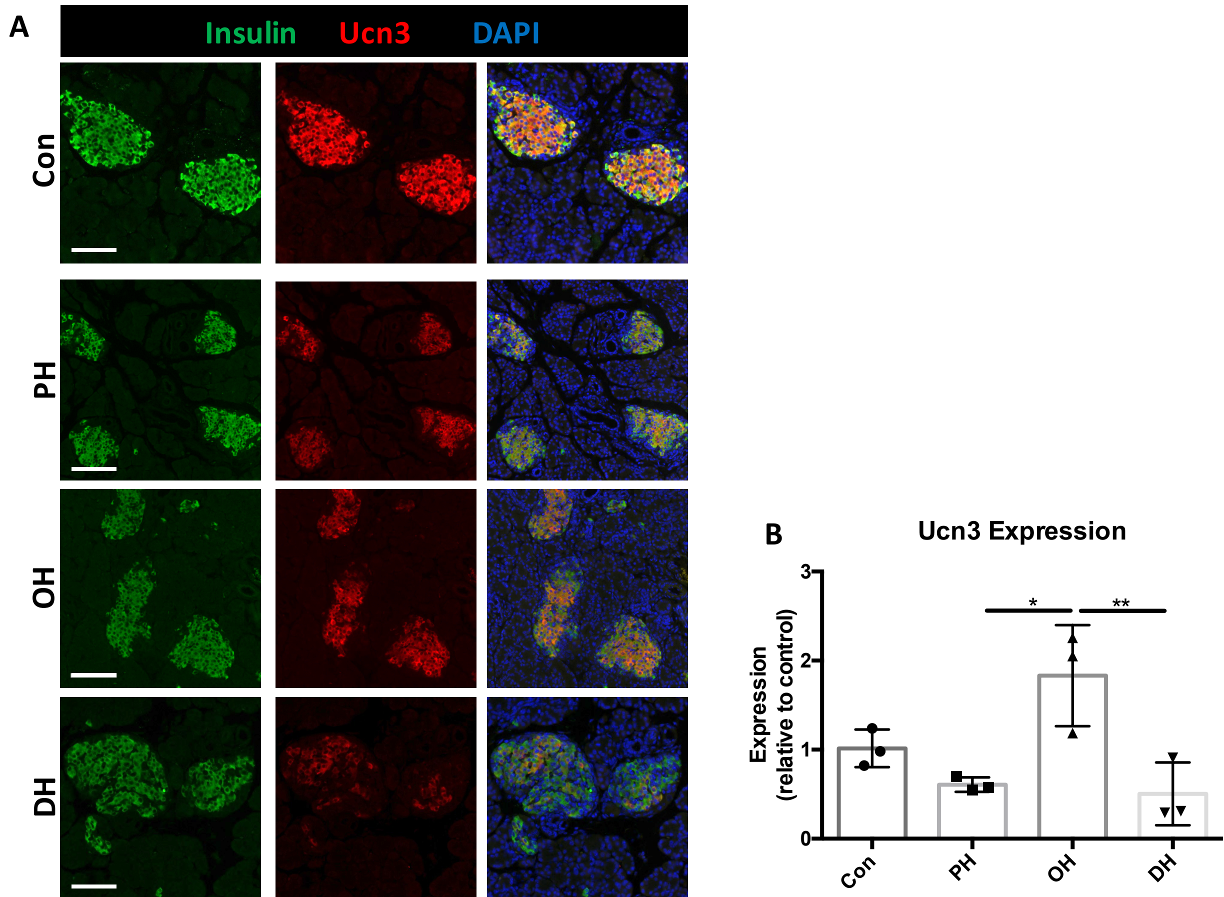


Figure 4-15: Reduced Ucn3 expression in DH islets. (A) Representative images of Ucn3 protein (red) with insulin (green) and DAPI (blue) shoes that it is reduced and diffuse in DH islets. (B) *Ucn3* mRNA is reduced is reduced in PH and DH islets compared to OH islets (n=3 for mRNA, images representative of 6 animals). Scale bar: 100 μm. *: $p \leq 0.05$; **: $p < 0.001$by One-Way ANOVA with Tukey correction for multiple comparisons. Figure modified from Kropp *et al*, 2017, in press.

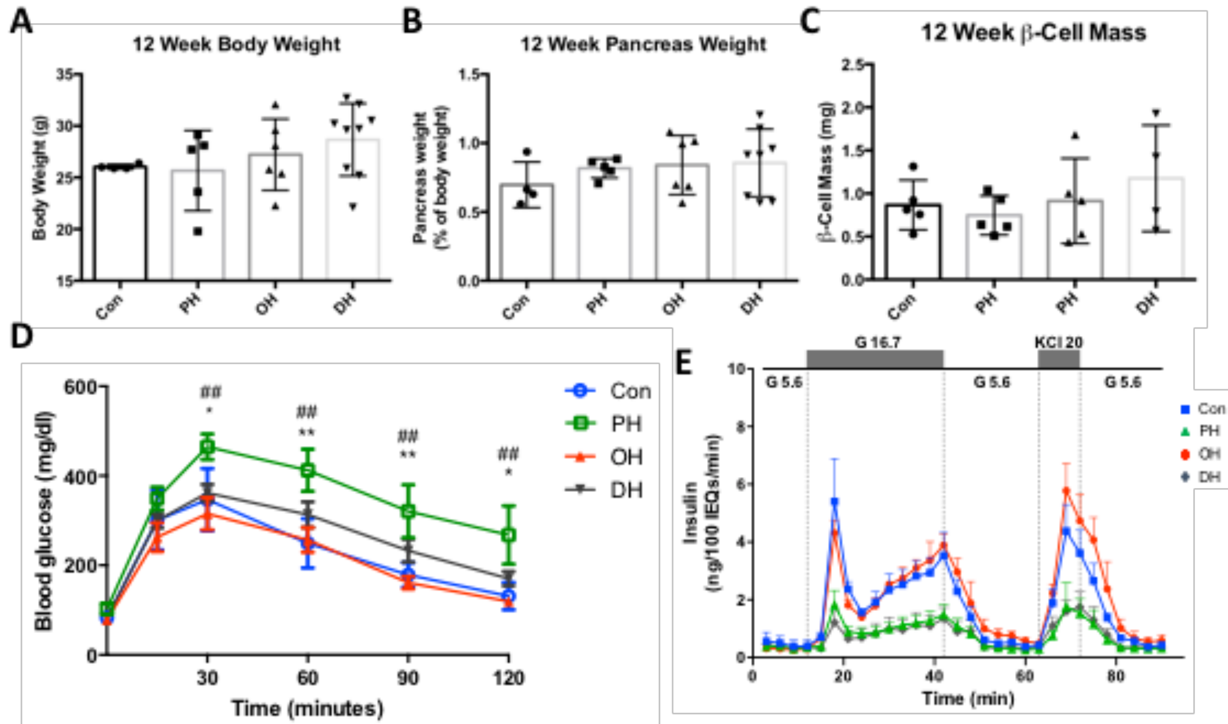


Figure 4-16: Altered β-cell function in adult PH and DH mice. Three-month old male DH have no difference in (A) body mass (n=4-9), (B) pancreas mass (n=4-8), or (C) β-cell mass (n=4-5). (D) Glucose tolerance after a 16-hour fast is impaired in PH; in contrast, DH have normal glucose tolerance (n=6-8). (E) Both PH and DH have impaired glucose-stimulated insulin secretion in a perifusion assay (n=3). One symbol: $p \leq 0.05$; two symbols: $p \leq 0.01$. *: Con v. PH; #: OH v. PH by Two-Way ANOVA with Tukey correction for multiple comparisons. Figure modified from Kropp *et al*, 2017, in press.

both PH and DH mice have significantly impaired GSIS in a perifusion assay (Figure 4-16E). The normal glucose tolerance observed in DH mice is likely due to increased liver glycogen content in DH mice compared with PH mice (Figure 4-17A), which I hypothesize is due to direct effects of decreased Oc1 activity in the liver (201,239,240). If the liver is acting as a glucose sink in DH animals, then the demand for insulin would be lower and DH β cells would not need to secrete as much insulin as control or PH β cells which I tested by measuring serum insulin during a GTT (Figure 4-17B, C). While this method is indirect, it does provide useful information about insulin dynamics in the blood. I found that there were no statistically significant differences in serum insulin levels in this assay, but Con animals had incredibly variable insulin measurements complicating the statistical analysis. In spite of the lack of statistical differences, there appears to be a trend toward reduced insulin in the DH animals as would fit my above prediction, however, these results do not allow for any definitive conclusions.

Impaired β-cell proliferation in adult Pdx1-Oc1 double heterozygotes in response to proliferative stimuli

Given that adult DH mice have impaired insulin secretion and reduced expression of *MafA*, I hypothesized that the underlying β-cell dysfunction would result in an impaired ability of DH β cells to respond to stress. To test this hypothesis, I placed 8-week-old male mice on a high fat diet (HFD) for 5 weeks. We have previously shown that 5 weeks of HFD is sufficient to induce insulin resistance (185). Insulin resistance places increased stress on β cells, which normally compensate with increased increase insulin production and proliferation (191). I observed that control, OH, and DH mice became glucose intolerant at the end of the 5 weeks of

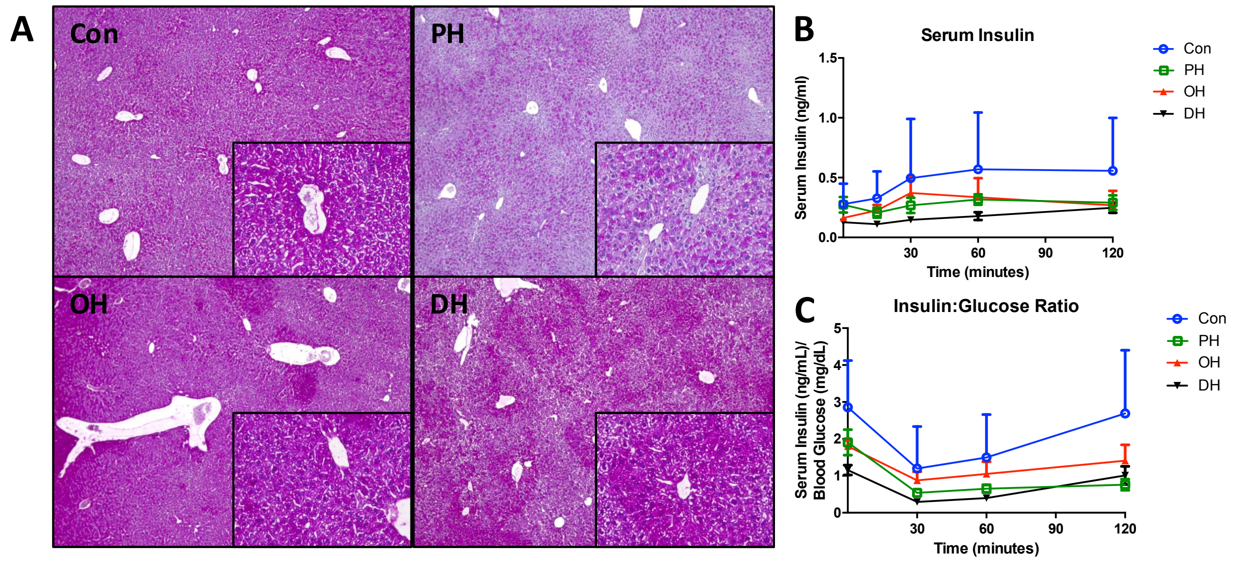


Figure 4-17: Liver glycogen offsets β-cell dysfunction in adult double heterozygotes. (A) Representative images of Periodic Acid Schiff staining for liver glycogen (purple) (n=5). Images acquired at 4x magnification; insets at 20x magnification. (B) Serum insulin measurements during IPGTT and (C) ratio of serum insulin to blood glucose. Figure modified from Kropp *et al*, 2017, in press.

HFD compared to age-matched, chow-fed mice (Figure 4-18). PH mice did not become any more glucose intolerant than their chow-fed counterparts with this duration of HFD treatment. Together, these results demonstrate that the HFD did effectively induce glucose intolerance.

As noted above, the normal response to insulin resistance is an increase in β-cell proliferation in order to increase the functional β-cell mass. β-cell mass increased significantly in HFD-fed control and PH mice, while OH mice showed a trend in increased β-cell mass (Figure 4-19A). DH mice showed no change in β-cell mass compared to chow-fed mice. This lack of β-cell mass expansion in HFD-fed DH mice was due to decreased β-cell proliferation (Figure 4-19B). DH mice had a similar impairment in glucose tolerance compared to control and OH mice (Figure 4-18), suggesting that DH β cells are exposed to a similar β-cell proliferative stimulus. When I compared HFD β-cell proliferation (from Figure 4-19B) to β-cell proliferation in chow-fed animals, only DH mice failed to increase β-cell proliferation in response to the diet (Figure 4-19C).

To further determine if the lack of an increase in β-cell proliferation in DH mice is due to an inherent inability to respond to proliferative stimuli, we performed an *ex vivo* proliferation assay using the growth factor placental lactogen as a stimulus for proliferation. Our group and others have shown that placental lactogen induces β-cell proliferation (218,253) and is one of the driving factors for increased β-cell proliferation and mass during pregnancy (254). Although the results were variable in controls and OH, it is clear that β cells from PH and DH mice do not respond to placental lactogen (Figure 4-19D) even though all groups express comparable levels of the prolactin receptor (Figure 4-19E). This result, paired with the lack of β-cell proliferation *in*

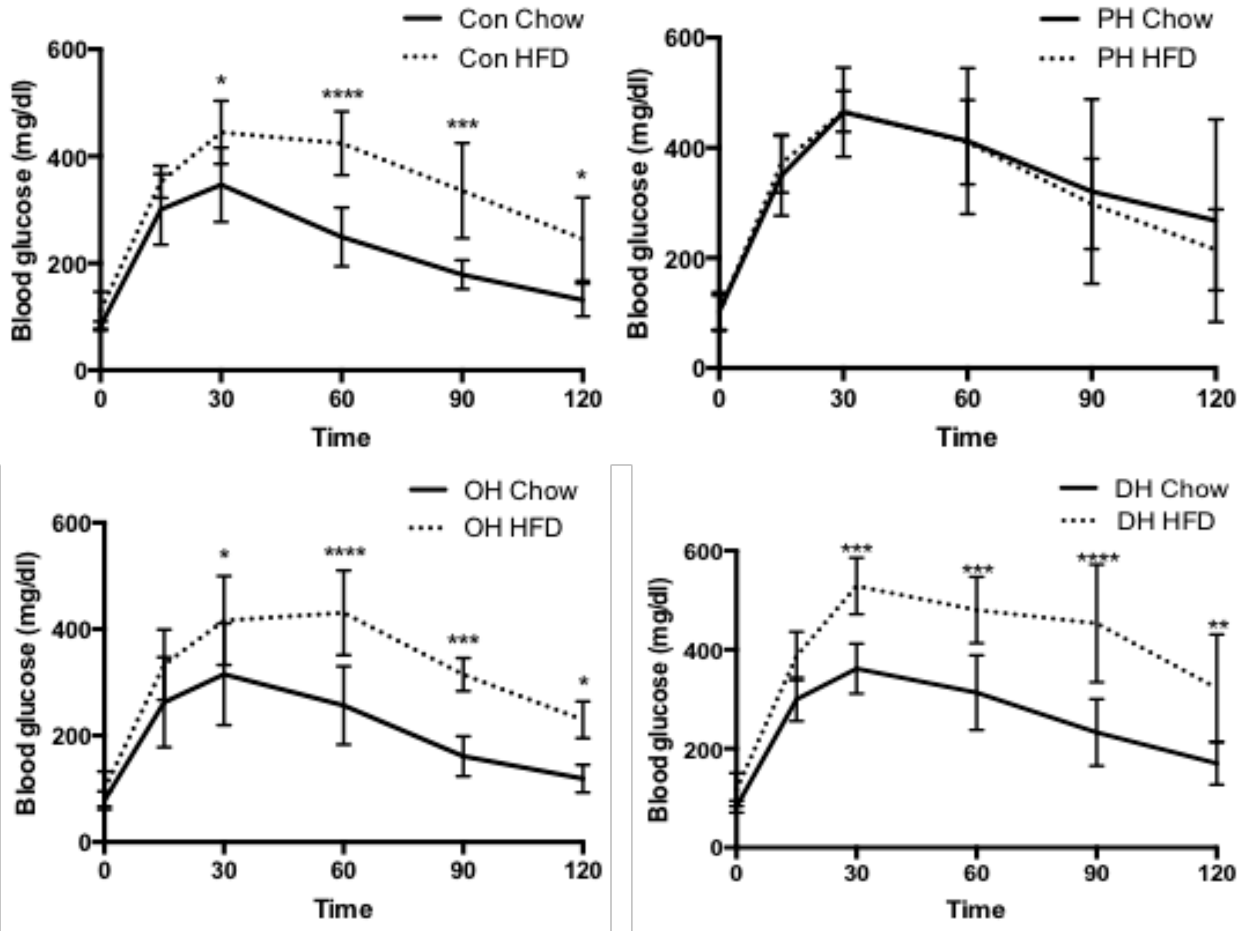


Figure 4-18: HFD glucose tolerance. PH mice do not become any more glucose intolerant than on chow diet. Con, OH, and DH become glucose intolerant. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<.0001$ by One-Way ANOVA with Tukey correction for multiple comparisons. Figure modified from Kropp *et al*, 2017, in press.

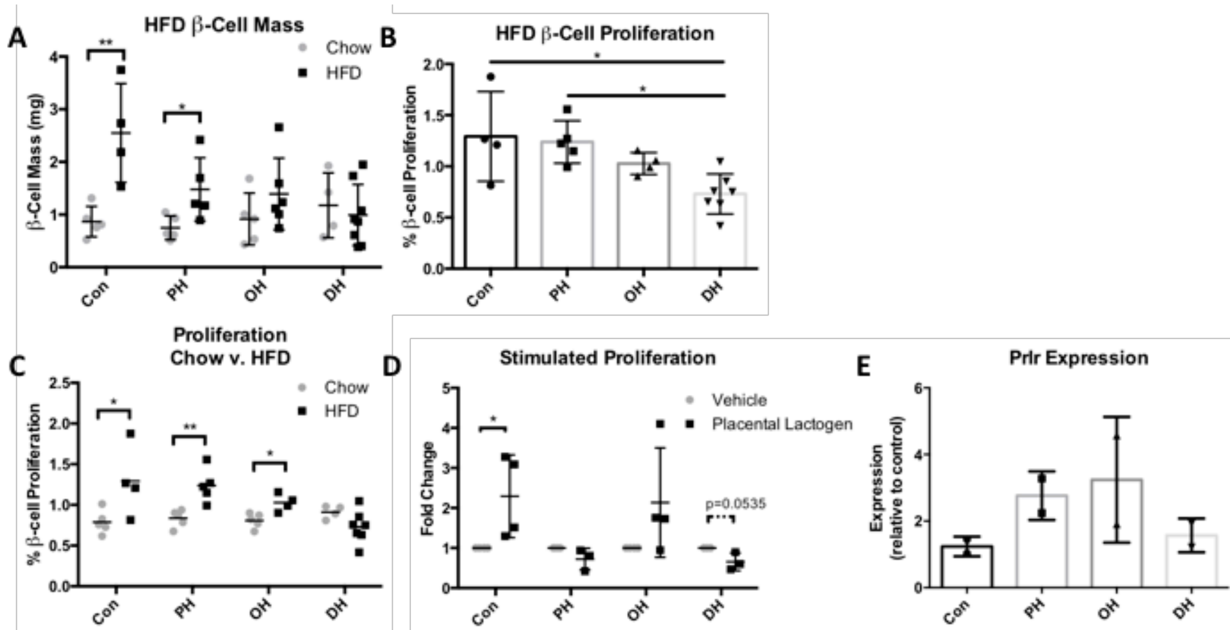


Figure 4-19: Impaired β-cell proliferation in adult DH mice. (A) Expansion of β-cell mass due to HFD compared to age-matched chow-fed animals (n=4-7). (B) β-cell proliferation following HFD (n=4-7). (C) Comparison of β-cell proliferation on chow diet versus HFD for each genotype (n=4-7). (D) Fold change in β-cell proliferation in response to placental lactogen in an *ex vivo* proliferation assay (n=3-4). (E) Prolactin receptor (*Prlr*) expression in 8-week old male islets (n=2). *: $p \leq 0.05$; **: $p \leq 0.01$; by Two-Tailed Students T-Test or One-Way ANOVA with Tukey correction for multiple comparisons. Figure modified from Kropp *et al*, 2017, in press.

vivo, suggests that DH β cells have a general impairment in their ability to respond to β-cell proliferative stimuli or stressors.

Discussion

Our previous studies demonstrated that the loss of one allele of both *Pdx1* and *Oc1* results in impaired β-cell specification, differentiation, and function. The functional delay observed at P1, paired with persistently reduced mRNA levels of *MafA* transcript at weaning, suggested that there could be an underlying defect in the ability of DH β cells to mature. I hypothesized that proper levels of Pdx1 and Oc1 in MPCs are necessary for postnatal β-cell maturation and adaptability. I thus examined the physiology and function of P14 islets and β cells since β cells undergo a transition in gene expression and function between the ages of P9 and P14 making them more similar to an adult β cell than a developing β cell (62,81,82,169). My findings demonstrate that P14 DH β cells remain immature, especially at the molecular level, and that adult DH β cells have a decreased proliferative capability in the face of a metabolic challenge.

