Deconstructing Pancreas Developmental Biology

Cecil M. Benitez[1,4], William R. Goodyer[1,4], and Seung K. Kim[1,2,3]

[1]Department of Developmental Biology, Stanford University School of Medicine, Stanford, California 94305-5329

[2]Department of Medicine (Oncology Division), Stanford University School of Medicine, Stanford, California 94305-5329

[3]Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, California 94305-5329

Correspondence: seungkim@stanford.edu

SUMMARY

The relentless nature and increasing prevalence of human pancreatic diseases, in particular, diabetes mellitus and adenocarcinoma, has motivated further understanding of pancreas organogenesis. The pancreas is a multifunctional organ whose epithelial cells govern a diversity of physiologically vital endocrine and exocrine functions. The mechanisms governing the birth, differentiation, morphogenesis, growth, maturation, and maintenance of the endocrine and exocrine components in the pancreas have been discovered recently with increasing tempo. This includes recent studies unveiling mechanisms permitting unexpected flexibility in the developmental potential of immature and mature pancreatic cell subsets, including the ability to interconvert fates. In this article, we describe how classical cell biology, genetic analysis, lineage tracing, and embryological investigations are being complemented by powerful modern methods including epigenetic analysis, time-lapse imaging, and flow cytometry-based cell purification to dissect fundamental processes of pancreas development.

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Outline

[4]These authors contributed equally.

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1 INTRODUCTION

Within the past two decades, there has been tremendous growth in our understanding of pancreas developmental biology (see the influential reviews by Pictet et al. 1972 and Slack 1995 for historical context). The considerable expansion of this field has been driven by incisive use of modern approaches to molecular embryology, cell lineage analysis, genetic network and signaling pathway analysis, and epigenetics, areas ably assessed in recent reviews and monographs (Oliver-Krasinski and Stoffers 2008; Gittes 2009; Pan and Wright 2011). Recently there has also been growing, ineluctable evidence of flexibility in fate decisions by pancreatic cells once thought "terminally differentiated," including conversion of non-β cells to fates resembling β cells (Puri and Hebrok 2010). Among visceral organs, the developmental biology of the pancreas has emerged as among the best understood, although much remains to be discovered, especially with respect to human pancreas development (McKnight et al. 2010). This intensive effort is driven, in part, by the devastating nature of pancreatic diseases, principally endocrine disorders like diabetes mellitus and exocrine disorders like pancreatic adenocarcinoma and pancreatitis. In our view, a more integrated picture of pancreas development has emerged, providing, for example, insights into timely subjects like islet β-cell biology through consideration of the developmental biology of non-β cells, nonislet cells, and nonepithelial pancreatic cell populations.

Here, we review fundamental aspects of pancreas developmental biology, while providing focused analysis of emerging areas that deserve attention. This includes fetal development of pancreatic exocrine cells, islet α cells, β cells, and β-cell maturation with a focus on organelle development. Other aspects of pancreas development, including definitive endoderm development, early pancreas induction, genetics of islet cell differentiation, intrinsic and extrinsic regulators of islet β-cell proliferation, and pancreas development in nonmammalian systems like zebrafish have been expertly reviewed elsewhere (Heit et al. 2006b; Zaret and Grompe 2008; Kinkel and Prince 2009; Tremblay 2010; Seymour and Sander 2011). Here we focus on studies from rodents, principally mice, which form the main basis of our understanding of pancreas developmental biology.

2 INITIATION OF PANCREAS DEVELOPMENT

Mouse pancreas development is operationally framed by three major periods: a primary transition from embryonic day (E) 9.5 to E12.5, a secondary transition from E12.5 to birth, and the postnatal period from birth to weaning (coinciding with the onset of adolescence). During the primary transition, pancreas development begins with thickening of the endoderm, proliferation of pancreatic progenitors, and evagination of a dorsal and ventral pancreatic bud at E9.5 and E9.75, respectively (panel 1 of Fig. 1) (see reviews by Jorgensen et al. 2007; Zaret and Grompe 2008). During bud evagination, transient epithelial stratification results in formation of microlumens, which subsequently coalesce and form continuous tubular structures characteristic of pancreas morphology (panels 2 and 3 of Fig. 1) (Villasenor et al. 2010). Although the molecular and cellular processes of tubulogenesis require further elucidation, elegant studies from Semb and colleagues suggest that Rho-GTPase family member Cdc42 may contribute to establishing tube formation given that in the absence of Cdc42, microlumens fail to coalesce into tubular structures (Kesavan et al. 2009). How Cdc42 coordinates tubule formation and other factors that contribute to tubulogenesis and microlumen formation represents an active and exciting area of research.