This work identifies the importance of combined Pdx1 and Oc1 function in MPCs for allowing β-cell maturation to proceed at the appropriate time. Whereas there are only minor differences between PH and DH mice at P7, there are four main differences between PH and DH mice at P14: 1. only DH mice showed an appreciable trend toward reduced serum insulin even though both displayed equally reduced pancreatic insulin content; 2. DH mice had reduced blood glucose whereas PH mice were normoglycemic; 3. only DH mice show significant decreases in expression of genes important for β-cell identity and function; 4. PH mice have

increased amino acid-stimulated gene expression. We did not observe a defect in GSIS using an *ex vivo* static incubation assay, but static incubations are cumulative end-point assays and do not allow for assessment of first and second phase insulin secretion that might be perturbed in DH β cells. For example, DH β cells may have defects in the first phase of insulin secretion, similar to what is observed in individuals with T2D, yet, their cumulative insulin secretion could match that of Control and SH mice over the time-course of the assay. Due to the low islet yield at P14, we were unable to perform perifusion assays to address this question. Notably, the increased amino-acid stimulated insulin secretion from PH islets could partially explain the difference in serum insulin that I observed. The mice were not fasted and thus amino acids would be present in the blood stream and potentially stimulating PH β cells to secrete insulin. Importantly, serum insulin levels reflect not only secretion but uptake in peripheral tissues especially the liver. Our previous and current results using this model suggest that Oc1 heterozygosity increases liver glycogen content, which may reduce blood glucose levels leading to a decreased need for insulin secretion, also explaining the difference in serum insulin. To eliminate any compounding phenotype due to Oc1 heterozygosity I would need to inactivate Oc1 specifically in the pancreas. Work by a previous graduate student using the Pdx1-Cre driver developed by Dave Tuveson (219) demonstrated that this Cre did not recombine the *Oc1*^{Fl} allele until after e11.5 and there was no phenotype in double heterozygotes. We feel that this finding demonstrates that the critical window for Pdx1/Oc1 interaction is between ~e9-e11.5.

Our studies define the window during which PH mice become hyperglycemic as being between P14 and weaning. It is possible that a terminal maturation step at weaning (169,255) is necessary to fully reveal the functional defects in PH β cells. Indeed, the idea that normal β-cell

function develops progressively (256) helps to explain the phenomenon observed here of progressive β-cell dysfunction in PH and DH mice. Dor and colleagues (169) showed that mice weaned onto HFD (mimicking high-fat milk) had inappropriately elevated insulin secretion at low glucose. This phenotype was in part due to a persistent increase in oxidative phosphorylation and impaired mitochondrial function. Pdx1 has been shown to regulate mitochondrial function in multiple ways (172,257), so it is very possible that reduced levels of Pdx1 prevent proper alterations in oxidative phosphorylation and mitochondrial function thereby resulting in the observed hyperglycemia and impaired GSIS.

The general trend toward reduced expression of many important β-cell genes (*Nkx6.1*, *Pax4*, *MafA*) (54,61,69,258) in DH reveals that reduced levels of Pdx1 and Oc1 in MPCs continue to impact the transcriptional network of β cells in the postnatal period. In particular, decreased expression of many genes vital for mature β-cell function indicated that there could be specific defects in the islet maturation state. For example, reduced Glut2 could decrease glucose entry into DH β cells since it is the primary glucose transporter in β cells. Lower levels of Glut2 and a mixed islet phenotype have been observed as secondary effects of hyperglycemia (75,155,259-261); however, DH mice are not hyperglycemic suggesting that these phenotypes are direct effects of Pdx1 and Oc1 double heterozygosity and not effects of glucose regulation. Further, reduced expression of *Gck*, responsible for glucose phosphorylation, the rate-limiting step in β-cell glucose metabolism, likely exacerbates the impairment in glucose sensing in DH β cells. Expression of genes necessary for insulin processing, packaging and secretion was also reduced. For example, decreases in the major pro-hormone convertase genes (*Pcsk1/2*) and the ZnT8 zinc transporter (*Slc30a8*) suggest that pro-insulin cleavage and insulin packaging could be

impaired which was supported by the altered distribution of proinsulin in DH β cells (Figure 4-13) (216,262), while reductions in ion channels (*Abcc8* and *Kcnj11*) could result in inefficient insulin release even though amino-acid-stimulated insulin secretion was unaffected in DH islets. Additionally, the decreased expression of Ucn3 in DH β cells (Figure 4-15) further suggests an impairment in the maturation state of DH β cells and a possible nuanced defect in GSIS. Together, these data suggest that many steps in GSIS may be impaired in DH β cells, thus impacting their ability to appropriately respond to glucose. As this is the hallmark of a mature β cell, our findings shed light on the importance of events in early endocrine development for mature β-cell function.

My previous work demonstrated that many of the phenotypes in DH mice resolve by weaning. However, DH islets had persistent reductions in *MafA* mRNA suggesting the possibility of further maturation/functional defects later in life. I questioned whether adult DH β cells were fully functional and if increased β-cell stress would unveil an underlying defect caused by Pdx1 and Oc1 double heterozygosity in MPCs. Adult DH β cells were indeed dysfunctional, similar to PH β cells, but the liver phenotype caused by Oc1 heterozygosity was epistatic to the β-cell dysfunction, thus masking that phenotype. In spite of functional defects, β-cell proliferation does increase in PH mice in response to HFD (174). In contrast, when DH mice were placed on HFD, β-cell proliferation and consequently β-cell mass did not increase. I hypothesized that this lack of proliferation could be due to an inherent defect in the proliferative capacity of DH β cells, which I tested *ex vivo*. Upon stimulation with placental lactogen, a known β cell mitogen, β cells in islets from control animals showed increased proliferation, whereas PH and DH β cells did not. Considering that β-cell proliferation increased

in PH in response to HFD, my *ex vivo* data demonstrates a context-dependent capacity of PH β cells to respond to a proliferative stimulus. Surprisingly, DH β cells show a trend toward reduced proliferation in response to placental lactogen. This proliferative defect in DH β cells is not due to reduced prolactin receptor expression. Rather, I propose that this is due to either an early (epi)genetic impact of Pdx1-Oc1 double heterozygosity on the ability to activate genes important for compensatory β-cell proliferation in adulthood, or an exhausted proliferative capacity due an earlier developmental increase in proliferation. I previously showed that DH β cells have a marked increase in proliferation at e18.5 to compensate for a decrease in endocrine specification (224). It has been suggested that β cells can only undergo a limited number of cell divisions in their lifetime, similar to the Hayflick principal (263). Perhaps the increased proliferation that occurs during late embryonic developmental precludes the adaptive proliferation in adult DH β cells. Such a defect would be secondary to the genetic alterations, but no less impactful when considering how developmental variations can result in susceptibility to adult disease.

Taken together, my data demonstrate that cooperative activity between Pdx1 and Oc1 in MPCs is necessary for proper β-cell maturation and adaptation. The molecular impairment in factors regulating, and necessary for, β-cell maturation is unique to DH mice and further demonstrates the lasting effect that early developmental regulators can have on a cell lineage. Pdx1 and Oc1 double heterozygosity successfully models how subtle, underlying defects might lead to a predisposition to T2D due to decreased β-cell functionality and an impaired responsiveness to proliferative stimuli. Whether the observed proliferative defects are direct or indirect results of double heterozygosity remains to be determined at the molecular level, and

most likely involves epigenetic modifications established in endocrine progenitors that persist into adult life. This study demonstrates the importance of β-cell developmental transcription factors for establishing a mature and responsive pool of β cells in adults.

CHAPTER V

REGULATION OF EXOCRINE PANCREAS DEVELOPMENT BY OC1

Introduction

The exocrine pancreas is composed of both digestive enzyme-secreting acinar cells and ductal cells that transport those enzymes to the duodenum. This function is vital for digestion as the absence of acinar cells or their loss of function results in exocrine pancreas insufficiency and severe digestive defects. The exocrine pancreas can also be the source of serious diseases including PDAC, pancreatitis, and interpapillary mucinous neoplasms (IPMN). PDAC is a particularly devastating cancer accounting for over 40,000 deaths in United States each year. During PDAC development and progression many developmental pathways (Notch, Wnt, and Hedgehog signaling; MPC-associated transcription factors) are reactivated thus contributing the observed metaplasia and neoplasia (reviewed in (264)). These pathways and factors may serve as therapeutic targets and it is thus necessary to understand their role in exocrine pancreas development, function, and metaplastic potential.

Although all cells of the exocrine pancreas derive from early primary MPCs, acinar cells differentiate from the 2° MPCs located at the tips of the branched pancreatic ductal tree, whereas duct cells differentiate from bipotential trunk cells (21). A number of transcription factors expressed in MPCs become restricted to acinar cells during differentiation including *Ptf1a* and *Nr5a2*, as well as *Mist1*, a transcription factor expressed in secondary MPCs (265-267). These transcription factors establish acinar cell identity by actively promoting the acinar gene expression program including digestive enzymes (e.g. *Amy1*, *Ela1/2*, *Cpa*) as well as the

secretory machinery (268-274). These factors appear to act together within a network of positive-regulatory relationships which subsequently promotes the acinar cell program (12-14,178,275). Loss of any one of these factors destabilizes acinar cell identity and increases susceptibility to development of PanINs and PDAC, especially when paired with an activating mutation of the *Kras* oncogene (135,273,276-279).

In spite of the important role that *Ptf1a*, *Nr5a2*, and *Mist1* play in acinar cell identity and homeostasis, they have different requirements during acinar cell differentiation. Ptf1a is required for pancreas development in both mice and humans. *Ptf1a*-null mice develop severely hypoplastic pancreatic rudiments lacking all mature pancreatic cell types (11,14). *Nr5a2*-null mice develop a pancreas, but it is severely hypoplastic with disproportionate defects in acinar cell development (134). Surprisingly, *Mist1* is dispensable for pancreas development and acinar cell differentiation even though it is important for maintaining acinar cell identity in the adult (135). The different requirements for these transcription factors highlights that, while operating within a cooperative network in mature acinar cells, they have at least partially independent roles in developing acinar cells. It is likely that other factors participate in the regulatory network to promote acinar differentiation and function and could modulate the expression or activity of the above-mentioned transcription factors.

Oc1, like the above-mentioned transcription factors, is expressed in MPCs as well as differentiated acinar cells, but unlike *Ptf1a*, *Nr5a2*, and *Mist1*, *Oc1* is also expressed in the pancreatic ducts (102,148). Absence of Oc1 from the ducts results in severe defects in duct differentiation and function. Dilated ducts and ductal cysts are apparent in *Oc1*-null pancreata as early as e15.5 and persist throughout development and into adulthood (102). These cysts are

likely due to the loss of primary cilia from ductal cells which contribute to impaired signaling and lack of sensory function (16,280), a finding supported by the evidence that Oc1 regulates many genes involved in primary cilia formation (149,281). We previously demonstrated that inactivation of *Oc1* in the developing pancreatic epithelium using a *Pdx1-Cre* driver ($Oc1^{\Delta panc}$) results in tortuous and hyper-proliferative ducts (16). Additionally, acinar defects were present at weaning including ADM, inflammation, fibrosis, and up-regulation of CTGF and matrix metallopeptidase 7 (MMP7) which are also up-regulated in pancreatitis (16,282,283). However, it was unclear if these acinar defects were the result of impaired differentiation or later defects in acinar function. Indeed, no specific function for Oc1 has been documented in differentiation of acinar cells.

The acinar dysplasia and increased expression of CTGF and MMP7 described above suggested that Oc1 may have a role in maintaining acinar homeostasis. Additionally, it has been demonstrated that Oc1 expression is absent from human PDAC samples further supporting this idea (284). I confirmed that *OC1* mRNA expression is significantly reduced in human PDAC samples compared to normal pancreas. Additionally, expression of multiple *OC1* downstream targets including *FOXA2*, *PDX1*, and *PKHD* were reduced in PDAC samples (Figure 5-1) (161). To determine the time course of OC1 loss during PanIN progression and PDAC, I examined OC1 protein expression in human tissue microarrays of normal, ADM, PanIN, and PDAC samples. This analysis revealed a slight increase in OC1 expression in ADM samples, but a progressive decrease in OC1 expression from PanIN1 to PanIN3 with a near complete absence of OC1 in PDAC samples (Figure 5-2) (161). These data suggest that OC1 has a role in maintaining acinar cell identity and preventing the progression of PanINs to PDAC. I predicted that the transient

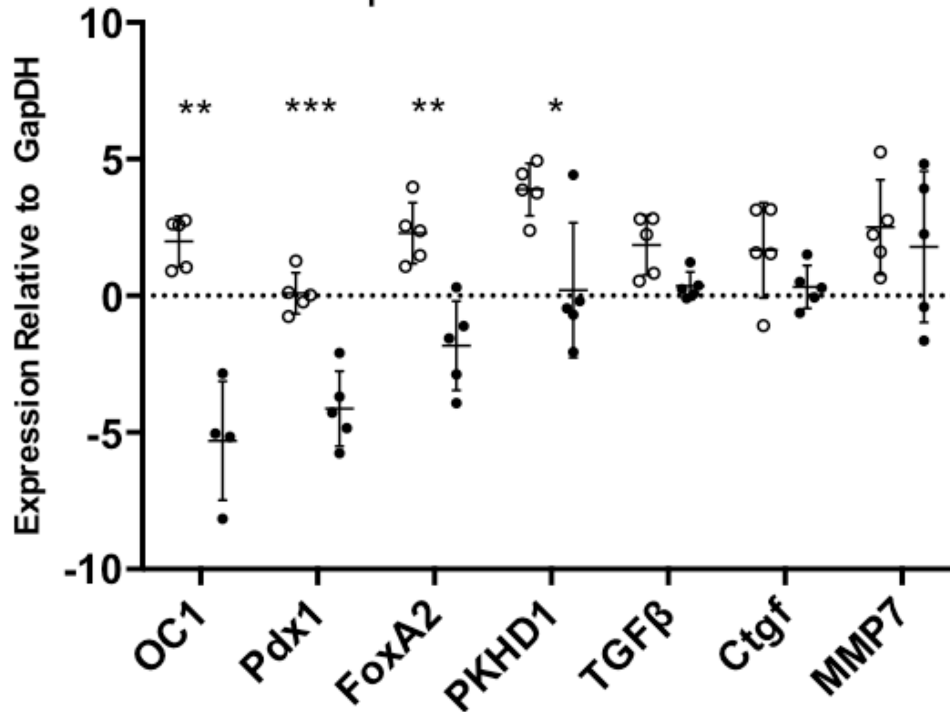


Figure 5-1: Expression of OC1 and co-regulated genes in normal pancreas and PDAC tumors. Gene expression analysis of normal (open circles) and PDAC tumor (closed circles) tissue samples (n=5 for each). qRT-PCR was performed on RNA extracted from samples and expression was normalized to GapDH. OC1 expression was significantly decreased in all of the PDAC tumors, and was undetectable in one sample. Expression of direct (FOXA2, PDX1) and indirect (PKHD1, TGF-b) HNF6 target genes, were also significantly decreased in PDAC tumor samples compared with normal pancreas. *P<0.05; **P<0.005; ***P<0.0005. Figure modified and reprinted with permission from Pekala *et al.*, 2014 (161).

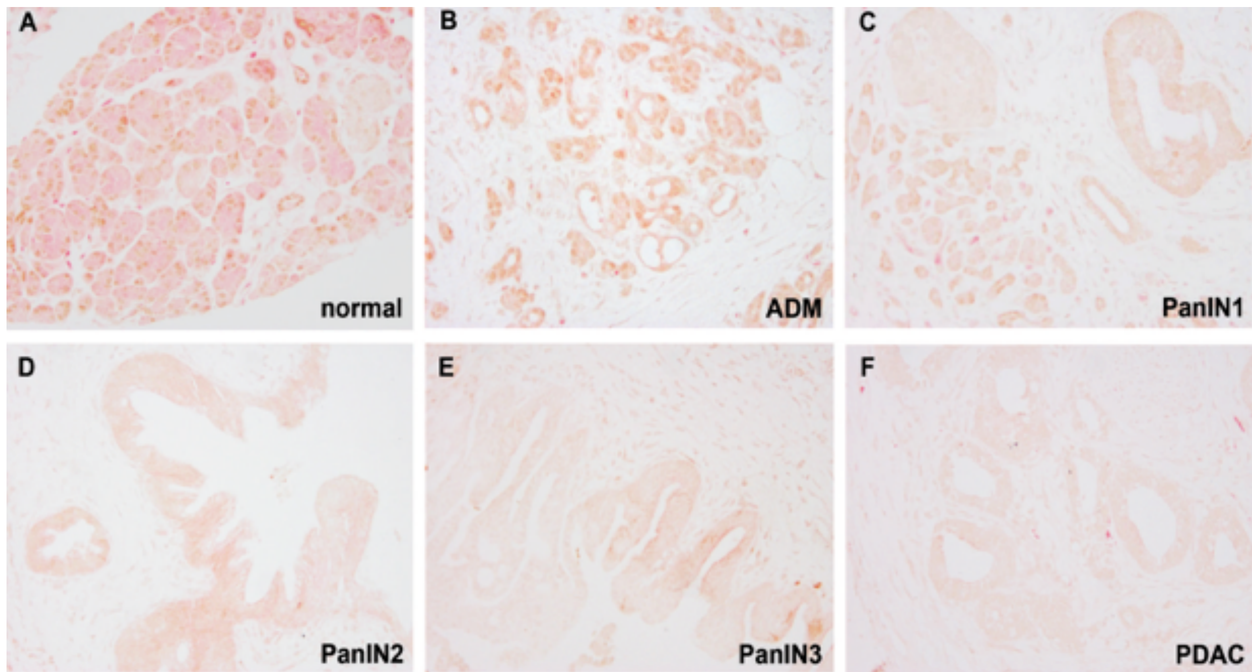


Figure 5-2: Survey of OC1 expression in normal and diseased human pancreas tissue using tissue microarrays. Representative examples from human tissue microarrays immunolabeled for OC1 protein expression (brown) against eosin contrast. OC1 expression is observed in normal pancreas (A), ADM (B), and PanIN1 (C). PanIN2 (D) and PanIN3 (E) showed reduced OC1 expression, while PDAC (f) lacked OC1 protein expression. Magnification: X200. Figure modified and reprinted with permission from Pekala *et al*., 2014 (161).

up-regulation of OC1 in ADM could be due to the increased ductal characteristics of metaplastic cells (as OC1 is naturally expressed higher in ducts than acini), but as metaplasia progresses to neoplasia OC1 is lost as a consequence of transformation.

The acquisition of duct characteristics in acinar cells during ADM is consistent with partial dedifferentiation and believed to be a protective measure allowing acinar cells to proliferate or redifferentiate following the injury (202,204). Given that this process results in the acquisition of developmental characteristics (*Sox9* expression, altered cell morphology, reduced or absent enzyme expression), it is important to further understand acinar cell development and the factors that regulate acinar differentiation. I hypothesized that Oc1 is necessary for acinar differentiation and that it regulates the gene expression network of acinar cells. Here I show that loss of Oc1 from the developing pancreatic epithelium results in both duct and acinar cell defects and that the gene expression of the exocrine pancreas is substantially altered. Additionally, I sought to identify both direct and indirect targets of Oc1 during pancreas development due to the lack of information about direct Oc1 targets in the pancreas. Only four targets are known in the pancreas including *Pdx1*, *Neurog3*, *MafA*, and *Hnf4*α which, with the exception of *Pdx1*, exclusively regulated endocrine differentiation and development. Thus, any findings for direct targets of Oc1 in the exocrine development would be completely novel.

Results

Loss of Oc1 from the pancreatic epithelium largely impacts endocrine gene expression at e15.5

To determine how the transcriptome of the developing pancreas is impacted by the loss of Oc1, I performed RNA-Seq on whole pancreata from control and Oc1$^{\Delta panc}$ (*Oc1$^{Fl/Fl}$; Pdx1*-Cre^{Tuv}) mice at e15.5. This RNA-Seq revealed variable changes in the transcriptome of Oc1$^{\Delta panc}$ samples, but consistently different gene expression from controls (Figure 5-3A) with more genes being down-regulated than up-regulated (Figure 5-3B). Pathway analysis of the Oc1$^{\Delta panc}$ transcriptome revealed that the most significantly altered pathway was Maturity Onset Diabetes of the Young (MODY) Signaling (Table 5-1). Included in this pathway are many genes important for endocrine differentiation including *Pdx1*, *Neurog3*, and *Hnf1*α. These findings are expected given that Oc1 directly binds to and activates the *Neurog3* promoter (153,154) and promotes endocrine specification. Upstream regulator analysis was used to determine how the hierarchy of pancreas development regulatory factors was altered due to the loss of Oc1. Transcription factors such as *Hnf1*α, *Hnf1*β, and *Pdx1* were all identified as top regulators of the pathways and genes affected in the RNA-Seq data (Table 5-1). These factors are all known to be downstream targets of Oc1 with Pdx1 being a direct target (15,145,285). Additionally, there were many changes in non-coding RNAs, however, these RNAs have not been characterized with any function in the pancreas. Together, these data indicate that Oc1 has a significant role in promoting the endocrine specification gene expression program in e15.5 pancreata; Oc1 does not appear to regulate genes directing acinar cell development at this time.

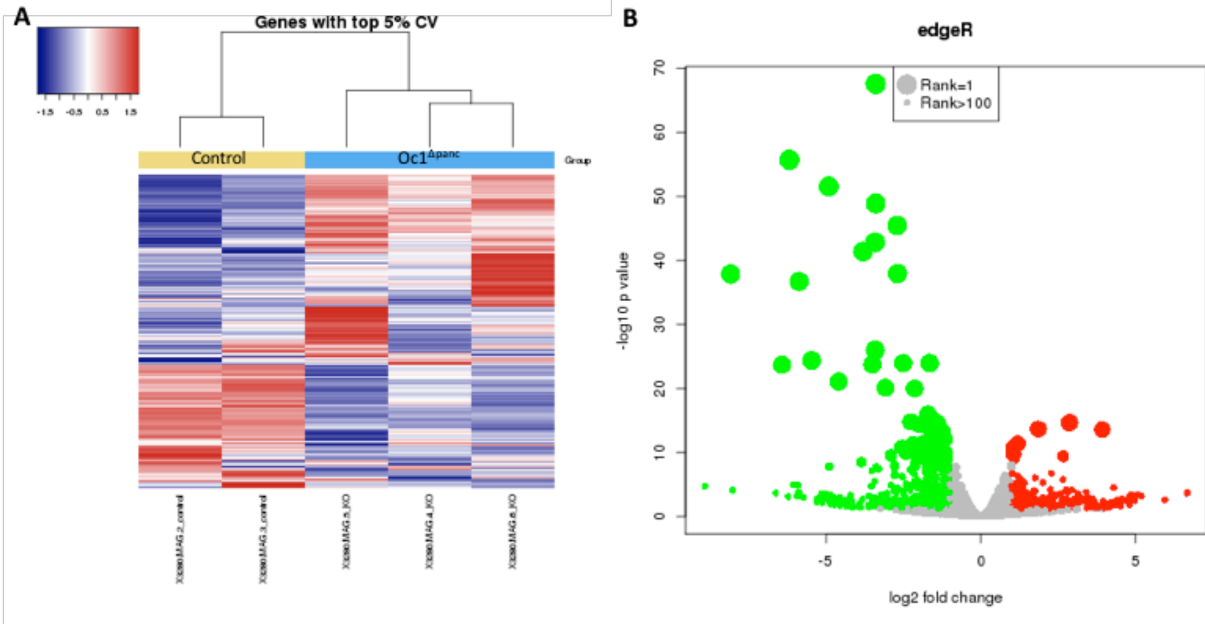


Figure 5-3: Trends in gene expression changes due to Oc1 inactivation in e15.5 pancreas. (A) Heatmap demonstrating gene expression changes in e15.5 Oc1$^{\Delta panc}$ pancreata compared to controls. Red indicates increased expression and blue represents decreased expression. (B) Volcano plot of differentially expressed genes in e15.5 Oc1$^{\Delta panc}$ pancreata. Significance was determined by log_2-fold change of 2 and p-value < 0.05. Green circles represent significantly decreased genes and red circles represent significantly increased genes.

Table 5-1: e15.5 RNA-Seq analyses. Most significantly altered pathways in e15.5 $Oc1^{\Delta panc}$ samples (above) and identification of upstream regulators based off of gene expression changes.

Pathway	P-Value (Percent Overlap)
Maturity Onset Diabetes of the Young (MODY) Signaling	3.47e-06 (28%)
FXR/RXR Activation	5.44e-06 (10.9%)
Human Embryonic Stem Cell Pluripotency	6.51e-06 (10.8%)
Axonal Guidance Signaling	1.35e-05 (6.6%)
Wnt/-catenin Signaling	1.86e-05 (9.4%)

Upstream Regulator	P-Value of Overlap
Hnf1α	5.45E-18
TGF-β1	3.57E-14
Hnf1β	1.72E-13
Tretinoin	3.32E-10
Pdx1	4.61E-09

Oc1$^{\Delta panc}$ pancreata have substantial morphology defects

We previously observed that e14.5 Oc1$^{\Delta panc}$ pancreata were hypoplastic (specifically, the pancreatic epithelium did not fully expand into the over-lying mesenchyme) (16). At e18.5, Oc1 pancreata were smaller to the naked eye and trended toward having reduced acinar area as indicated by amylase immunolabeling (Figure 5-4A). Additionally, there was a trend toward increased ductal area (as assessed by CK19 labeling) compared to acinar area consistent with a loss of acinar tissue (Figure 5-4B). This finding could also be attributed to duct hyperplasia, a phenotype that we previously observed in P21 Oc1$^{\Delta panc}$ mice (16) but not quantified at e18.5. We previously observed ADM and fibrosis in the pancreata of P21 Oc1$^{\Delta panc}$ mice (16). I sought to determine if these exocrine phenotypes manifested during development and thus examined the histology of e18.5 pancreata from both control and Oc1$^{\Delta panc}$ mice. Morphological analysis using hematoxylin and eosin staining (H&E) revealed abnormal pancreas morphology in Oc1$^{\Delta panc}$ mice with smaller acinar lobes, dilated ducts, and altered acinar-cell morphology suggestive of ADM (Figure 5-4C). To assess for fibrosis, I used Sirius Red/Fast Green to label collagen and the pancreatic epithelium, respectively (Figure 5-4D). There appeared to be increased collagen deposition in e18.5 Oc1$^{\Delta panc}$ pancreata, but this increase did not reach statistical significance due to the low number of pancreata examined and the variability in the control samples (Figure 5-4E). These data demonstrate that defects in the acinar compartment of Oc1$^{\Delta panc}$ mice exist prior to birth and are suggestive of differentiation defects in both duct and acinar cells.

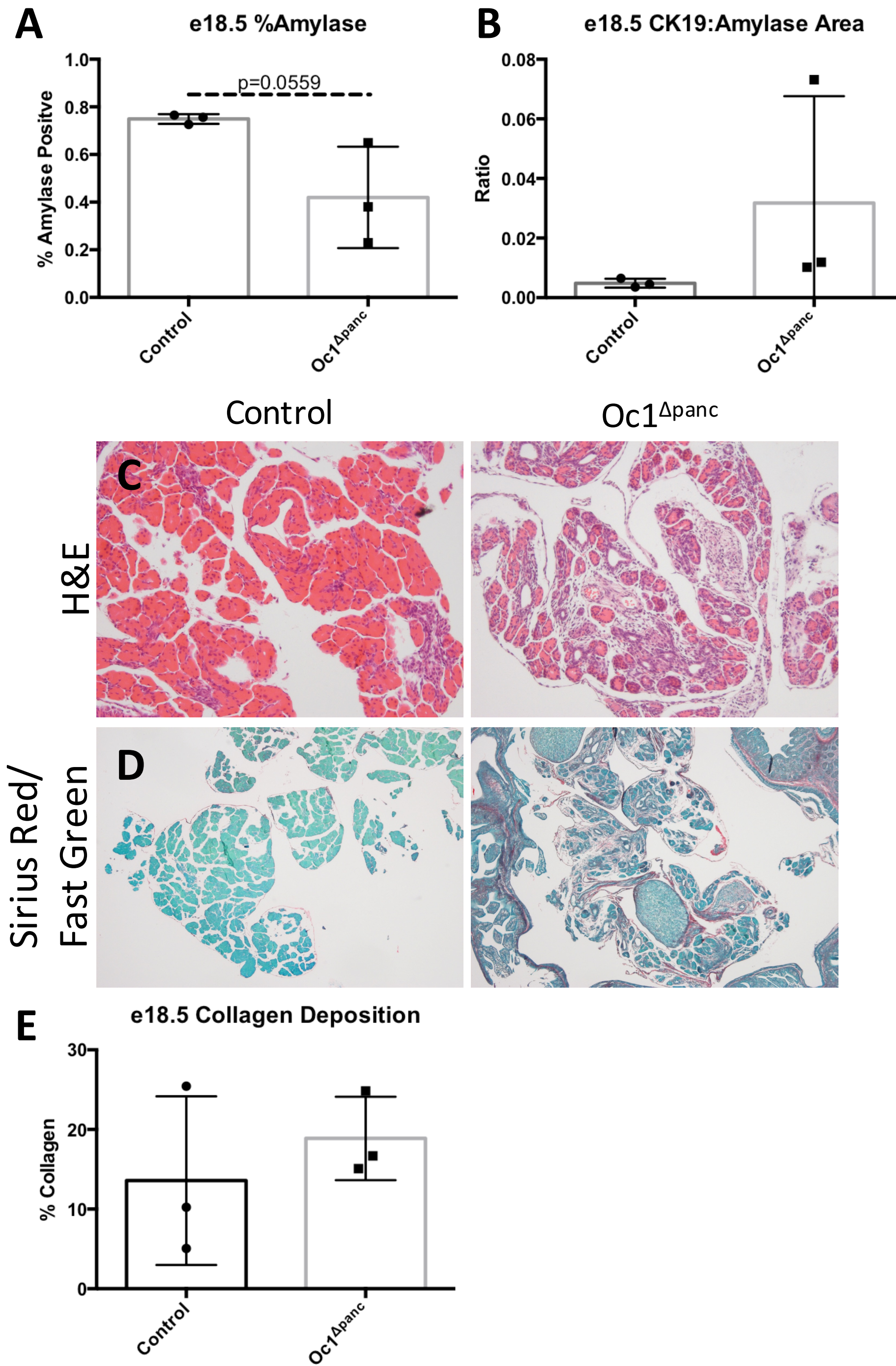


Figure 5-4: Morphology of e18.5 Oc1$^{\Delta panc}$ pancreata. (A) Amylase+ area trends toward a reduction in Oc1$^{\Delta panc}$ samples without a statistical increase in the ratio of CK19+:Amylase+ area (B). (C) H&E staining and (D) Sirius Red/Fast Green staining for collagen. (E) Quantification of Sirius Red/Fast Green staining. Images captured at 20X. Students T-Test for statistics.

The next question asked was how Oc1 inactivation affected the exocrine pancreas after birth, when acinar cell function becomes critical for secretion of enzyme zymogens to assist in the digestion of milk. As at e18.5, H&E staining revealed substantial morphological defects with evidence of ADM and a further increase in fibrosis in $Oc1^{\Delta panc}$ mice (Figure 5-5A,B). Indeed, there was significant collagen deposition in $Oc1^{\Delta panc}$ pancreata at this age (Figure 5-5C). Immunofluorescence analysis (Figure 5-6A) using antibodies against amylase and CK19 revealed that the acinar compartment was significantly reduced in $Oc1^{\Delta panc}$ mice compared to controls with a concomitant increase in the duct compartment (Figure 5-6B,C). The increase in CK19+ area is likely due to a combination of duct hyperplasia and decreased acinar area thereby resulting in a relative increase in duct area. Additionally, cells positive for both CK19 and amylase were observed supporting the development of ADM (Figure 5-6D). Together, these data suggest that loss of Oc1 expression from the developing pancreatic epithelium results in both duct and acinar defects during development that persist and are exacerbated postnatally. These defects are reminiscent of pancreatitis and consistent with the defects observed at P21 (16).

To better understand the mechanism for reduction in the acinar compartment, I analyzed acinar death (using TUNEL) and proliferation (using Ki67) at both e18.5 and P2. Acinar-cell death in $Oc1^{\Delta panc}$ mice trended towards an increase at e18.5, but was variable between samples (Figure 5-7A,B). Acinar-cell death was significantly increased in $Oc1^{\Delta panc}$ mice at P2 (Figure 5-7C) suggesting a continued loss of acinar cells after birth. In addition to increased acinar-cell death, another mechanism possibly reducing acinar area is decreased acinar-cell proliferation. Immunofluorescence analysis of acinar-cell proliferation at both e18.5 and P2

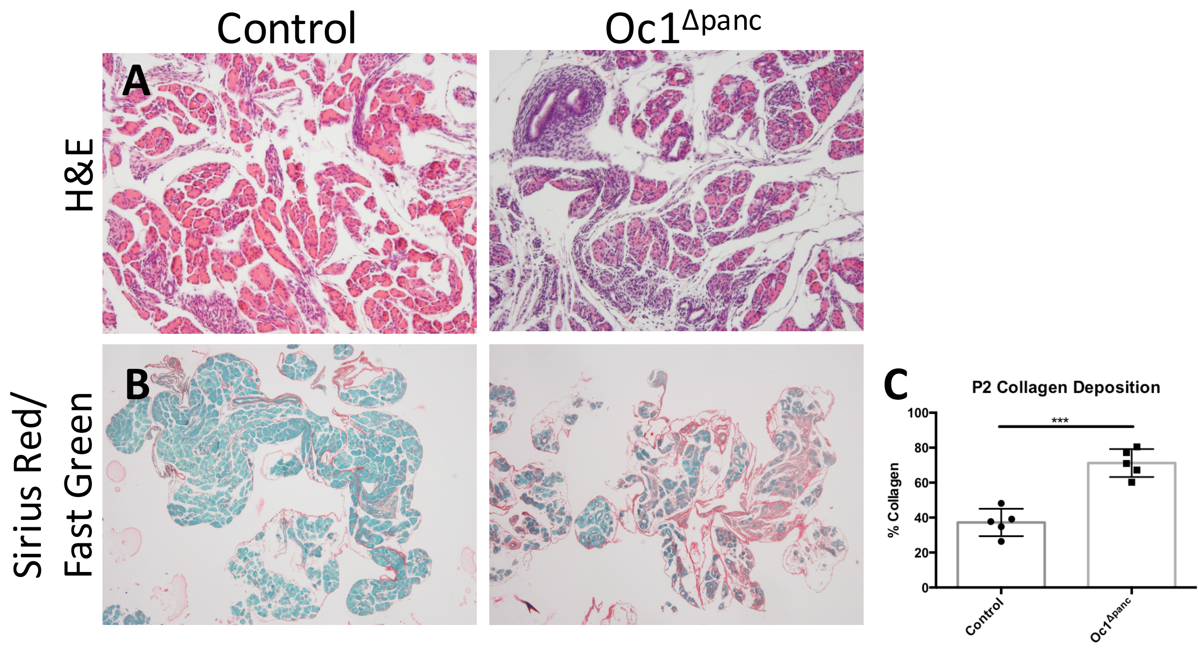


Figure 5-5: Morphological changes of P2 Oc1$^{\Delta panc}$ pancreata. (A) H&E staining and (B) Sirius Red/Fast Green staining for collagen. (C) Quantification of collagen deposition from B. Images captured at 20X. ***: $p<0.001$ by Students T-Test.

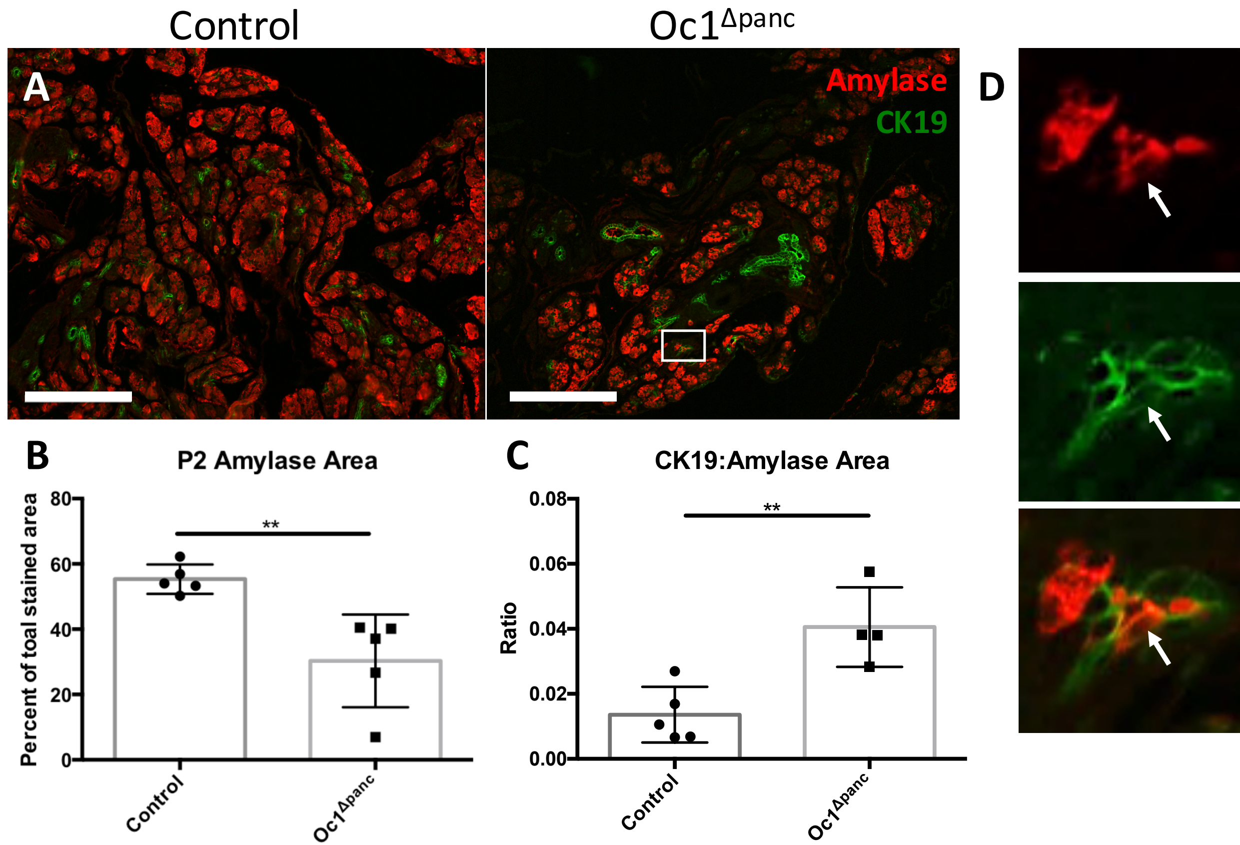


Figure 5-6: Exocrine dysplasia in P2 Oc1$^{\Delta panc}$ pancreata. (A) Representative images of amylase (red) and CK19 (green) immunofluorescence in control and Oc1$^{\Delta panc}$ pancreata. (B) Quantification of amylase+ area and (C) ratio of CK19+:amylase+ area. (D) Higher magnification of amylase+/CK19+ cell (arrow). Image from box in A. Scale bar represents 200 μm. **: $p<0.01$ by Student's T-Test.

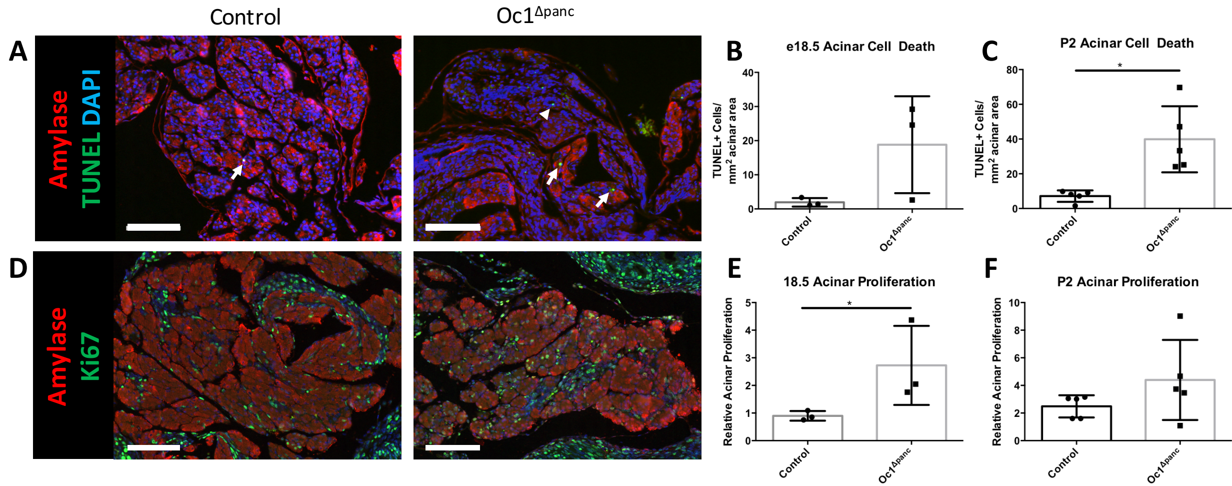


Figure 5-7: Variable acinar-cell death and proliferation in P2 Oc1$^{\Delta panc}$ pancreata. (A) Representative images of immunofluorescence labeling for amylase (red), dying cells (TUNEL; green), and DAPI (blue). Arrows: TUNEL+ acinar cells; arrowhead: TUNEL+ duct cell. Quantification of acinar-cell death at (B) e18.5 and (C) P2. (D) Representative images of immunofluorescence labeling for amylase (red) and proliferating cells (Ki67; green). Quantification of acinar-cell proliferation at (E) e18.5 and (F) P2. Scale bars represent 200 μm. *: $p<0.05$ by Student's T-Test.

revealed that, surprisingly, proliferation was elevated at e18.5 and trended toward an increase at P2 (Figure 5-7C-E). It is possible that this increased proliferation is an attempt at compensation for the reduced acinar compartment. Such a compensatory mechanism has been documented in other instances of decreased acinar populations (286-288). Together, these data suggest a complex balance between acinar-cell loss and an attempt to compensate for decreased acinar-cell differentiation.

Oc1 inactivation results in an impaired exocrine transcriptome at e18.5 and P2

The morphological alterations in $Oc1^{\Delta panc}$ pancreata at e18.5 and P2 suggested an impairment in exocrine pancreas differentiation as a consequence of Oc1 loss. I hypothesized that Oc1 regulates the gene expression of developing acinar cells and thus the loss of Oc1 would result in significant alterations to the transcriptome of developing acinar cells. To test this hypothesis, RNA was extracted from exocrine-enriched samples which were predominantly composed of acinar cells but also contained some ducts. Samples were collected from control and $Oc1^{\Delta panc}$ mice at both e18.5 and P2. RNA-Seq revealed substantial gene expression changes at both time points with consistent types of alterations to the gene expression program. For example, molecular function analysis indicated that with ligands of signaling pathways ("Binding") and enzymes ("Catalytic Activity) were predominantly affected (Figure 4-8).

Pathway analysis using Ingenuity Pathway Analysis (IPA) revealed that the most affected pathways in $Oc1^{\Delta panc}$ samples were all associated with increased inflammation ("Granulocyte Adhesion and Diapedesis", "Agranulocyte Adhesion and Diapedesis", "Altered B and T Cell Signaling in Rheumatoid Arthritis") (Table 5-2). These changes suggested that there was

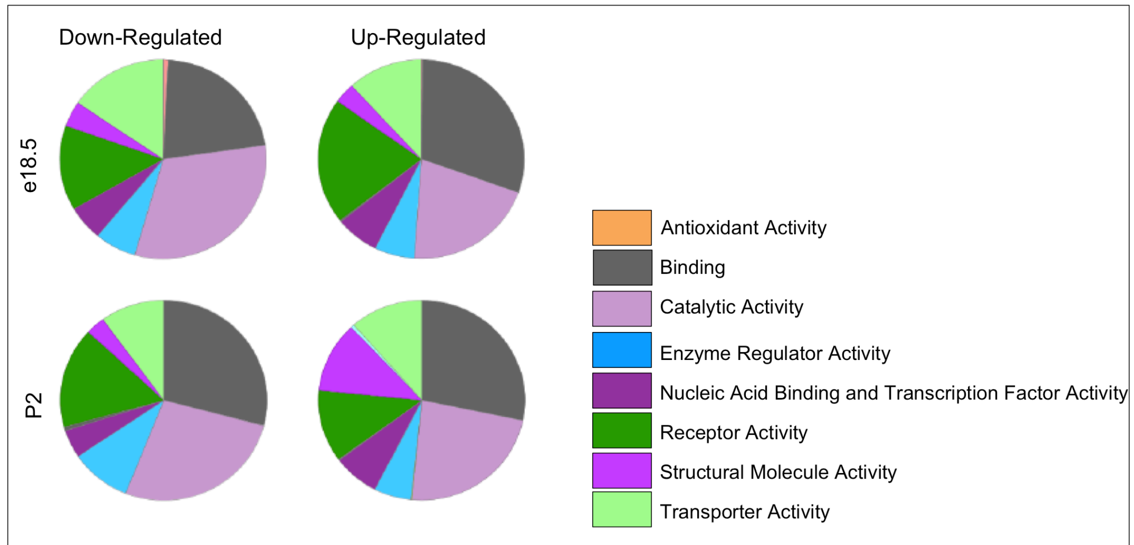


Figure 5-8: Biological function analysis of differentially expressed genes. Panther Gene analysis of biological function of genes altered in e18.5 and P2 Oc1$^{\Delta panc}$ RNA-Seq data.