Coincident with tubulogenesis, specification and patterning of pancreatic progenitors results in formation of a bipotent stalk or "trunk" domain and multipotent "tip" domain (panel 3 of Fig. 1). Cells residing at the tip, or most distal area of a branch, are thought to include multipotent progenitor cells (MPCs) that give rise to both endocrine and exocrine cells, whereas progeny of cells residing in the trunk produce duct and endocrine cells (panel 3 of Fig. 1). After the primary transition, tip progenitors lose their multipotency and become preacinar cells at about E13 (panel 4 of Fig. 1; also see the following section). Cell-fate specification and pancreatic morphogenesis are intricately coordinated by cell–cell interactions and extrinsic signals emanated from nonpancreatic tissues, such as mesenchyme and blood vessels (Puri and Hebrok 2010; Magenheim et al. 2011). The coordination of extrinsic signals and cell intrinsic mechanisms of transcriptional and chromatin regulation in the specification of cell fate is an area of intensive exploration (for example, see Xu et al. 2011).

3 EXOCRINE PANCREAS DEVELOPMENT

The exocrine compartment constitutes nearly 95% of pancreatic mass and is composed of two major cell types: acinar and duct cells. Acini secrete digestive enzymes, such as amylase, that are channeled into the duodenum through a branched network of duct cells. Development of acinar and duct cells is intertwined with tubulogenesis and branching morphogenesis (Hick et al. 2009; Kesavan et al. 2009; Villasenor et al. 2010), but the cell interactions and mechanisms coordinating morphogenesis with acinar and ductal cell-fate specification are not well understood. Here, we focus on the transcriptional cascades that influence

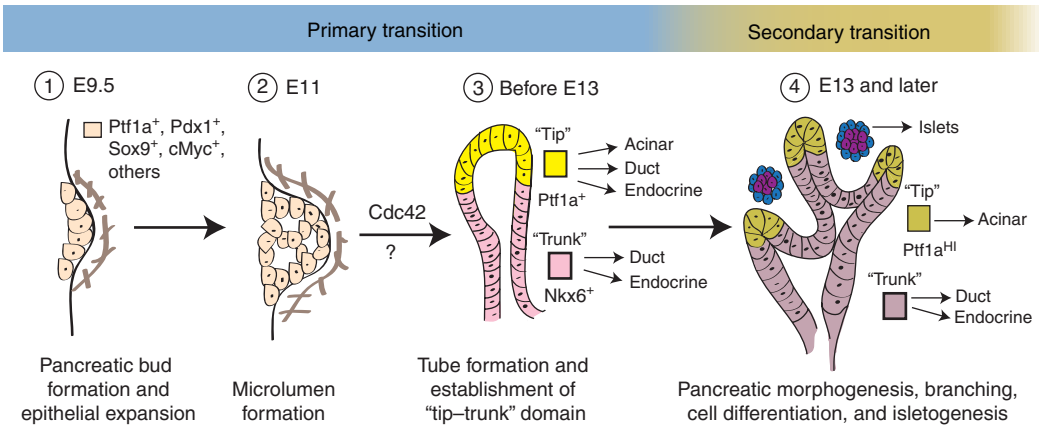

Figure 1. Pancreatic morphogenesis and developmental regulation. Mouse pancreatic development is characterized by a "primary transition" from embryonic day (E) 9.5 to E12.5 and a "secondary transition" from E13 to birth. (*Panel 1*) Pancreatic budding and pancreatic proliferation occur at approximately E9.5 and a subset of epithelial cells at that stage express *pancreas-specific transcription factor 1a* (*Ptf1a*), *pancreatic and duodenal homeobox 1* (*Pdx1*), *sry-box 9* (*Sox9*), *cMyc*, and other transcription factors. (*Panel 2*) Proliferation of epithelial cells results in the formation of microlumens (empty white spaces) at E11. Mesenchymal cells (brown crosshatch) overlie the developing pancreatic bud and secrete a variety of growth and differentiation factors (see text). Before E13, Cdc42 influences microtubule coalescence and formation of a continuous branched tube. (*Panel 3*) Coincident with tubulogenesis, multipotent "tip" and bipotent "trunk" domains establish. The multipotent Ptf1a$^+$ tip progenitors derive acinar, duct, and endocrine cells, whereas the bipotent *Nkx6 homeobox* (Nkx6$^+$) trunk progenitors produce duct and endocrine cells. (*Panel 4*) After E13 and during the secondary transition, pancreatic branching, cell differentiation, acinar cell expansion, and islet formation drive pancreatic morphogenesis. Islets represent clusters of endocrine cells. At this stage, the "tip" domain will derive acinar cells, whereas the "trunk" domain will derive duct and endocrine cells.

acinar cell-fate lineage allocation and duct cell biology (MacDonald et al. 2010).