Table 5-2: Summary of gene expression changes and altered pathways from e18.5 $Oc1^{\Delta panc}$ RNA-Seq. Green text represents increased expression or pathway activation. Red text represents decreased expression.

<table>
<tr><th>Genes Altered In $Oc1^{\Delta panc}$</th><th>Top 5 Altered Pathways in $Oc1^{\Delta panc}$</th></tr>
<tr><td rowspan="3">358 up-regulated</td><td>Granulocyte Adhesion and Diapedesis</td></tr>
<tr><td>Agranulocyte Adhesions and Diapedesis</td></tr>
<tr><td>Altered T Cell and B Cell Signaling in Rheumatoid Arthritis</td></tr>
<tr><td rowspan="2">146 down-regulated</td><td>B Cell Development</td></tr>
<tr><td>Role of Macrophages, Fibroblasts, and Endothelial cells in Rheumatoid Arthritis</td></tr>
</table>

substantial infiltration of immune cells as a consequence of Oc1 inactivation. Immunolabeling for the macrophage marker F4/80 indeed revealed a slight increase in macrophages in the pancreas of e18.5 Oc1$^{\Delta panc}$ mice (Figure 5-9). The most significantly altered pathway that was not specifically related to inflammation was "Transcriptional Regulatory Network of Embryonic Stem Cells". Within this pathway were up-regulated genes including *Cdx2*, *HoxB1*, *Eomes*, and *Sox2* which all have important roles in endoderm patterning and differentiation of the organs derived from the foregut endoderm. Expression of *HoxB1*, *Eomes* and *Sox2* suggests the persistence of undifferentiated endoderm and expression of *Cdx2* suggests misappropriation of pancreatic cells to a non-pancreas lineage. Notably, *Cdx2* is an important regulator of duodenal cell fates and is normally excluded from the presumptive pancreatic endoderm (289,290).

Interestingly, the second most significantly altered non-immune pathway in e18.5 Oc1$^{\Delta panc}$ samples was "Basal Cell Carcinoma Signaling" which contained many elevated Wnt ligands as well as *Shh* and the Shh effector *Gli1*. During pancreas development, Shh expression is specifically repressed in the presumptive pancreatic endoderm by secreted notochord-derived factors (see Chapter I). Mis-expression of *Shh* in the pancreatic epithelium results in "intestinalization" of the pancreas (44). Wnt signaling is important for pancreas development and particularly important for expansion of the developing acinar cells (125,286,288). Additionally, Wnt signaling is required for increased acinar-cell proliferation during recovery from injury (287), so the increased Wnt signaling could be responsible for the increased acinar-cell proliferation observed at e18.5. The alterations to these pathways suggest an impairment in exocrine pancreas differentiation and a potential misappropriation of some intestinal characteristics within the pancreas.

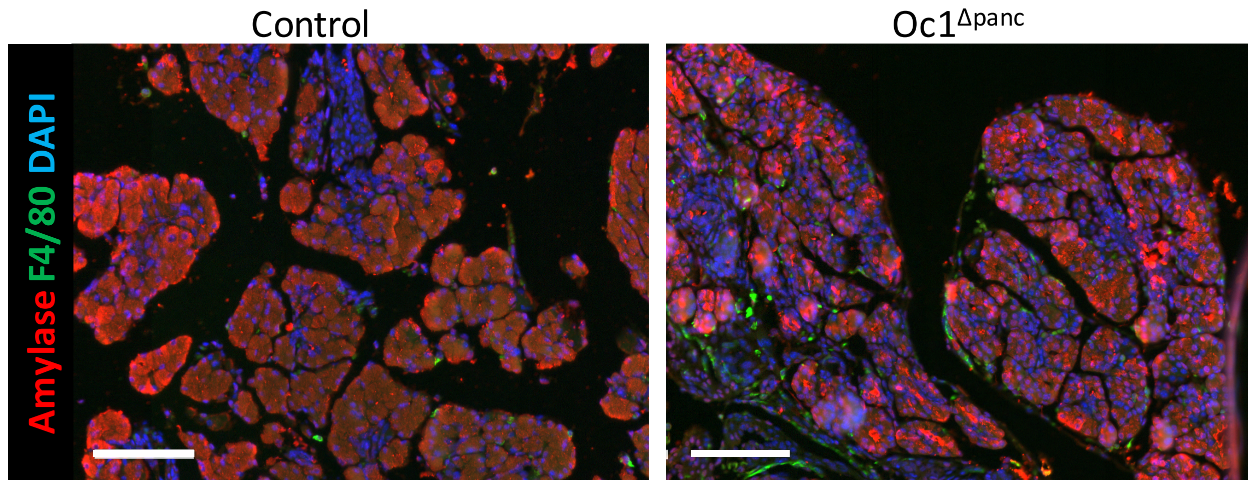


Figure 5-9: Macrophage infiltration in e18.5 Oc1$^{\Delta panc}$ pancreata. Representative images of immunofluorescence labeling for amylase (red), F4/80 (green) and DAPI (blue). Scale bar represents 100 μm.

Manual analysis of the RNA-Seq data revealed changes in gene expression also consistent with an impairment in acinar differentiation and expression of intestinal regulatory factors. The essential acinar transcription factor *Ptf1a* was reduced ~1.6-fold suggesting an impairment in the identity of $Oc1^{\Delta panc}$ acinar cells at e18.5. Additionally, many genes associated with digestive enzyme production/regulation (*Prss1*, *Prss3*, *SerpinF2*, *SerpinI2*) were also reduced in $Oc1^{\Delta panc}$ samples further suggesting an impairment in the identity or differentiation state of $Oc1^{\Delta panc}$ acinar cells. Transcription factors associated with the developing intestinal epithelium including *Iroquois 4* (*Irx4*) and *Intestinal-specific Homeobox* (*Isx*) were up-regulated in $Oc1^{\Delta panc}$ samples. Interestingly, the *Oc1* paralog *Onecut2* (*Oc2*) was also up-regulated in $Oc1^{\Delta panc}$ samples. This increased *Oc2* expression is likely a compensatory increase due to the loss of *Oc1*, but previous studies suggest that increased *Oc2* cannot fully compensate for Oc1 (150).

RNA-Seq performed on exocrine-enriched samples from P2 control and $Oc1^{\Delta panc}$ mice was evaluated with IPA to identify affected pathways and upstream regulators. As at e18.5, one of the most significantly affected pathways at P2 was "Agranulocyte Adhesion and Diapesis" (Table 5-3) suggesting an increase in inflammation which was supported by F4/80 immunofluorescence (Figure 5-10). The most significantly altered pathway was "Calcium Signaling" which included many myosins and actins, but also calcium dependent factors (e.g. *Tnnc1/2*, *Tnnc3, CamK2a*) (Table 5-3). Components of this pathway were generally up-regulated which is interesting considering that calcium signaling is an important component of digestive enzyme secretion from acinar cells. The remaining pathways in the top 5 most altered ("Cellular effects of Sildenafil", "Actin Cytoskeleton Signaling", "Epithelial Adherens Junction Signaling")

Table 5-3: Summary of gene expression changes and altered pathways from P2 Oc1$^{\Delta panc}$ exocrine-enriched RNA-Seq. Green text represents increased expression or pathway activation. Red text represents decreased expression.

<table>
<tr><th>Genes Altered In Oc1^{Δpanc}</th><th>Most Significantly Increased Pathways in Oc1^{Δpanc}</th></tr>
<tr><td rowspan="2">572 up-regulated</td><td>Calcium Signaling</td></tr>
<tr><td>Cellular Effects of Sildenafil (Viagra)</td></tr>
<tr><td rowspan="3">438 down-regulated</td><td>Agranulocyte Adhesion and Diapedesis</td></tr>
<tr><td>Actin Cytoskeleton Signaling</td></tr>
<tr><td>Epithelial Adherens Junctions Signaling</td></tr>
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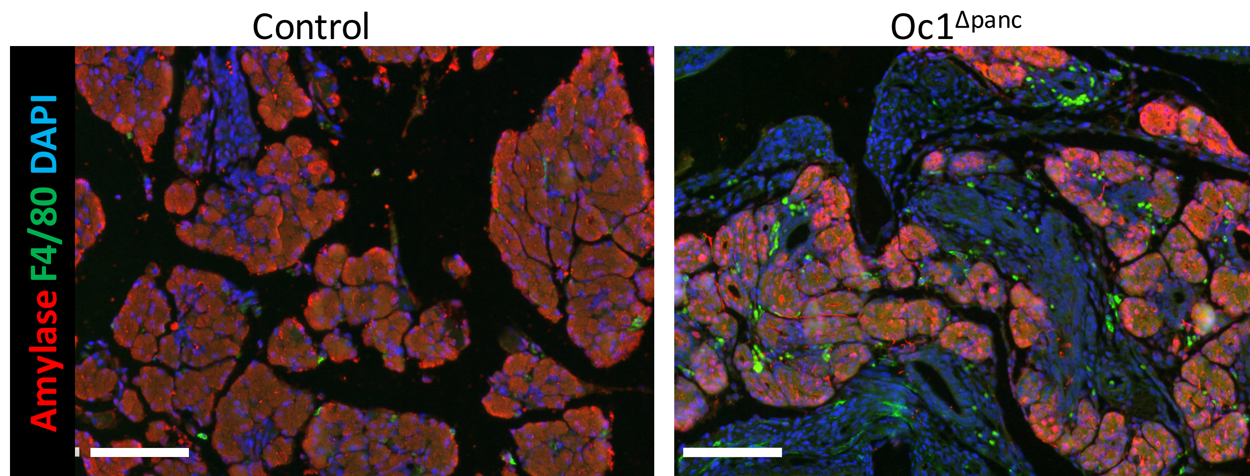

Figure 5-10: Macrophage infiltration in P2 Oc1$^{\Delta panc}$ pancreata. Representative images of immunofluorescence labeling for amylase (red), F4/80 (green) and DAPI (blue). Scale bar represents 100 μm.

also contained many myosins and actins which were up-regulated. Thus, loss of Oc1 from the developing epithelium results in increased expression of cytoskeletal and calcium signaling components potentially due to acinar dysfunction.

Upstream Regulator Analysis identified the muscle lineage transcription factor *MyoD1* as the most prominent activated regulator in P2 Oc1$^{\Delta panc}$ samples (Table 5-4). Conversely, *Nr5a2* was identified as the most significantly inhibited regulator (Table 5-4). *Ptf1a* and *RbpjL* (an essential component of the PTF1 transcription complex in mature acinar cells) were also identified as upstream regulators that were inhibited in Oc1$^{\Delta panc}$ samples (Table 5-4). The identification of these factors suggests that loss of Oc1 resulted in a more specific impairment in acinar-cell identity at P2 than at e18.5. Examination of the genes that lie downstream of these factors indeed reveals significant reductions in many digestive enzymes including *Amy1* (amylase), *Cela1* (elastase), *Pnlip* (pancreatic lipase), and *Cpa1/2*. Additionally, *Mist1* was significantly reduced in Oc1$^{\Delta panc}$ samples, which was confirmed by immunohistochemistry (Figure 5-11). Notably, *Hnf1β* was also identified as an upstream regulator that was inhibited in Oc1$^{\Delta panc}$ samples (Table 5-4). As noted earlier, Hnf1β is a key regulator of pancreatic duct development and of genes that promote establishment and maintenance of primary cilia (104,291,292). Additionally, *Hnf1β* is a direct target of Oc1 in cholangiocytes and known to be downstream of Oc1 in the pancreas (102,144). It is likely that this relationship also results in the loss of 1° cilia in the pancreas. Thus, loss of Oc1 from the developing pancreatic epithelium results in decreased expression of both regulators and functional genes of acinar and duct cells. Whether regulation of these genes by Oc1 is direct or indirect remains to be determined.

Table 5-4: Upstream regulator analysis from P2 Oc1$^{\Delta panc}$ RNA-Seq.

Upstream regulator	Activation z-score	P-value of overlap
MyoD1	4.227	$1.85e^{-18}$
Nr5a2	-3.021	$6.05e^{-10}$
RbpjL	-2.000	$5.98e^{-8}$
Ptf1a	-1.925	$1.17e^{-6}$
Hnf1β	-1.046	$1.29e^{-9}$

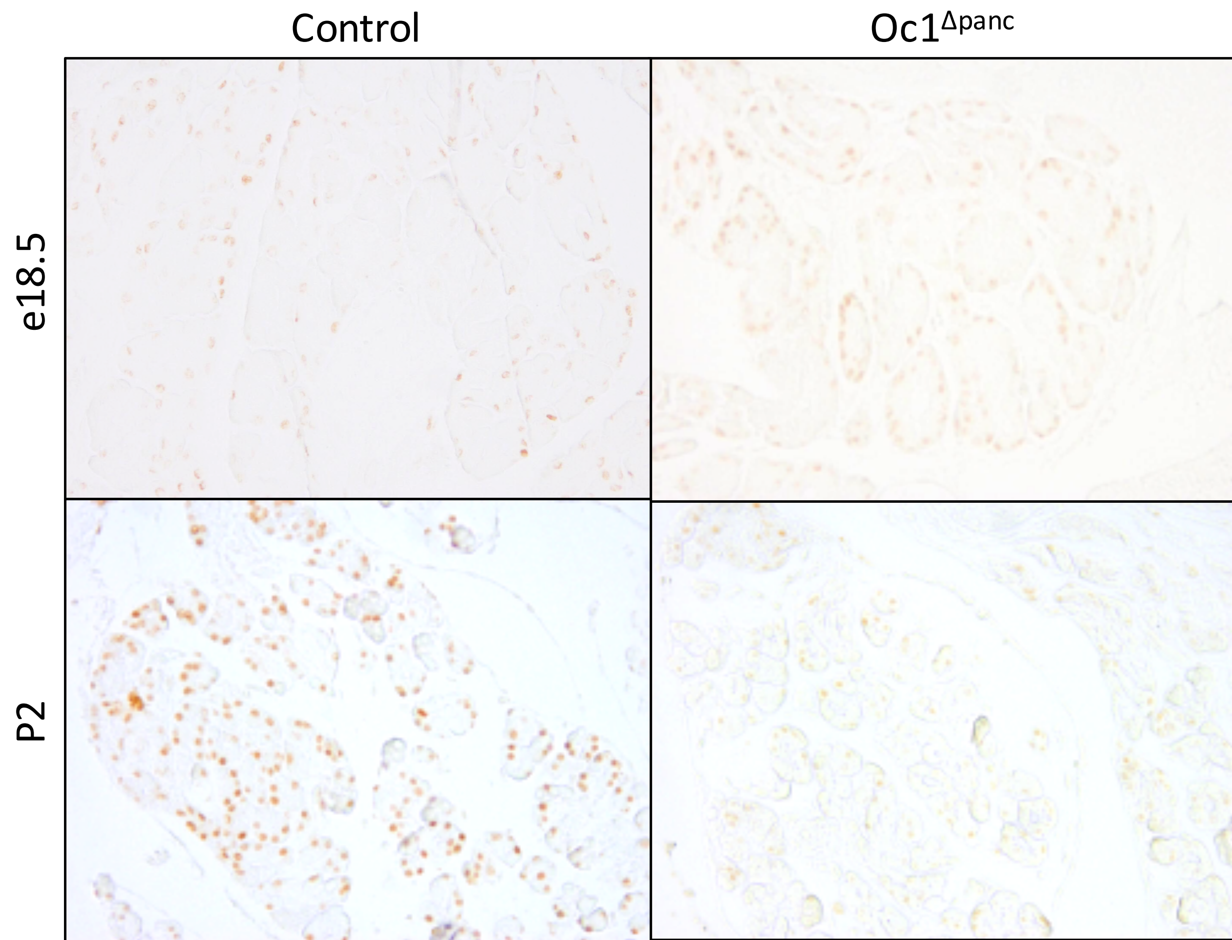

Figure 5-11: Mist1 expression in Oc1$^{\Delta panc}$ pancreata. Mist1 immunolabeling (brown) in e18.5 (above) and P2 (below) samples. Light diffraction used to outline tissue.

Oc1 appears dispensable in committed acinar cells

As noted above, Oc1$^{\Delta panc}$ pancreata have many acinar cell defects at both the transcriptional and morphological level. I hypothesized that these observed defects were due to the specific requirement for Oc1 in acinar development. This distinction is important because ductal defects can lead to secondary acinar cell defects and pathologies (293,294). In order to determine the requirement for Oc1 specifically in the committed acinar cells, I utilized an unpublished acinar-specific *Elastase-Cre*$^{ER\text{-}T2}$ (*Ela-Cre*^{ER}) driver to inactivate *Oc1*. This *Cre* transgenic line was developed by Dr. Steve Konieczny at Purdue University using a ~500 bp fragment of the rat *elastase1* promotor to drive *Cre*$^{ER\text{-}T2}$ expression (276,295). In addition to the *Ela-Cre*^{ER}, I utilized a *Rosa26*$^{LoxStopLox\text{-}eYFP}$ knock-in allele to lineage trace those cells that had Cre activity (220). Notably, this Cre has some tamoxifen-independent activity and immunolabeling for YFP reporter expression at e15.5 revealed a small number of YFP+ cells, demonstrating some Ela-Cre^{ER}-mediated recombination at e15.5 even without administration of tamoxifen (Figure 5-12). In order to determine the specificity of the Ela-Cre^{ER} for acinar cells, I performed immunofluorescence analysis for YFP on pancreatic tissue sections from 6-week old mice (chosen to try to capture even rare recombination in non-acinar cells). Co-labeling these tissue sections for YFP and amylase (acinar cells), synaptophysin (endocrine cells), or cytokeratin 19 (CK19, duct cells) revealed YFP expression exclusively in amylase+ acinar cells (Figure 5-13A-C). By two measures, both fluorescent area and counting cells, it was confirmed that the Ela-Cre^{ER} had activity in ~50% of acinar cells independent of tamoxifen administration (Figure 5-13D). Finally, immunohistochemical labeling for Oc1 revealed that Oc1 was not detectable in approximately half of acinar cells in Oc1$^{\Delta acini}$ mice (*Oc1*$^{Fl/Fl}$*; Ela-Cre*^{ER}) while its expression was

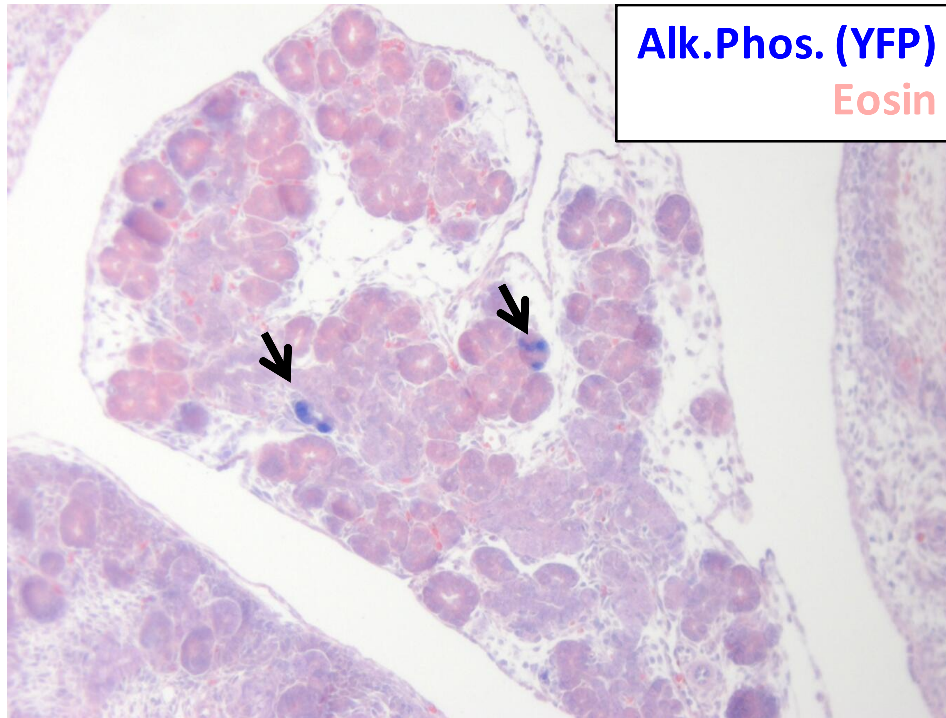

Figure 5-12: YFP expression in e15.5 Elas-Cre^{ER}+ pancreata. Representative image of alkaline phosphatase (blue) labeling of YFP in e15.5 Elas-Cre^{ER}+ pancrereata. Scattered YFP+ cells are indicated with arrows.

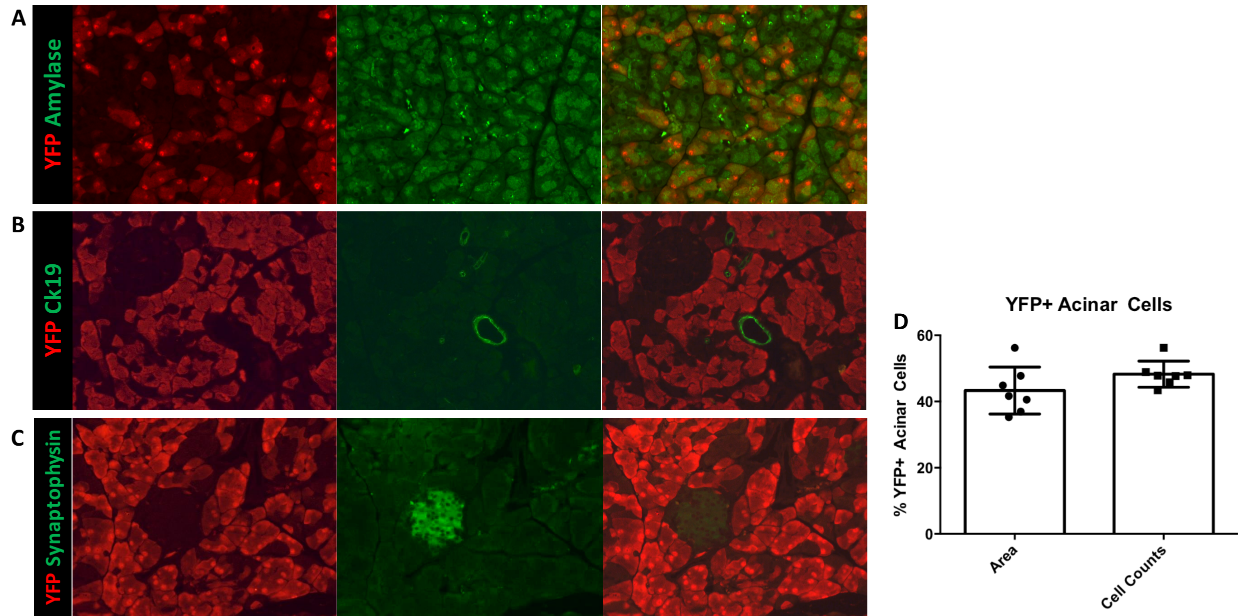

Figure 5-13: YFP expression in acinar cells of 6-week old mice. Representative images of immunofluorescence labeling for YFP in red with amylase (A, green), CK19 (B, green), or synaptophysin (C, green). (D) YFP is expressed in ~50% of acinar cells when quantified by either immunofluorescent area or number of positive cells.

unaffected in the pancreatic ducts (Figure 5-14). These results indicated that the *Ela-Cre*^{ER} driver could be used to effectively inactivate Oc1 in developing acinar cells.

I first examined the phenotype of Oc1$^{\Delta acini}$ mice at P2, the time at which there were the most dramatic exocrine defects in Oc1$^{\Delta panc}$ mice. P2 Oc1$^{\Delta acini}$ mice were slightly, but significantly, heavier than control mice, but there was no difference in pancreas weight (Figure 5-15A,B). H&E analysis of tissue sections did not reveal any differences in pancreas morphology nor did Sirius Red/Fast Green reveal any evidence of increased fibrosis as had been observed in Oc1$^{\Delta panc}$ pancreata at this time point (Figure 5-15C,D). When aged to six weeks, Oc1$^{\Delta acini}$ mice no longer had any differences in body weight and pancreas weight remained unchanged from controls (Figure 5-16A,B). H&E analysis revealed that pancreas morphology was largely normal in Oc1$^{\Delta acini}$ mice, but I did observe some acinar lobes that were noticeably paler than others; this was never observed in controls (Figure 5-16C). This difference in eosinophilia has been documented in pancreatic allografts (296), but no functional consequence has ever been reported. While these findings suggest that Oc1 is dispensable in differentiated acinar cells, it is only inactivated in ~50% of cells using this Cre model and the acinar compartment is known to be refractory to incomplete insult/injury (287,297). To increase the activity of the Ela-Cre^{ER} and inactivate Oc1 in a greater proportion of acinar cells I administered 1 mg tamoxifen to pregnant dams at gestational day (gd) 15.5. Females given tamoxifen gave birth to pups in normal Mendelian ratios, but the mothers consistently failed to nurse their pups resulting in perinatal death and precluding any postnatal analyses. Analysis of YFP expression in e18.5 pancreata did reveal that nearly all acinar cells were YFP+ (Figure 5-17), but no further analysis was carried out.

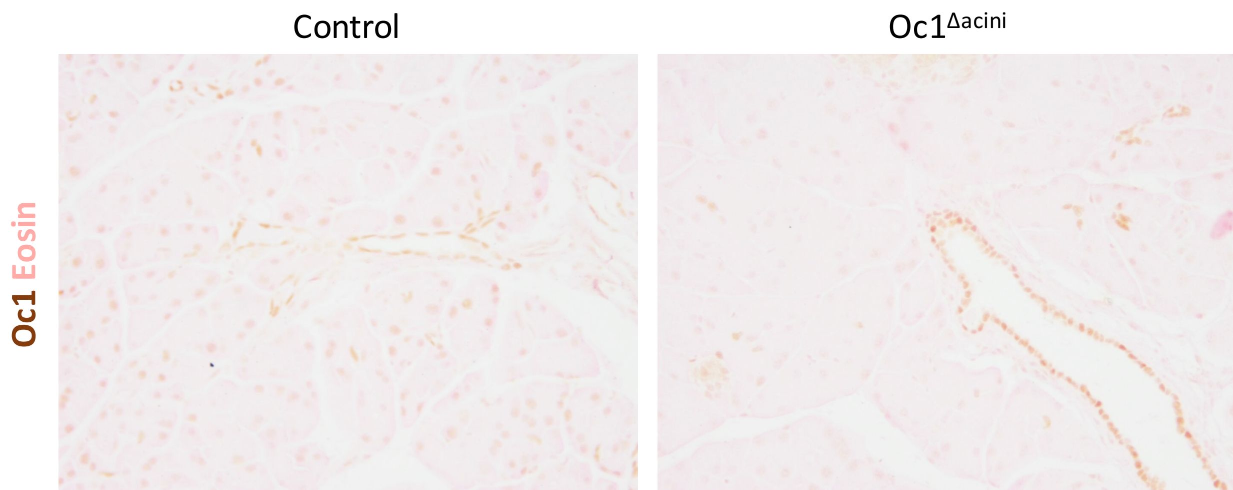

Figure 5-14: Oc1 recombination in 6-week old Oc1$^{\Delta acini}$ pancreata. Representative immunolabeling for Oc1 (brown) shows expression in nuclei of acinar cells and ducts. Oc1 expression persists in ducts of Oc1$^{\Delta acini}$ but is absent from many acinar cells. Eosin (pink) labels all pancreatic tissue.

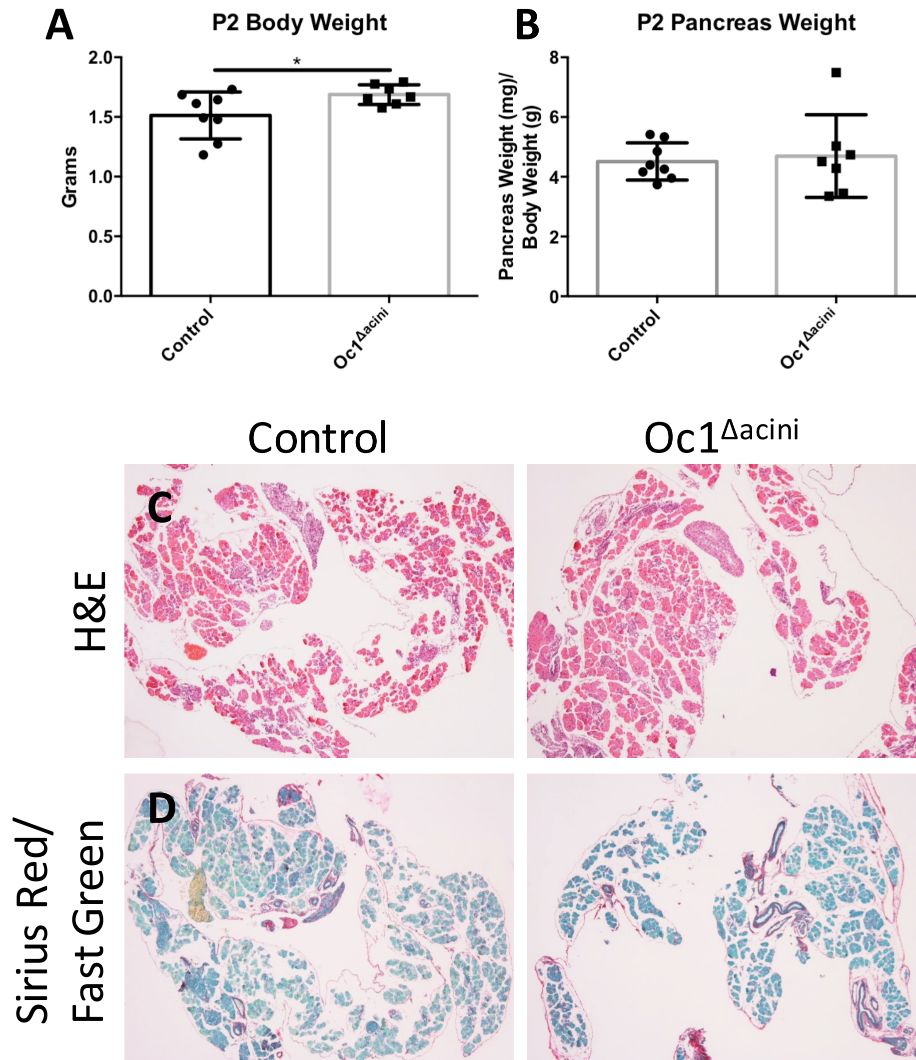

Figure 5-15: Analysis of P2 Oc1$^{\Delta acini}$ mice. (A) P2 Oc1$^{\Delta acini}$ mice are slightly heavier than littermate controls, but have no difference in pancreas weight (B). Neither H&E staining (C) or Sirius Red/Fast Green staining (D) for collagen revealed any differences in Oc1$^{\Delta acini}$ pancreata. Images captured at 4X. *: $p<0.05$ by Student's T-Test.

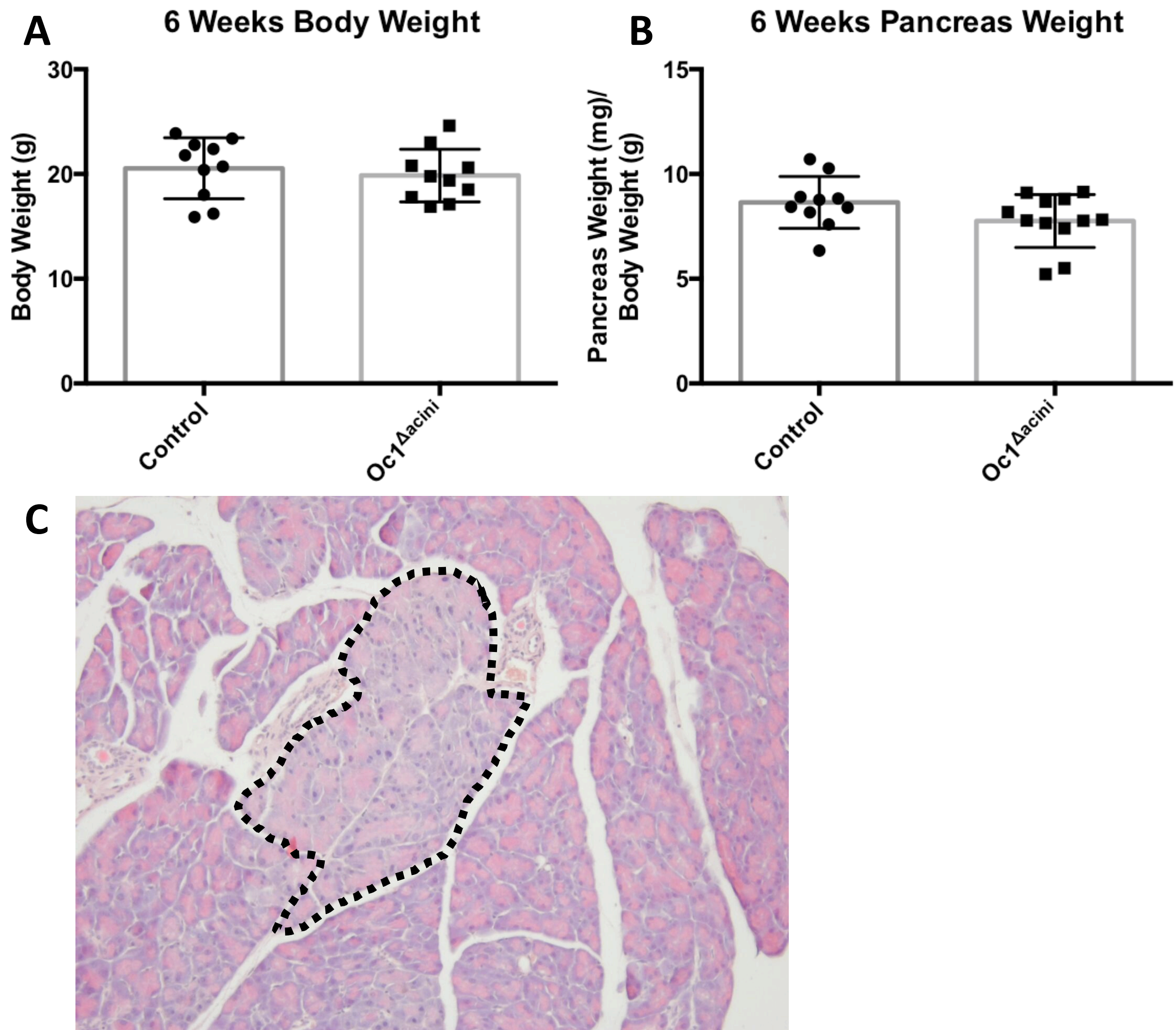

Figure 5-16: Analysis of Oc1$^{\Delta acini}$ mice at 6-weeks old. No difference in body weight (A) or pancreas weight (B) in 6-week old Oc1$^{\Delta acini}$ mice. (C) H&E staining of Oc1$^{\Delta acini}$ pancreas with pale acinar area outlined.

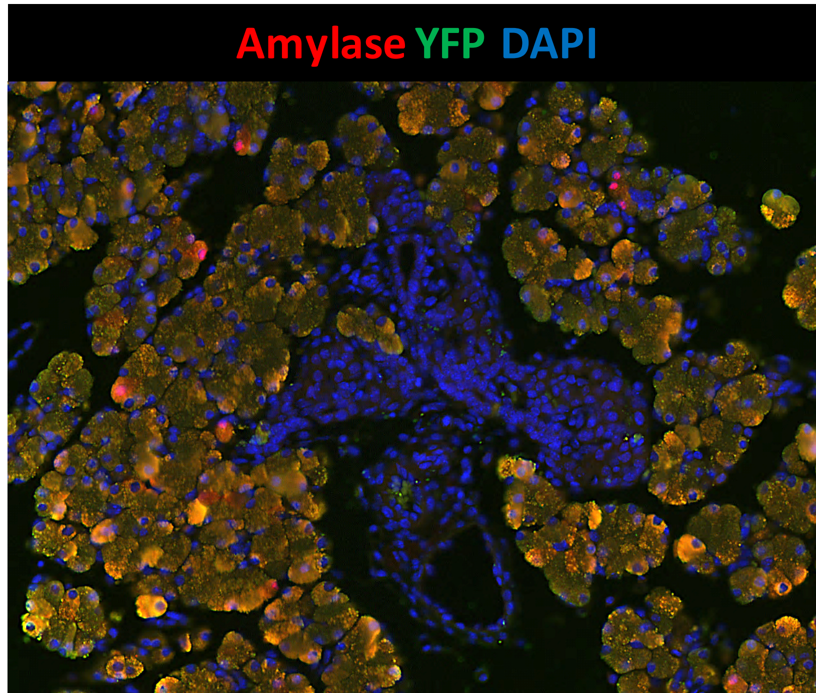

Figure 5-17: YFP expression after tamoxifen administration. Representative immunofluorescence image of an e18.5 pancreas label for amylase (red), YFP (green), and DAPI (blue). Tamoxifen was administered to the pregnant dam at gd15.5.

Identification of direct Oc1 targets during pancreas development

Oc1 clearly has a role in pancreas development, especially with respect to directing endocrine and duct fates, however, the mechanism by which it promotes these fates remains unclear. RNA-Seq performed at e15.5, e18.5, and P2 identified potential targets of Oc1, but those targets may be direct or indirect. In fact, only four direct targets of Oc1 are known in the pancreas (*Pdx1*, *Ngn3*, *MafA*, and *Hnf4α*), which are all also components of the endocrine development program (15,145,153,154,285,298). For that reason, I sought to identify novel, direct targets of Oc1 during pancreas development that would thereby allow for greater understanding of its role in directing pancreas development and, in particular, exocrine development. I chose to perform chromatin-immunoprecipitation followed by high-throughput sequencing (ChIP-Seq) at e18.5 because at this time Oc1 is expressed in both acinar and duct cells, but excluded from all hormone+ cells thereby allowing for a focus on the factors important for exocrine pancreas development. Additionally, our collaborator, Dr. Doris Stoffers at the University of Pennsylvania, is performing ChIP-Seq for Oc1 at earlier time points in development. Thus, this ChIP-Seq experiment will identify novel targets of Oc1 late in development and predominantly in acinar and duct cells.

To first ensure that I could successfully enrich for known Oc1 targets, I performed a ChIP for Oc1 in the human hepatoma cell line HepG2 in which Oc1 was over-expressed. Using published ChIP data from HepG2s as a guide (285), enrichment of the Oc1 targets *FoxA2*, *Hnf4α*, and *Glut2* was measured by PCR. There was not enrichment of *FoxA2*, but there was detectable enrichment of both *Hnf4α*, and *Glut2* (~18- and ~8-fold, respectively; Figure 5-18A). To determine whether similar results could be obtained from pancreas tissue rather than a cell

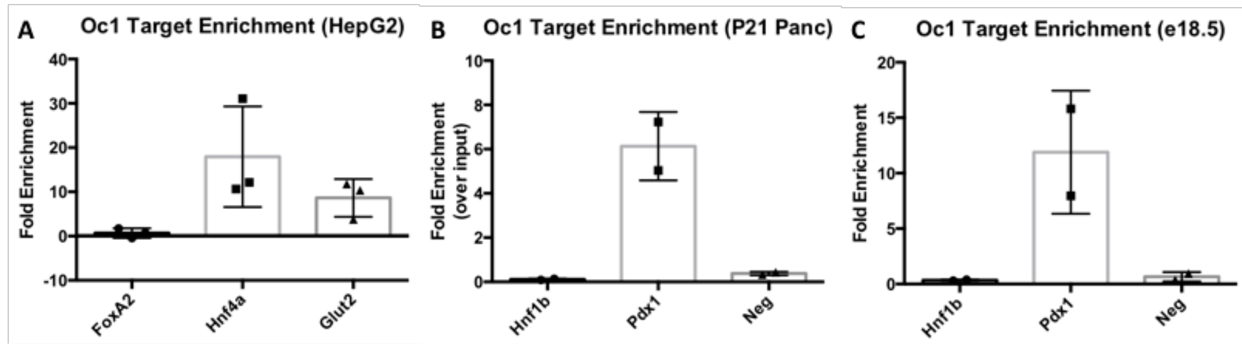

Figure 5-18: ChIP-PCR results for Oc1 targets. Enrichment of Oc1 targets in Oc1-ChIP samples from HepG2 cells (A), P21 pancreas (B), and e18.5 pancreas (C).

line over-expressing Oc1, a ChIP for Oc1 from P21 mouse pancreata was performed and the DNA was tested for enrichment of *Pdx1* and *Hnf1β*. *Pdx1* is one of the few known direct targets of Oc1 in the pancreas (15) and is expressed in adult acinar cells thus making it a likely target. *Hnf1β* is a known direct target of Oc1 in cholangiocytes (144) and is expressed in adult pancreatic ducts, thus it is a possible target of Oc1 in adult duct cells. PCR analysis of the ChIP samples demonstrated that there was no enrichment of *Hnf1β*, but there was ~6-fold enrichment of *Pdx1* (Figure 5-18B). This encouraging result led me to finally test if I could enrich for Oc1 targets in the same type of sample that would ultimately be used, e18.5 pancreata. Pooling 3-4 pancreata was sufficient to provide enough material to ChIP for Oc1 and again detect enrichment of *Pdx1* (Figure 5-18C). These results demonstrated that performing ChIP-Seq for Oc1 from e18.5 pancreata was feasible.

ChIP-Seq analysis of e18.5 mouse pancreas from Oc1 identified ~7,500 peaks significantly enriched over input. These peaks were most commonly in the proximal promoters (<500 bp upstream of the transcription start sites [TSS]s) or 5' untranslated regions (UTR)s of genes (Figure 5-19A), and HOMER motif analysis identified "Hnf6" as the most significantly represented transcription factor binding motif occurring in ~90% of all peaks ($p=1e^{-8679}$). Of the top 10 most significantly-enriched peaks, none of the closest genes are known to have a role in pancreas development; however, regulatory elements can be very distant from the associated genes. It is possible that these most-enriched peaks are in previously uncharacterized enhancer or repressor regions of genes known to regulate pancreas development.

Many of the peaks called to specific genes make sense and merit further investigation. As expected from the ChIP-PCR data, a peak was associated with Area III of the *Pdx1* promoter

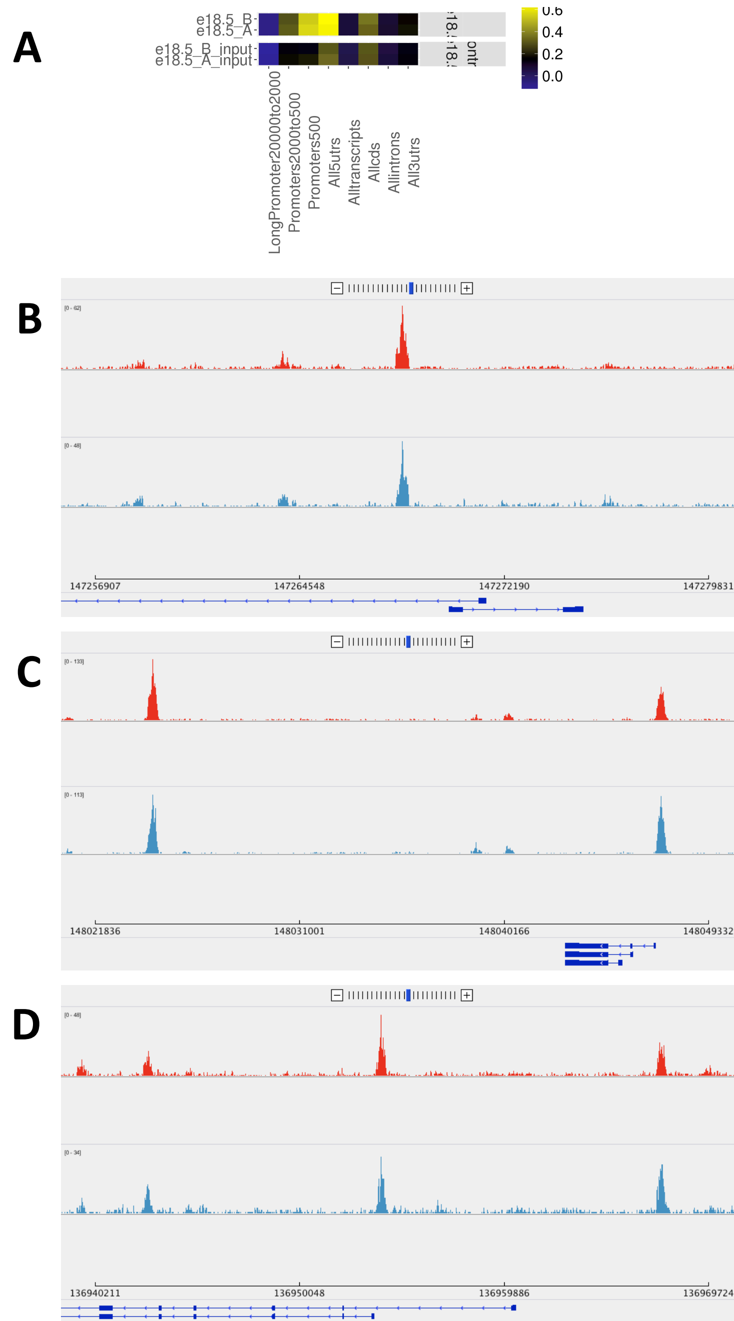

Figure 5-19: Oc1 ChIP-Seq findings. (A) Peaks of Oc1 binding were predominantly enriched in proximal promoters and 5' UTRs of genes. Peaks were identified in Area III of the *Pdx1* promoter (B), promoter and downstream of *FoxA2* (C), and many regions of *Nr5a2* (D). Both biological replicates shown in B-D. Coding regions of genes represented in blue (including splice variants) are shown below the peaks.

(Figure 5-19B). This area is essential for *Pdx1* expression, but does not confer β-cell specific expression and thus is logical that Oc1 would bind to this region late in development. Two substantial peaks were associated with *FoxA2* (Figure 5-19C) which is expressed in both β cells and the pancreatic ducts. One of these peaks was immediately upstream of the TSS and the other was ~18 kb downstream of the TSS. These binding events are almost certainly in differentiated duct cells since Oc1 is not expressed in β cells. Interestingly, no peaks were identified in the *Hnf1β* promoter even though Oc1 is known to bind to the *Hnf1β* promoter and activate its expression in cholangiocytes (144). Additionally, multiple peaks were associated with *Nr5a2* (Figure5-19D), the transcription factor important for acinar-cell specification and identity maintenance. Together, these findings support a role for Oc1 in regulating genes important for both duct and acinar-cell identity.

Discussion

Exocrine pancreas diseases are often characterized by activation of developmental pathways and sometimes reactivation of developmental transcription factors that promote a protective dedifferentiated state. For example, in later stages of PDAC, acinar-derived tumor cells lose expression of important regulatory factors such as *Ptf1a* or *Mist1* (135,273). It is thus important to understand the role of regulatory factors that promote both exocrine cell development and identity. I and others have demonstrated that Oc1 is absent from PDAC whereas it is normally expressed in acinar cells (161,284). Its continued expression and absence in transformed acinar cells led me to hypothesize that Oc1 was necessary for acinar cell development and function through regulating the transcriptome of developing acinar cells.

I first demonstrated that loss of Oc1 from the developing pancreatic epithelium results in substantial exocrine dysplasia including hypoplasia, ADM, fibrosis, and inflammation. The presence of many of these morphological differences at e18.5 suggests that they are a consequence of differentiation defects since there is not active secretion of zymogens at that time. Additionally, these observations are reminiscent of phenotypes observed with the inactivation of transcription factors (*Ptf1a*, *Nr5a2*) that are important for specification and differentiation of acinar cells rather than transcription factors important in mature function (*Mist1*, *Gata6*) (14,131,134,135,272,299). As such, the defects observed at e18.5 and P2 could be due to early impairments in acinar cell specification and differentiation which have subsequently evolved into the observed phenotypes. In such a scenario, Oc1 could be dispensable for acinar cells following differentiation. Further, if Oc1 actively regulates acinar cell identity then I would expect consistent reductions in the expression of many acinar-specific genes in $Oc1^{\Delta panc}$ at the different timepoints when I performed RNA-Seq. Most gene expression changes at e15.5 can be attributed to impaired endocrine specification and differentiation, the known role of Oc1 in pancreas development. However, many of the gene expression changes between e18.5 and P2 are unique to the individual time point rather than consistently altered expression at both e18.5 to P2. For example, the signaling pathway alterations at e18.5 (increased Hh and Wnt) did not persist at P2 and many of the changes observed at P2 were related to calcium signaling or increased expression of cytoskeletal factors including many myosins and actins. There were consistent reductions in some acinar-specific genes such as *Ptf1a* and *Prss1* suggesting that acinar-cell differentiation or identity is impaired in $Oc1^{\Delta panc}$ mice. There remains the possibility that these acinar-cell defects are secondary to duct defects.

Oc1 has a known role in promoting the differentiation of pancreatic ducts and it is well documented that the loss of Oc1 results in many ductal defects including loss of primary cilia and ductal cysts (16,102). It is likely that the increased acinar-cell death at P2 is due to continued damage to the exocrine pancreas as a result of premature activation of acinar-derived digestive enzyme zymogens within the pancreatic ducts. Indeed, the tortuous nature of the ducts in Oc1$^{\Delta panc}$ mice at P2, and the loss of primary cilia from duct cells (16), would likely result in a dramatic decrease in transport of enzyme zymogens to the duodenum thereby providing a means of autodigestion should the enzymes become active. Such ductal defects causing secondary acinar defects have been documented (293,294). Most notably, mutations in the *cystic fibrosis transmembrane conductance regulator* (*CFTR*) gene result in exocrine pancreas insufficiency due to impaired digestive enzyme transport. *CFTR* is expressed in the pancreatic ducts where it is necessary for regulation of bicarbonate levels in the lumen of the main pancreatic duct (300). Mutations in *CFTR* result in a lower pH of the pancreatic duct as well as impaired movement of fluids through the duct. Both of these factors contribute to inappropriate activation of acinar-derived digestive enzymes, autodigestion of the pancreatic parenchyma, and pancreatitis. This condition thus demonstrates that a defect in the pancreatic ducts can have cell-nonautonomous effects on the acinar cells resulting in an acinar pathology.

To determine if Oc1 was necessary specifically in developing acinar cells, I used the acinar-specific *Ela-Cre*^{ER} to inactivate Oc1 in differentiated acinar cells. Although Oc1$^{\Delta acini}$ mice were slightly heavier than controls at P2, no other overt phenotype existed at P2 or later at 6 weeks. These data suggest that Oc1 is dispensable for development and maturation of committed acinar cells. Interestingly, a duct-specific inactivation of Oc1 (using Sox9-Cre^{ER}

driver) very closely phenocopies the Oc1$^{\Delta panc}$ phenotype with acinar defects including ADM, fibrosis, an inflammation (280). Together, these findings support the conclusion that acinar-cell defects in Oc1$^{\Delta panc}$ mice are cell-nonautonomous and a consequence of defects in the pancreatic ducts. Further, it has been documented that over-expression of Oc1 in acinar cells can induce ADM and a ductal gene expression program (205). This finding must be tempered by the consideration that Oc1 is endogenously expressed at a much higher level in duct cells than acinar cells, thus over-expression could simply promote the ductal fate because of a stoichiometric increase in Oc1 and binding to duct-associated genes otherwise unavailable in acinar cells. Together, these data suggest that Oc1 has a more significant role in duct development and identity than acinar development and identity.

The ongoing work to identify Oc1 targets during development helps to clarify the role that Oc1 plays in acinar and duct cells with promotion of either respective cell fate. Oc1 binding suggests that it directly regulates genes such as *Pdx1*, *Nr5a2*, *Hnf1β*, and *FoxA2* which are all expressed in the exocrine compartment. *Pdx1* and *Nr5a2* are both expressed in differentiated acinar cells. These two transcription factors also have significant roles in acinar-cell development. Regulation of either, or both, *Pdx1* and *Nr5a2* early in development could be important for specification of acinar cells from MPCs thereby promoting acinar-cell fates. In particular, the regulation of *Nr5a2* by Oc1 early in development should be a point of focus for future work since these two factors have very similar knockout phenotypes and could function within a co-regulatory network. Oc1 could regulate development of the pancreatic ducts through regulation of *Hnf1β* and *FoxA2*. Oc1 is known to regulate both of these factors in hepatobiliary development, so it is fitting that it regulates their expression in the pancreas.

Perhaps the most important role of Oc1 in differentiated duct cells is the continued promotion of *Hnf1β* and *FoxA2* expression thereby maintaining duct identity. Regardless of the specific role of Oc1 in either duct or acinar cells, the identification of novel Oc1 targets during pancreas development is important information for the field for improving the understanding of Oc1's function.

CHAPTER VI

SUMMARY AND FUTURE DIRECTIONS

Oc1 is an important transcription factor for pancreas development especially with respect to the endocrine compartment of the pancreas. Oc1 is vital for activation of *Neurog3* and the subsequent endocrine specification pathway, a role it plays in cooperation with Pdx1. Oc1 is also essential for the overall growth of the pancreas and the development of the pancreatic ducts (16,102,153,224). These functions are critical since both compartments of the pancreas must develop properly in order to prevent development of, or predisposition to, diseases such as T2D and PDAC. In this thesis, I have sought to improve our understanding of the role of Oc1 in the development of both compartments of the pancreas through molecular and physiological analyses. The work presented here provides greater insight into regulation of pancreas development and how dysregulation can result in disease phenotypes. These findings are significant because they provide a deeper understanding of regulation of development which can be applied for understanding adult disease. This point will be discussed below.

As noted above, one of the essential roles of Oc1 in pancreas development is activation of *Neurog3* which it performs in cooperation with Pdx1. In collaboration with Dr. Doris Stoffers's laboratory, I have characterized the Pdx1/Oc1 double heterozygosity model which presents with impairments in β-cell specification and differentiation during embryogenesis, and postnatal β-cell maturation and adaptability ((224) and Kropp *et al* (in press)). In summary, my thesis addressed the question of whether proper levels of Pdx1 and Oc1 are necessary for endocrine development and function. I discovered that:

1. DH mice have reduced gene expression of factors necessary for endocrine specification and differentiation.
2. Specification and differentiation of α and β cells are significantly impaired in DH mice.
3. DH mice have persistent, postnatal impairments in islet gene expression and impaired insulin production.
4. β-cell maturation is impaired in DH mice with evidence of improper insulin processing.
5. Stimulated proliferation of DH β cells is impaired in adults.

The work presented here demonstrates that Pdx1/Oc1 double heterozygosity has a substantial impact on the development of β-cells and results in novel and unique phenotypes. The function of these two factors goes beyond specification of the endocrine lineage and has lingering impacts on postnatal β-cell maturation and function.

Since this work has established the physiological importance of Pdx1 and Oc1, future work should focus on identifying the mechanism of function and to what extent it is context (developmental stage and cell type) specific. The finding that double heterozygosity has a greater impact than single heterozygosity for either factor suggests that these two factors act together at a distinct subset of their targets; *in vitro* data supports this hypothesis (154). Current work by Dr. Stoffers's group is identifying direct Pdx1 and Oc1 targets in e13.5 pancreata using ChIP-Seq. They are comparing these new findings to our published RNA-Seq data at e15.5 to infer the consequences of single or double heterozygosity on embryonic gene expression. Through their work they will be able to determine which targets are unique to Pdx1 or Oc1 and which targets are most likely to be jointly regulated by both factors.

While Dr. Stoffers's work currently focuses at e13.5, pancreas development is an exceptionally dynamic process with cellular heterogeneity increasing as MPCs become specified and differentiated. It is very likely that RNA-Seq and ChIP-Seq analyses at other time points (e.g. e11.5 or e15.5) would provide very different results and would thus be worthwhile. This point is especially pertinent since expression of both Pdx1 and Oc1 is dynamic during the e11.5-e15.5 window. At e11.5 most cells of the pancreas are MPCs and co-express both Pdx1 and Oc1, so ChIP-Seq analysis would be the most informative for identification of common targets. By e13.5 many MPCs still persist, but a substantial portion of cells have already become specified to different fates and Oc1 expression would be lost from any cells specified to the endocrine lineage. At e15.5, the time point we previously analyzed by RNA-Seq, Pdx1 and Oc1 are not co-expressed in cells committed to the endocrine lineage but remain co-expressed in the trunk epithelium and tip cells. ChIP-Seq is thus more likely to identify their target genes associated with exocrine differentiation than endocrine differentiation. The heterogeneity of the pancreas at this time does complicate the ability to determine in which cell types Pdx1 and Oc1 cooperate to regulate common targets. For that reason, correlating the ChIP-Seq analysis with single-cell RNA-Seq could provide the clearest picture of how these two factors impact development of specific populations of cells. If, for example, we identified *Nkx6.1* as a common target through ChIP-Seq and observed decreased *Nkx6.1* expression in a specific population of DH cells with single-cell RNA-Seq, then we could infer the direct regulation of *Nkx6.1* by Pdx1 and Oc1 in that population. However, there are limitations to such approaches. ChIP-Seq requires entire populations of cells whereas single-cell RNA-Seq inherently does not. Thus, it is impossible to know exactly how transcription factor binding events correlate with changes in

gene expression. Methods that more directly associate transcription factor binding with transcription start sites such as chromatin conformation capture would provide more direct evidence of regulation than any correlative approach. In all, combinations of these assays could be utilized to great effect for understanding the complex role of Pdx1 and Oc1 cooperativity.

Not only could Pdx1 and Oc1 directly regulate transcription of targets by binding to and activating their promoters and/or enhancers, but they could also alter the chromatin environment through histone modifications. Indeed, both Pdx1 and Oc1 are known to interact with chromatin modifying factors including the p300 histone acetyl-transferase, the Swi/Snf nucleosome remodeling complex, and the NuRD (Nucleosome Remodeling Deacetylase) complex (86,140,141,177); thus, a role for these factors in epigenetic regulation of genes involved in pancreas development is reasonably likely. Analysis of the epigenetic landscape (methods described below) during pancreas development and postnatally would not only provide clarity into how Pdx1 and Oc1 regulate their common targets, but also provide insight into how they can have a prolonged impact on gene expression even after they are no longer co-expressed. This question could be addressed through assays such as ATAC-Seq (Assay for Transposase-Accessible Chromatin) that would reveal what regions of the genome are alternatively accessible or inaccessible as a consequence of double heterozygosity. ATAC-Seq does not provide any information about the specific histone modifications altered in a given context, so useful information would also be obtained from ChIP-Seq analysis of histone modifications ($H3K27^{Ac}$, $H3K4^{3me}$, $H3K9^{Ac}$ for active regions; $H3K27^{3me}$, $H3K9^{3me}$ for repressed regions) which would give more insight into specifically which types of epigenetic modifications are mediated by Pdx1, Oc1, or both during pancreas development. Finally, whole genome

bisulfite sequencing could reveal if Pdx1 and Oc1 have a role in regulating DNA methylation during development. As Dr. Anil Bushan's group has shown, DNA methylation plays an important role in regulating the cell identity and maturation state in the postnatal pancreas (82,99), so if Pdx1 and/or Oc1 interact with DNA methyltransferases they could impact the methylation state of specific targets during development. Combining analyses of these different epigenetic modifications would unveil the many roles of Pdx1 and Oc1 in regulation of pancreas development. A model depicting these potential mechanisms of regulation and the consequences of double heterozygosity is provided in Figure 6-1.

Analyses of these different epigenetic marks would be most informative if carried out at different points during development and postnatally (e.g. e11.5, e15.5, P14, 12 weeks). By examining these different time points we could identify when certain epigenetic modifications are established and also whether they persist throughout development and after. Regulation by long-lasting epigenetic modifications is consistent with, although not examined in, my findings that DH mice have continued impairments in β-cell proliferation. The early establishment of a permissive epigenetic environment could be necessary for the later activation of genes regulating β-cell proliferation in response to stimuli such as HFD or pregnancy hormones. If such an environment were not established in DH MPCs, then increased expression of genes necessary for stimulated β-cell proliferation could be prevented. Analysis of the epigenetic landscape throughout development and into adulthood would help to clarify this possibility. While the described sequencing experiments would be most informative if performed globally, targeted approaches (e.g. ChIP-PCR) could be used based off of information gathered from RNA-Seq and the proposed Pdx1 and Oc1 ChIP-Seq experiments. Together, analysis of the

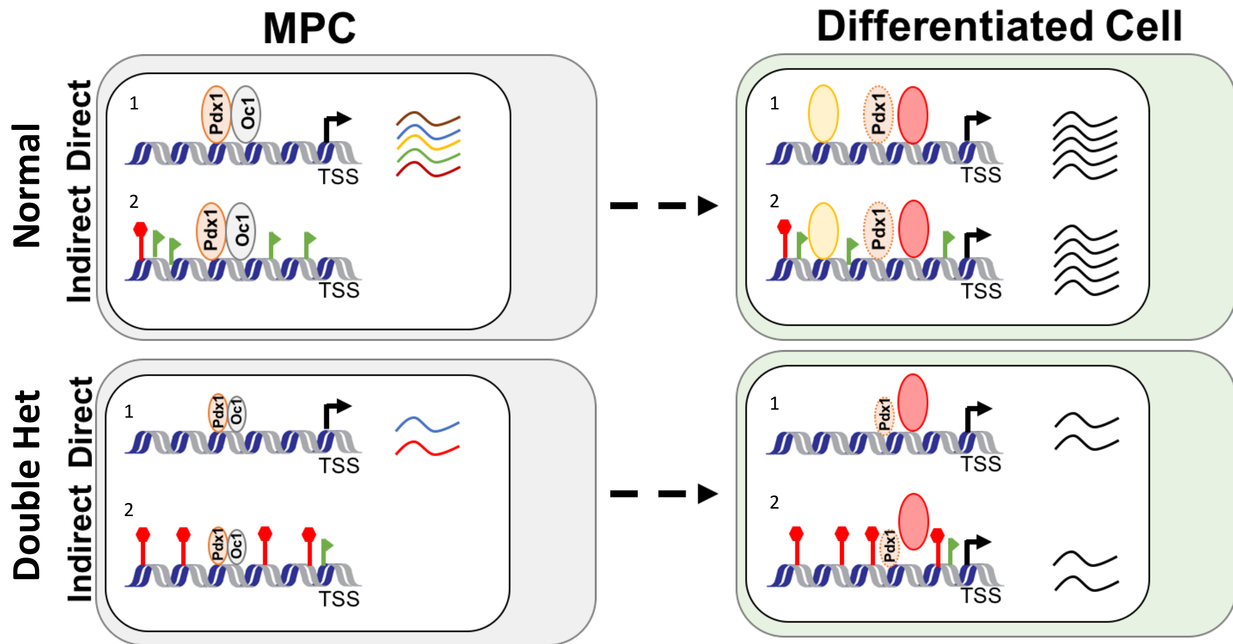

Figure 6-1: Working model for Pdx1/Oc1 regulation. Top row: In normal development, Pdx1 and Oc1 function in MPCs (left) in two potential ways: 1. through direct binding to and activation of promoters of downstream transcriptional regulators for endocrine development (represented by different colored transcripts). Those downstream transcription factors (colored ovals) would promote expression of functional genes (represented by black transcripts) within differentiated endocrine cells (right). 2. Pdx1 and Oc1 cooperate in MPCs to establish a permissive epigenetic environment of activating histone modifications (green symbols) with few repressive histone modifications (red symbols) at key endocrine genes. This permissive epigenetic environment allows for the later expression of functional genes in differentiated endocrine cells. Bottom row: In the context of double heterozygosity, reduced levels of Pdx1 and Oc1 result in reduced expression of downstream transcription factors and thus reduced expression of functional genes in differentiated cells. Alternatively, reductions in Pdx1 and Oc1 could prevent the establishment of a permissive epigenetic environment at functional genes in MPCs, resulting in inaccessibility of these promoters later in differentiated endocrine cells.

epigenetic profile of DH pancreata and postnatal β-cells would provide substantial clarity into the mechanism by which Pdx1 and Oc1 regulate pancreas development.

In my second aim, I sought to determine the necessity for Oc1 in committed acinar cells and to identify direct targets of Oc1. My data and other's data suggest that Oc1 is dispensable for acinar cell differentiation and that acinar-cell defects that occur in the absence of Oc1 are secondary to defects in development of the pancreatic ducts. It is known that Oc1 is an important regulator of duct development (16,102), but the mechanism by which it promotes duct-cell fate remains unknown. Oc1 is known function in the biliary tract where it regulates *Hnf1β* and primary cilia development (104,144,301), and the ChIP-Seq analysis identified two Oc1 binding sites in the *Hnf1β* gene body. Additionally, the ChIP-Seq analysis identified two binding sites associated with *FoxA2* which is expressed in the mature ducts. Just as with my first aim, it will be important to determine the mechanism by which Oc1 regulates duct development. The ongoing ChIP-Seq analysis at e18.5 have the potential to identify direct targets of Oc1 in the ducts, but the power of this assay will be limited by the relatively low abundance of duct cells in an e18.5 pancreas. Studies in duct/duct-like cell lines (HPDE6 [human pancreatic ductal epithelium] and IMPE [Immortalized Mouse Pancreas Epithelial Cells]) would provide a more homogenous population of cells to identify the targets of Oc1 in ductal cells. Any findings from *in vitro* studies using cell lines could be followed up *in vivo* with analysis of gene expression from sorted duct cells and immunohistochemical approaches.

Additionally, it would be valuable to identify Oc1 co-regulators through techniques such as reversible crosslinking immunoprecipitation (ReCLIP) followed by mass spectrometry performed either in cell lines or tissue samples. This information would be informative because

very little is known about the Oc1's interaction with other transcription factors or regulators. I hypothesize that the Oc1 co-regulators will be different in different cell types (MPCs, bipotent progenitors, differentiated ducts) and the identification of these co-regulators will further our understanding of how Oc1 operates within a regulatory network to promote different pancreatic cell fates. Overall, the future directions for both of these projects will rely on probing the molecular mechanism(s) of Oc1 activity in the establishment and maintenance of cell identity in the pancreas.

While the future work on these projects will have a decidedly molecular focus, it is important to consider the role of Pdx1 and Oc1 within the greater context of human health and disease. The establishment and maintenance of cell identity is of vital importance for preventing development and/or progression of disease. The work presented here has established a role for Oc1 in establishing endocrine cell identity through its necessary cooperation with Pdx1 on promoting endocrine development. It shows that reductions in two transcription factors early in development can have a lasting impact on cell identity and function. The maturation defects in P14 DH β cells show that underlying defects can persist in spite of normal β-cell mass and glucose responsiveness. Additionally, the proliferation defect observed in DH β cells demonstrates how adaptability can be impaired although the β cells themselves appear outwardly normal. Should an individual have such a defect, they would be predisposed to development of T2D. When faced with a situation requiring β-cell adaptation, there would be no such expansion. Thus, this model of double heterozygosity functions as a model of T2D predisposition and demonstrates how relatively subtle defects in expression of transcription factors during development can have long-term consequences on physiology.

Further, T2D is rarely a disease caused by single polymorphisms. Rather, T2D is caused by a combination of complex genetic susceptibility and environmental influences. Subtle perturbations to the genetic makeup of β cells likely poise these cells for later failure and T2D development. This work provides insight into the consequences of such a process.

Beyond predisposition to disease, the work presented here provides means to better understand T2D pathogenesis and potentially treatment. T2D is characterized by dedifferentiation and loss of β cells. Transient re-expression of Oc1 in dedifferentiated β cells could function to promote the endocrine development pathway and reverse dedifferentiation. It is unknown if this process occurs endogenously, but if a mechanism existed to induce transient expression of Oc1 in dedifferentiated β cells then such an approach could be used therapeutically. Currently, directed differentiation of ES or iPS cells is a promising strategy for generating new β cells and will likely be used more frequently for β-cell replacement therapy. My work demonstrates that cooperation between Pdx1 and Oc1 is important for attainment of fully mature β cells, yet Oc1 is rarely, if ever, included in directed differentiation strategies. Incorporation of Oc1 into such strategies could improve the yield of fully functional β cells available to therapy thereby improving the efficacy of the treatment plan.

Additionally, OC1 protein expression is reduced or absent in human PDAC suggesting that it might play a role in maintaining acinar-cell identity. This finding was initially supported by the observation that loss of Oc1 from the developing pancreatic epithelium resulted in severe acinar-cell defects, but partial loss of Oc1 from committed acinar cells did not result in any overt phenotype. Rather, Oc1 appears to be most important for development of the pancreatic ducts, especially since loss of Oc1 from the ducts results in a phenotype nearly

identical to Oc1$^{\Delta panc}$ mice (280). Still, loss of Oc1 expression from acinar cells during the development and progression of acinar lesions can be used to score those lesions for severity. The ability to use Oc1 as a new marker of PanIN and PDAC progression could be utilized by pathologists and physicians to help determine the best treatment strategy for individuals with pre-cancerous lesions or PDAC. Since Oc1 appears to be dispensable from committed acinar cells, it could have a more significant role in the development of pancreatitis or IPMNs through its regulation of duct cell identity. These diseases not only affect the duct cells themselves, but also the rest of the pancreas and overall health of the affected individual, so the consequences are far reaching. For example, improper development or maintenance of primary cilia in duct cells as a consequence of reduced or absent Oc1 would result in cell-nonautonomous defects similar to the *CFTR* mutations described in Chapter V. Oc1, through its regulation of duct cell function and identity, likely has a more significant role in pathogenesis of human pancreatic diseases than is currently appreciated.

Knowledge gained from developmental biology aids in understanding how predisposition to adult disease can be manifested very early in development and how we can use our knowledge of development to better understand and treat such diseases. Collectively, this work shows that Oc1 has an important role in directing differentiation of multiple pancreatic cell types and promoting a functional mature state. The establishment of these mature and functional cells is essential for prevention of diseases such as T2D, pancreatitis, and possibly PDAC. As such, Oc1, alone or in cooperation with other factors, has a considerable role in preventing pancreatic diseases and future studies could identify novel ways to utilize Oc1 for therapeutic purposes.

Appendix

Part 1: Tables associated with Chapter III

Table A-1: IPA analysis of e15.5 RNA-Seq associated with Chapter III. Table modified and reprinted with permission from Henley *et al*., 2016 (224).

Diseases and disorders

Pdx1$^{LacZ/+}$	p-value	Molecules	*Oc1*$^{+/-}$	p-value	Molecules	*Pdx1*$^{Lacz/+}$;*Oc1*$^{+/-}$	p-value	Molecules
Cancer	5.01E-06	40	Cancer	1.70E-22	595	**Endocrine System Disorders**	1.18E-09	42
Organismal Injury and Abnormalities	2.36E-05	29	Neurological Disease	8.57E-15	394	Gastrointestinal Disease	1.18E-09	67
Reproductive System Disease	2.36E-05	25	Hereditary Disorder	6.14E-11	138	Hereditary Disorder	1.18E-09	39
Gastrointestinal Disease	5.30E-05	34	Psychological Disorders	6.14E-11	274	Metabolic Disease	1.18E-09	47
Developmental Disorder	2.81E-04	13	Skeletal and Muscular Disorders	6.14E-11	297	Neurological Disease	4.01E-08	55

Molecular and cellular functions

Pdx1$^{LacZ/+}$	p-value	Molecules	*Oc1*$^{+/-}$	p-value	Molecules	*Pdx1*$^{Lacz/+}$;*Oc1*$^{+/-}$	p-value	Molecules
Gene Expression	8.07E-07	5	Cellular Development	3.81E-14	327	**Carbohydrate Metabolism**	1.14E-10	40
Cellular Growth and Proliferation	1.90E-05	29	Cellular Movement	9.83E-12	309	Cell Death and Survival	1.34E-10	68
Cellular Assembly and Organization	1.18E-04	14	Cell Death and Survival	2.50E-11	458	Molecular Transport	3.04E-10	73
Cellular Function and Maintenance	1.18E-04	16	Cellular Growth and Proliferation	2.63E-10	462	Small Molecule Biochemistry	3.04E-10	67
Molecular Transport	1.18E-04	12	Lipid Metabolism	9.51E-10	171	Nucleic Acid Metabolism	7.49E-10	24

Physiological System Development and Function

Pdx1$^{LacZ/+}$	p-value	Molecules	*Oc1*$^{+/-}$	p-value	Molecules	*Pdx1*$^{Lacz/+}$;*Oc1*$^{+/-}$	p-value	Molecules
Embryonic Development	5.71E-06	16	Embryonic Development	3.90E-11	198	**Endocrine System Development and Function**	9.28E-11	33
Organismal Development	5.71E-06	20	Organismal Development	3.90E-11	303	Tissue morphology	9.28E-11	50
Skeletal and Muscular System Development and Function	5.71E-06	15	Tissue Morphology	4.63E-11	242	Cardiovascular System Development and Function	7.76E-06	37
Connective Tissue Development and Function	1.13E-04	17	Cardiovascular System Development and Function	5.69E-10	158	Hematological System Development and Function	7.76E-06	33
Organ Development	1.13E-04	11	Organismal Survival	4.31E-09	241	Nervous System Development and Function	8.80E-06	32

Table A-2: Downstream effector analysis from e15.5 RNA-Seq associated with Chapter III. Table modified and reprinted with permission from Henley *et al*., 2016 (224).

Downstream Effects Analysis IPA – Endocrine Development and Function

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Categories	Diseases or Functions Annotation	p-Value	Activation z-score	Molecules	# Molecules
Endocrine System Development and Function, Tissue Morphology	quantity of beta islet cells	1.76E-09	-1.414	INS,Ins1,KCNJ11,MNX1,NKX2-2,NKX6-1,PAX4,PCSK2	8
Endocrine System Development and Function, Tissue Morphology	quantity of endocrine cells	1.66E-09	-1.128	GAST,INS,Ins1,KCNJ11,MAFB,MNX1,NEUROG3,NKX2-2,NKX6-1,PAX4,PAX6,PCSK2	12
Cellular Development, Endocrine System Development and Function	differentiation of endocrine cells	1.26E-04	-0.865	NKX2-2,NKX6-1,PAX4,PDX1	4
Endocrine System Development and Function, Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry	concentration of corticosterone	1.69E-05	-0.735	CRHR2,GCK,GLP1R,INS,KLB,NPY,SCG5,SPP1	8
Endocrine System Development and Function	glucose tolerance	1.51E-04	0.447	ABCC8,GLP1R,IAPP,INS,MAFA,PDX1,SPP1,SYTL4,TGFB3	9
Endocrine System Development and Function, Tissue Morphology	quantity of alpha islet cells	7.57E-06	1.000	INS,Ins1,NKX2-2,PCSK2	4
Endocrine System Development and Function, Organ Morphology	area of islets of Langerhans	1.92E-03		INS,Ins1	2
Cellular Development, Endocrine System Development and Function	differentiation of beta islet cells	2.71E-05		NKX2-2,NKX6-1,PDX1	3
Cell Cycle, Endocrine System Development and Function	entry into cell cycle progression of endocrine cell lines	5.36E-05		Ins1,SST,TF	3
Cellular Development, Cellular Growth and Proliferation, Digestive System Development and Function, Embryonic Development, Endocrine System Development and Function, Organ Development, Organismal Development, Tissue Development	formation of islet cells	3.96E-03		PAX6,PDX1	2
Cell Death and Survival, Cellular Development, Endocrine System Development and Function, Organ Morphology	regeneration of islet cells	1.92E-03		NKX6-1,PCSK2	2
Cell Cycle, Endocrine System Development and Function	replication of beta islet cells	5.88E-04		GAST,REG1A	2

Part 2: Examining whether co-transduction of HPDE6 cells with Pdx1 and Oc1 activates the endocrine lineage program

Preface

A primary goal of the diabetes-research community is to generate functional β cells from non-β-cell sources. This can be achieved through directed differentiation of stem cell populations (208,221,302,303), or transdifferentiation of mature cell types (reviewed in (304). Of particular interest are the pancreatic ducts which retain some characteristics of the embryonic bipotent trunk progenitors from which endocrine cells are specified, including the expression of MPC-associated transcription factors such as *Sox9* and *Oc1*. Multiple groups have investigated the potential of adult pancreatic ducts to function as facultative endocrine progenitors, but the results have been mixed and remain inconclusive. For example, ectopic expression of *Neurog3* in human duct cells increases the expression of factors associated with endocrine cells including insulin, Pax6, and chromogranin A (305,306). Additionally, some studies have shown that the partial duct ligation (PDL) model of pancreas injury induces generation of Neurog3+ and/or insulin+ cells in the pancreatic duct (307,308), but, in contrast, two other studies have shown that PDL is insufficient to induce endocrine differentiation from duct cells (309,310). The latter findings are supported by lineage tracing studies in mice demonstrating that adult duct cells do not give rise to endocrine cells after birth (130,186). The lack of consistency may result in part from differences in genetic models and penetrance of lineage tracing, but also suggests that further investigation into duct plasticity is merited.

One strategy to initiate transdifferentiation of duct cells to β cells is activation of *Neurog3* which can in turn activate the endocrine specification cascade. Our collaborators and I

have shown that Pdx1 and Oc1 have a cooperative role in promoting expression of *Neurog3* (153,154,224), so I hypothesized that Pdx1 and Oc1 can cooperate to activate the endocrine specification program in adult duct cells. Oc1 expression is maintained in adult pancreatic ducts in vivo, but Pdx1 is absent. I predicted that reintroduction of Pdx1 in ductal cells would activate *Neurog3* expression and the downstream components of the β-cell differentiation pathway such as *MafA*, *MafB*, and *Insulin*.

Materials and methods

Cell Culture

Human pancreatic ductal epithelial cells (HPDE6) were provided by Dr. Ming-Sound Tsao (Ontario Cancer Institute) (311). HPDE6 ells were cultered 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). HEK293T cells were provided by Roland Stein (Vanderbilt University). HEK293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS.

Lentiviral transduction (see below for virus production) of HPDE6 cells was carried out in triplicate as follows: Cells were grown to 70% confluency in 6-well plates. Each well received 10 μL of virus (for each virus) and 4 μL Polybrene (8 mg/mL, Sigma Aldrich) in 2 mL Keratinocyte Basal Medium + 10% tetracycline-free FBS (Clonetech). Plates were spun 1 hour at 1,200 RPM at room temperature and incubated 72 hours. After 72 hours, media was changed to Keratinocyte Basal Medium + 10% tetracycline-free FBS additionally supplemented with 0.1 μg/ml puromycin and 1 μg/ml doxycycline (dox). Media was subsequently changed every 48

hours. Groups were as follows: Tet3G (Control); Pdx1, Tet3G (Pdx1-transduced); Oc1, Tet3G (Oc1-transduced); Pdx1, Oc1, Tet3G (Pdx1/Oc1-transduced). Cells received doxycycline (dox) for either three or seven days. Those receiving dox for only three days were then switched to Keratinocyte Basal Medium + 10% tetracycline-free FBS for the remaining four days.

Lentivirus generation

The *Pdx1* (*pLVX-Pdx1-IRES-mCherry*) (Figure A-1) and *Oc1* (*pLVX-TRE3G-myc-Hnf6*) (Figure A-2) vectors were generated by a previous graduate student in the lab (Kathryn Henley). The *pLVX-TRE3G* promoter regulator is part of the Lenti-X Tet-On 3G Inducible Expression system (Clonetech) that included the *pLVX-Tet3G* regulator plasmid. In the presence of dox, the Tet3G protein produced from the *pLVX-Tet3G* plasmid binds to the *tet* operator sequences in the *pLVX-TRE3G-myc-Hnf6* and activates gene expression. Lentiviral vectors were packaged in HEK293T cells as follows using reagents from the Lenti-X Tet-On 3G Inducible Expression System (Clonetech): One tube containing 7 μL vector DNA (1 μg/μL), 36 μL Lenti-X HTX Packaging Mix 2, and 557 μL Xfect Reaction buffer was missed with a second tube containing 7.5 μL Xfect Polymer and 592.5 μL Xfect Reaction Buffer. Mixture incubated for 10', was dropped onto a 70% confluent plate (10 cm) of HEK293T cells, and incubated at 37°C overnight. Media was changed to DMEM + 10% FBS following overnight incubation. Transfected HEK293T cells were allowed to produce virus for 72 and then the media was collected, filtered through a 0.45 μm filter and mixed 3:1 with Lenti-X Concentrator (Clonetech). Lenti-X Concentrator mix was incubated overnight at 4°C then spun for 15' at 1,500 RPM at 4°C. Supernatant was aspirated

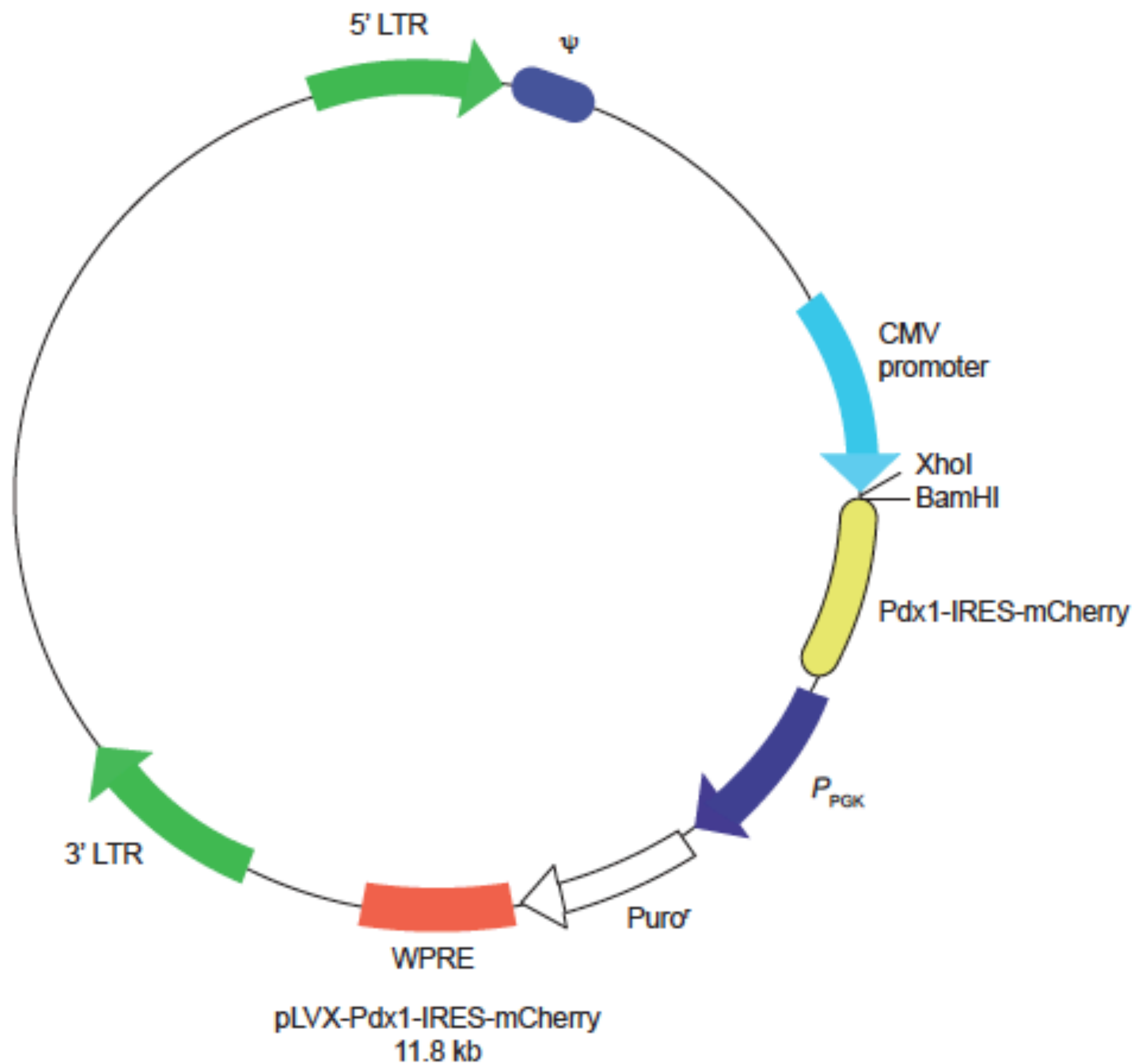

Figure A-1: Schematic of pLVX-Pdx1-IRES-mCherry lentiviral plasmid. To generate a bicistronic vector for lentiviral over-expression of murine *Pdx1*, the 1.4 kb Pdx1-IRES insert was digested with *XhoI* and *BamHI* cloned into the pLVX-N3-G2-mCherry vector. Schematic includes components relevant to lentiviral production (ψ, packaging signal) and transcriptions (RRE, Rev-response element, P_{PGK}, phosphoglycerate kinase promoter; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element). This plasmid map was modified from the pLVX-N3-mCherry vector map (Clontech, Dr. Matthew Tyska laboratory) by Kathryn Henley, PhD, and is not drawn to scale.

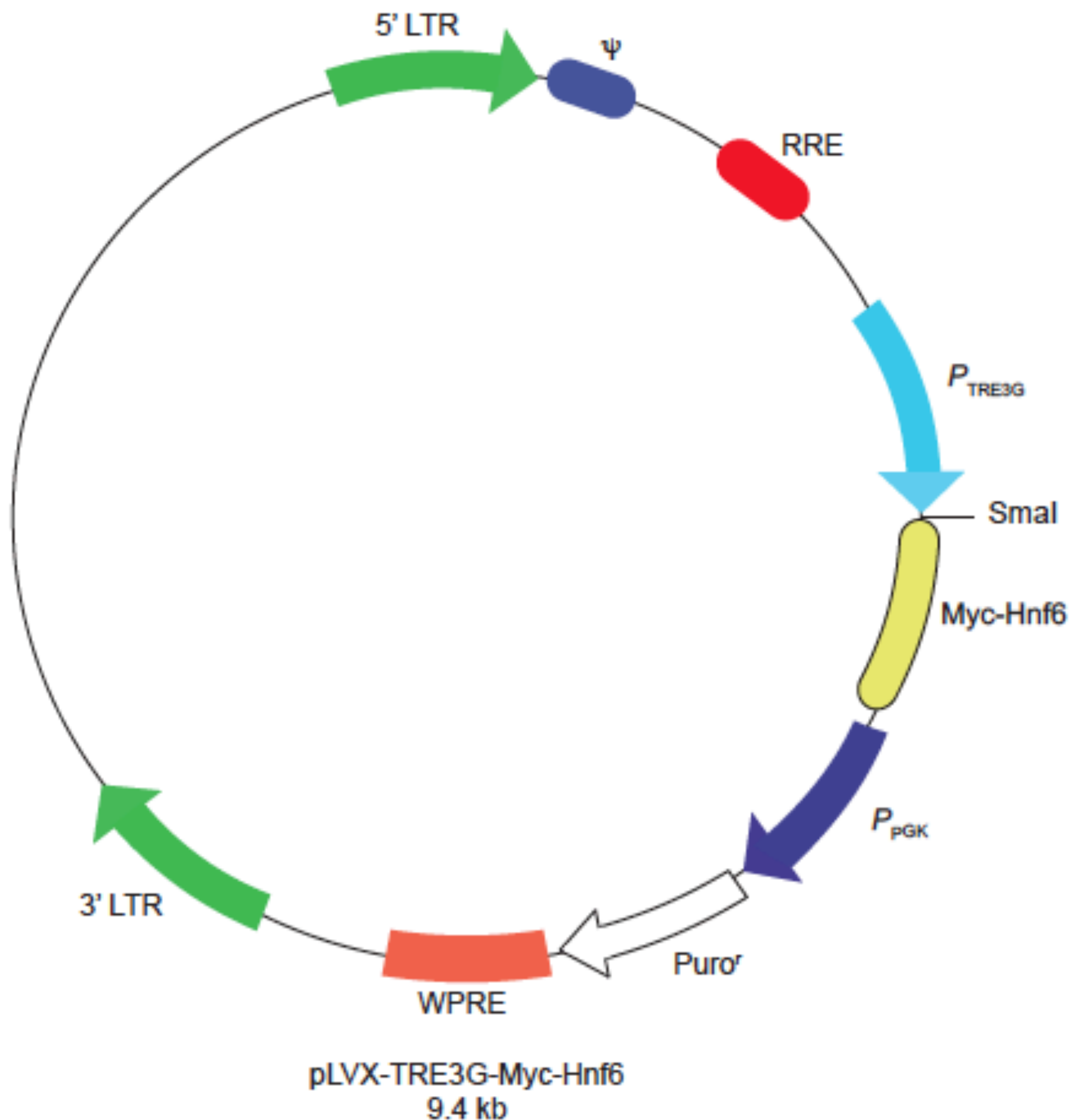

Figure A-2: Schematic of pLVX-TRE3G-Myc-Hnf6 lentiviral plasmid. To generate an inducible model of murine *Oc1* (*Hnf6*) lentiviral over-expression, the 1.6 kb Myc-Hnf6 insert was digested with *SmaI* and cloned into the pLVX-TRE3G vector. Schematic includes components relevant to lentiviral production (ψ, packaging signal) and transcriptions (RRE, Rev-response element, P_{PGK}, phosphoglycerate kinase promoter; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element). This plasmid map was modified from the pLVX-TRE3G vector map (Clontech) by Kathryn Henley, PhD, and is not drawn to scale.

and virus pellet was resuspended in 250 μL of water. All virus production was performed by pooling five 10 cm plates. Viruses were stored at -80°C until used.

RNA isolation and gene expression analysis

To collect total RNA from transduced cells, media was aspirated and 500 μL Trizol reagent was added directly to the cells, pipetted up and down to lyse cells, and frozen at -80°C until isolation. RNA isolation was carried out as described earlier. cDNA was generated as described earlier, but to a final amount of 500 ng. qRT-PCR was carried out as described earlier with the primers listed in Table A-3.

Results and Discussion

To determine if Pdx1 and Oc1 together are capable of initiating transdifferentiation of duct cells to β cells, I chose to express both of these factors in an immortalized, but non-transformed, human pancreatic ductal cell line (HPDE6) (311,312). This cell line is often used as a normal control duct cell line in studies of pancreatic cancer (312-314). *OC1* is endogenously expressed in HPDE6 cells but at a much lower level than the duct-specific transcription factor *SOX9* (Figure A-3). I anticipated that this level of OC1 might be insufficient to activate the endocrine lineage program once Pdx1 was reintroduced. PDX1 was undetectable in this cell line (Figure A-3). The *Pdx1* expression construct contained the mouse *Pdx1* cDNA driven by the ubiquitious minimal CMV promoter and an *mCherry* sequence following an internal ribosome exit sequence (IRES) thereby allowing for monitoring of cells expressing *Pdx1* (Figure A-1). The *Oc1* expression construct contained a Myc-tagged mouse *Oc1* cDNA under control of a dox-

Table A-3: Primers used for gene expression analysis in Appendix: Part 2

Gene	Forward Primer	Reverse Primer
GAPDH	TCA ACG ACC ACT TTG TCA AGC T	AGC CAA ATT GGT TGT GTC ATA CCA
PDX1	GTC CAG CTG CCT TTC CCA T	TCC GCT TGT TCT CCT CCG
OC1	GAG TTC CAG CGC ATG TCC	TGT TGC CTC TAT CCT TCC A
NEUROG3	TAT TCT TTT GCG CCG GTA GA	ACT TCG TCT TCC GAG GCT CT
INS	AGA GGC CAT CAA GCA GAT CAC TGT	AGG TGT TGG TTC ACA AAG GCT
GCG	AGC ATT TAC TTT GTG GCT GGA	GCT TGT CCT CGT CTG GAT TT
MAFA	TGA GCG GAG AAC GGT GAT TTC TAA GG	GGA ACG GAG AAC CAC GTT CAA CGT A
MAFB	ACC TTG GCT AAG GCG AGA GTA G	CTT CAG CCT GGA GAG AAG TTA CTC
SOX9	ACT CGC CAC ACT CCT CCT C	GCT TCA GGT CAG CCT TGC
PAX6	CTT GGG AAA TCC GAG ACA GA	TAG CCA GGT TGC GAA GAA CT

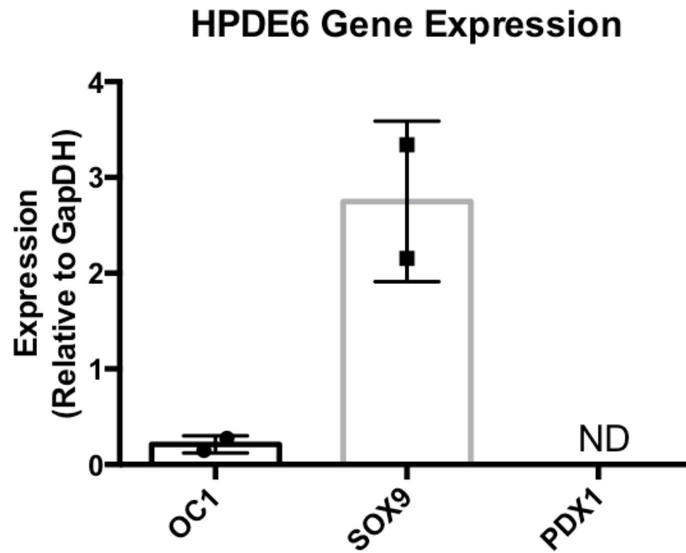

Figure A-3: Endogenous expression of pancreatic transcription factors in HPDE6 cells. OC1 and SOX9 are expressed in HPDE6 cells, albeit at very different levels, in HPDE6 cells. PDX1 was not detected.

inducible promotor allowing for temporal regulation of *Oc1* expression (Figure A-2). The Myc tag allowed for labeling with an anti-Myc antibody to confirm Oc1 expression and differentiate it from endogenous OC1 expression in HPDE6 cells. Cells were transduced with the *Pdx1* lentivirus, the *Oc1* lentivirus, or both, grown in antibiotic-selection media containing dox and then RNA was extracted for gene expression analysis.

One week in antibiotic+/dox+ culture resulted in increased expression of *PDX1*, *MAFA*, *MAFB*, and *SOX9* specifically in cells that had been transduced with both Pdx1 and Oc1 (Figure A-4), suggesting activation of the endocrine development pathway. The finding that *NEUROG3*, *INS*, and *GCG* were unchanged was not wholly surprising. In this experiment, dox was present continuously in the culture, thus maintaining OC1 expression. Previous studies from our lab demonstrated that maintained expression of *Oc1* prevents proper islet development (155,157). I decided to test if a shorter duration of *Oc1* expression would result in increased *NEUROG3* expression and endocrine characteristics by exposing transduced cells to antibiotic+/dox+ media for three days and then switching to dox-free media for the remaining four days. In this scheme, expression of either *Pdx1* or *Oc1* alone had no significant impact on gene expression, but expression of both factors resulted in increased expression of *NEUROG3*, *GCG*, and *MAFA* (Figure A-5). The finding that three days of combined *Pdx1* and *Oc1* expression can activate *NEUROG3* expression was encouraging and supported results from our collaborators in which a *Neurog3-luciferase* construct could be activated by combined Pdx1 and Oc1 expression in HepG2 cells (154). This is the first *in vitro* evidence of Pdx1 and Oc1 cooperating to promote the endocrine program in adult pancreatic duct cells. Increased expression of *GCG* but not *INS* may be due to the fact that, in mice, Gcg-expressing cells differentiate from Neurog3-expressing

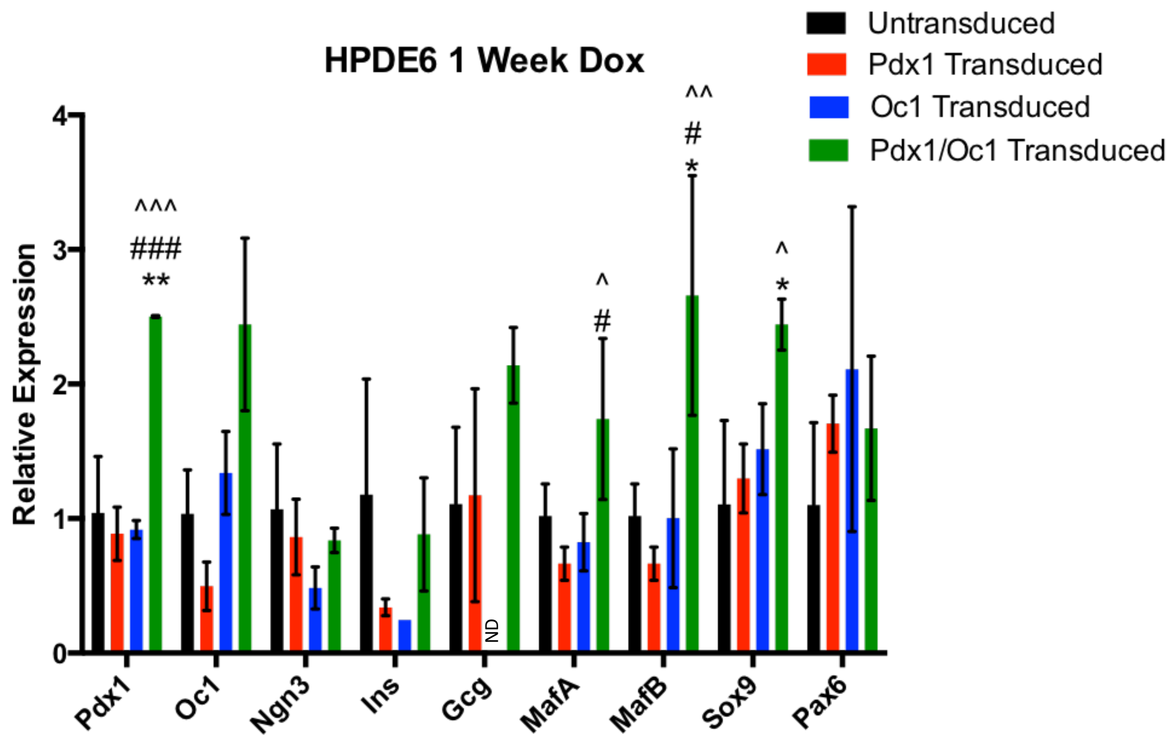

Figure A-4: Gene expression of HPDE6 cells after 1 week of Pdx1/Oc1 expression. Gene expression of transcription factors and hormones associated with endocrine development. For each gene, the control is set to one and expression is relative to the control. One symbol: $p<0.05$, two symbols: $p<0.01$, three symbols: $p<0.001$. ^: Pdx1 transduced vs. Pdx1/Oc1 transduced; #: Oc1 transduced vs. Pdx1/Oc1 transduced; *: Untransduced vs. Pdx1/Oc1 transduced.

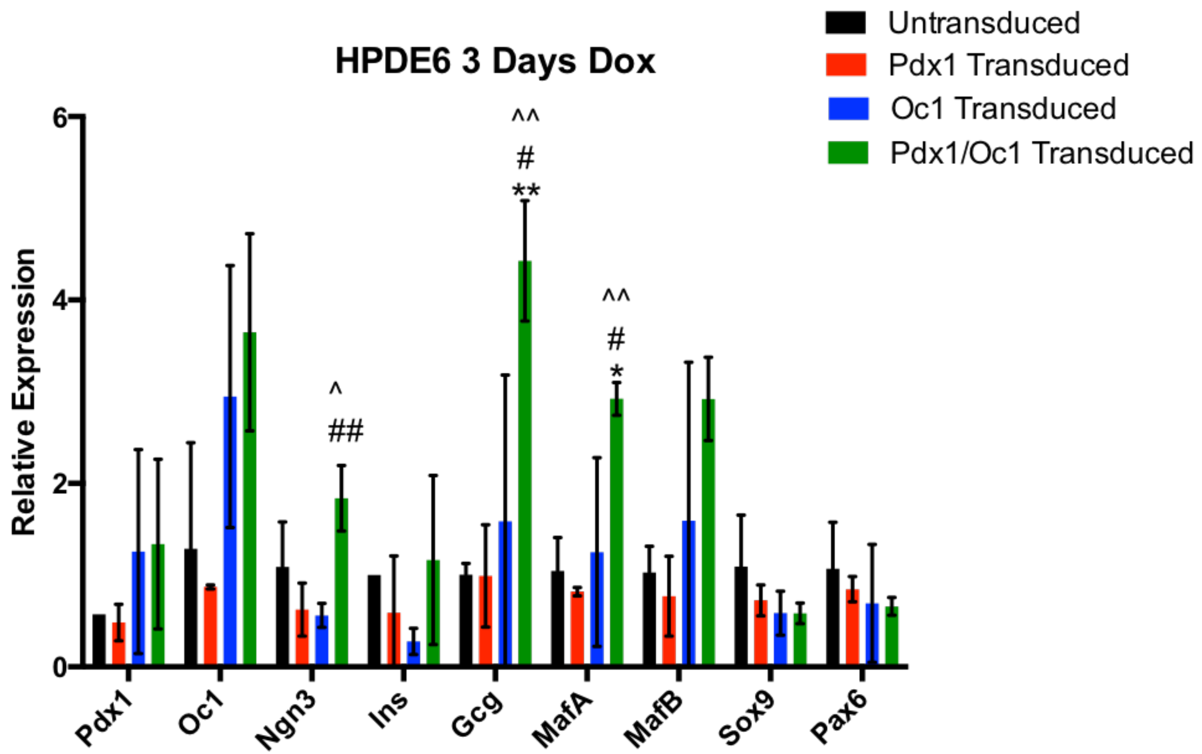

Figure A-5: Gene expression of HPDE6 cells after three days of Oc1 expression and one week of Pdx1 expression. Gene expression of transcription factors and hormones associated with endocrine development. For each gene, the control is set to one and expression is relative to the control. One symbol: $p<0.05$, two symbols: $p<0.01$. ^: Pdx1 transduced vs. Pdx1/Oc1 transduced; #: Oc1 transduced vs. Pdx1/Oc1 transduced; *: Untransduced vs. Pdx1/Oc1 transduced.

cells earlier in development than Ins-expressing cells (46). My results suggest that *Pdx1* and *Oc1* co-expression is sufficient to activate the initial stages of the endocrine specification and differentiation program. It was unexpected that *MAFA* expression would be increased without a concomitant increase in *MAFB* since in mice it is known that *MafB* expression precedes *MafA in vivo* during endocrine cell differentiation and development (61,62). However, *MAFB* levels were variable in this assay and thus did not reach statistical significance. Additionally, it is possible that there was a transient increase in *MAFB* prior to the seven-day time point and thus not detected. Despite the discrepancy in *MAFA* and *MAFB* expression these findings are consistent with a role for transient Oc1 up-regulation in *NEUROG3* activation and a requirement for *Oc1* to be inactivated in order for endocrine development to proceed.

Collectively, these data suggest that Pdx1 and Oc1 are capable of altering the identity of adult duct cells and initiating a process of transdifferentiation to the endocrine lineage. These findings are consistent with those of other groups showing that over-expression of select transcription factors (predominantly *Pdx1*, *Neurog3*, and *MafA*) can direct mature cells of the exocrine pancreas to a β-cell-like fate (315-318) and reviewed in (319), although the findings reported here are more modest than in other publications. Further investigation into the capacity of Oc1 and Pdx1 cooperativity in transdifferentiation of mature cell types into β cells is necessary, but these findings support a potential role of these two factors in redirecting the identity of differentiated cells. In complement to this work, the Stoffers lab is currently characterizing a mouse model of *Pdx1* and *Oc1* over-expression during pancreas development. They are utilizing a “Dox-off” system to temporally regulate both *Pdx1* and *Oc1* transgene expression and determine the impact of transient of transient over-expression on endocrine

development *in vivo*. Finally, future work both *in vivo* and *in vitro* will determine the specific temporal regulation of Pdx1 and Oc1 cooperation for promotion of the endocrine fate.

Part 3: Dietary ivermectin can suppress Cre^{ER} activity

Preface

Although animal facilities at research institutions are closely monitored for parasitic infections, outbreaks can and do occur. The most common amongst those are fur mites and pin worms. The animal facility at Vanderbilt University had such an outbreak of both fur mites and pin worms and thus decided to perform a global treatment with dietary ivermectin, an anti-parasitic delivered through the food and effective against both fur mites and pin worms. While mice fed a diet containing ivermectin are not known to experience deleterious health-related side effects, ivermectin has been documented to initiate the activity of $Cre^{ER\text{-}T2}$ (UBC-$Cre^{ER\text{-}T2}$) in the offspring of pregnant dams fed the diet (320). I thus sought to determine if there were any effects of dietary ivermectin on the activity of the Ela-Cre^{ER} used in my studies.

Materials and methods

Mice contained the *Elastase-Cre*$^{ER\text{-}T2}$ transgene and *Rosa26*$^{LSL\text{-}eYFP}$ knock-in allele. All mice used for this study are described in Chapter IV and maintained as previously described with the exception of diet containing ivermectin (12ppm). Female mice were either maintained on a chow-diet during pregnancy and nursing or placed on diet containing ivermectin three days after breeding and maintained on ivermectin-diet until the pups were weaned. Tissues were collected from 3-week old mice and processed as described earlier. Immunofluorescence analysis was carried out as described earlier.

Results and Discussion

Analysis of YFP expression in pancreatic acinar cells was used to determine $Cre^{ER\text{-}T2}$ activity in the presence or absence of dietary ivermectin. Six animals fed normal chow (three Cre+, three Cre-) were analyzed as well as nine animals on ivermectin+ diet (six Cre+, three Cre-). Regardless of maternal diet, YFP expression was never detected in pancreata from Cre- animals. Pancreata from Cre+ animals whose mothers were fed normal chow displayed YFP expression in ~50% of acinar cells as described earlier. In the Cre+ animals whose mothers had been fed the ivermectin-containing diet, 4/6 displayed no YFP expression while 2/6 displayed YFP expression in ~50% of acinar cells (Figure A-6). These results demonstrate that ivermectin in the maternal diet can limit the ubiquitous activity of $Cre^{ER\text{-}T2}$ by an unknown mechanism. It was untested whether tamoxifen administration could activate the $Cre^{ER\text{-}T2}$. Corbo-Rodgers *et al* hypothesized that ingestion of ivermection results in the generation of a metabolite capable of being transferred transplacentally and/or via lactation that in turn affects $Cre^{ER\text{-}T2}$ activity (320). My findings are consistent with such a mechanism even though there is no experimental evidence to support or disprove it. Notably, my studies suggest that maternal dietary ivermectin prevents activity of $Cre^{ER\text{-}T2}$ whereas Corbo-Rodgers *et al* reported that maternal dietary ivermectin activates $Cre^{ER\text{-}T2}$. The reason for this difference is unknown, but discussion with other groups at Vanderbilt performing similar studies also found that $Cre^{ER\text{-}T2}$ activity was suppressed or prevented by ivermectin treatment. These findings demonstrate that the common anti-parasitic drug ivermectin can affect the activity of $Cre^{ER\text{-}T2}$ in the offspring of mothers exposed to the drug and it should thus be used with caution.

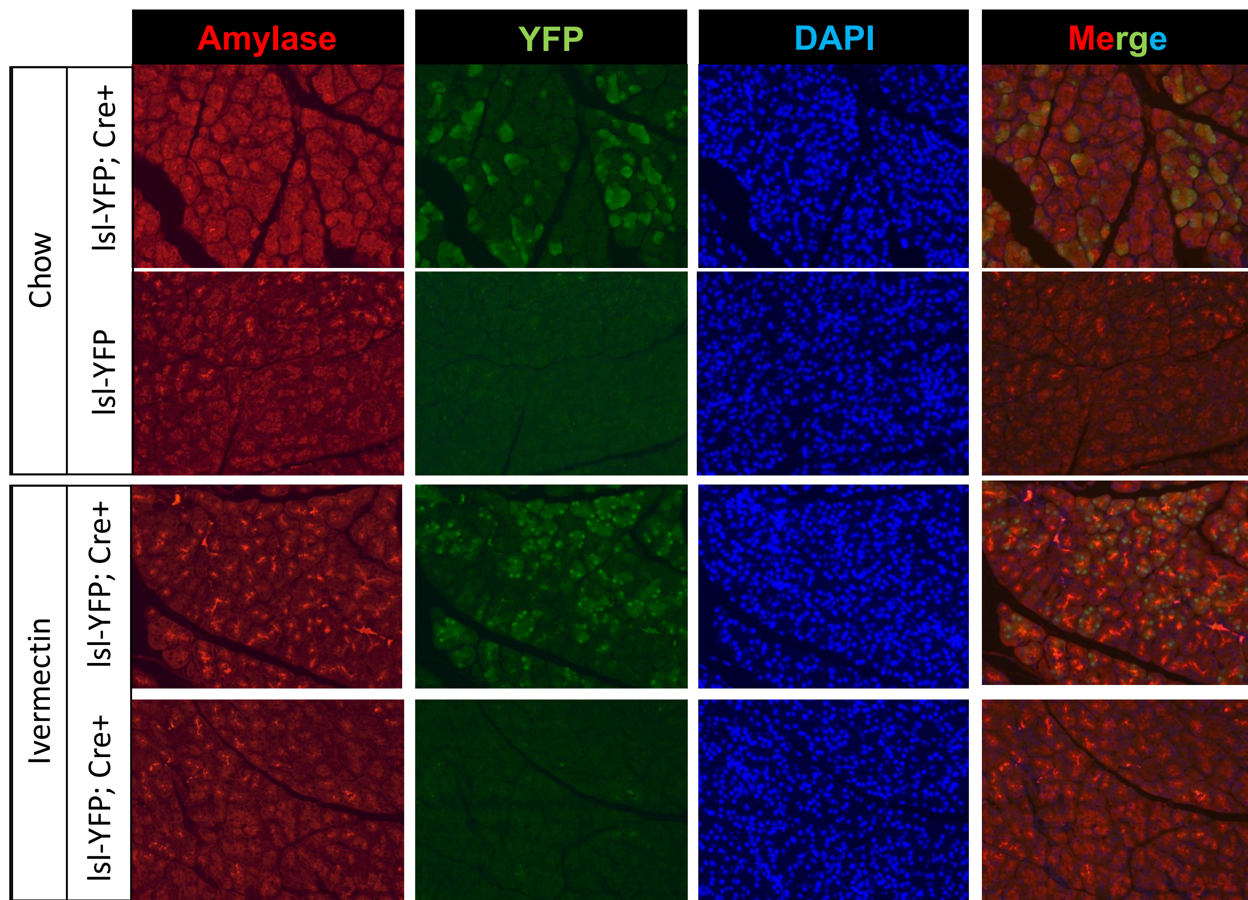

Figure A-6: Effect of dietary ivermectin on Elas-Cre^{ER} activity. Tissue sections from 3-week old mice exposed to ivermectin *in utero* were immunolabeld for amylase (red), YFP (green), and DAPI (blue). Mice harbored either the *Rosa26*$^{lsl\text{-}eYFP}$ allele (lsl-YFP), *Elas-Cre*^{ER} allele (Cre+), or both. Images are captured at 40X and are representative of 3-6 biological replicates.

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