3.1 Acinar Specification

Acinar cells derive from multipotent progenitors that express *Ptf1a*, a basic helix–loop–helix (bHLH) transcription factor initially expressed in pancreatic progenitors and later maintained in acini (Krapp et al. 1998; Kawaguchi et al. 2002; Hald et al. 2008). Absence of *Ptf1a* results in failure of acinar cell formation. Recent studies in zebrafish suggest that reduced *Ptf1a* expression permits endocrine allocation, whereas increased *Ptf1a* expression commits pancreatic progenitors to an acinar fate (Zhou et al. 2007; Dong et al. 2008; Hesselson et al. 2011). Thus, *Ptf1a* may have dual roles, dependent on its expression levels or activity. Mice permitting temporal and conditional *Ptf1a* inactivation or expression should yield further insight into acinar cell specification and development, and also to determine if levels of *Ptf1a* expression can adjust the fate of multipotent progenitors.

Studies by Schaffer et al. (2010) show that the interaction between Ptf1a and Nkx6 transcription factors contributes to developmental acinar cell allocation. The investigators suggest that a mutual inhibitory interaction between Ptf1a and Nkx6 operates during a competence window in MPCs before E14. In this model, Ptf1a represses *Nkx6* expression to promote acinar development, whereas Nkx6 factors like Nkx6.1 or Nkx6.2 repress *Ptf1a* expression to promote duct or endocrine development (Fig. 2A). Because endocrine and duct cells are present in *Nkx6.1/Nkx6.2* double mutants (Henseleit et al. 2005), it is likely that additional factors may repress acinar cell fate and drive endocrine and ductal development in MPCs. In vitro studies have shown that histone deacetylation also influences acinar cell fate (Haumaitre et al. 2008). Thus, further studies should reveal mechanistic connections between transcriptional and epigenetic regulation of acinar cell allocation from pancreatic MPCs.

Acinar development is also regulated by multiple extrinsic signals and cell–cell interactions. For example, pancreatic mesenchymal cells secrete FGF10 and follistatin, which promote acinar specification by activation and inhibition of the FGF and TGFβ signaling pathways, respectively (Miralles et al. 1998; Duvillie et al. 2006). In addition, other studies suggest Notch and Wnt signaling can repress acinar cell fate (Apelqvist et al. 1999; Miyamoto et al. 2003; Esni et al. 2004). In general, the link between signaling, transcriptional, and epigenetic control of acinar cell specification remains far less well understood than those involved in

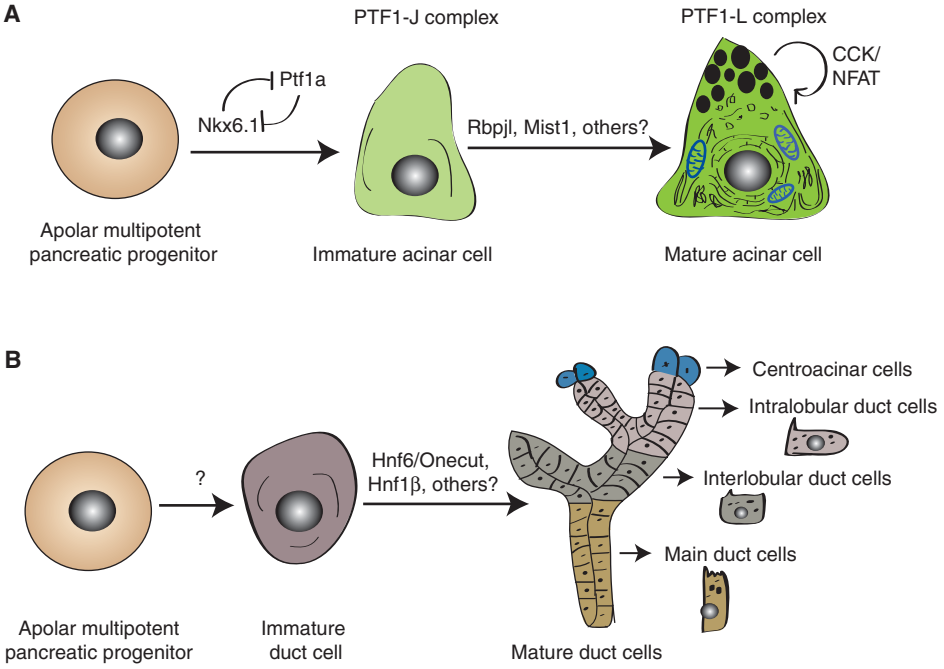

Figure 2. Pancreatic acinar and ductal cell differentiation. Apolar multipotent progenitors develop into three distinct progeny: acinar cells (top, green), duct cells (bottom, brown), and endocrine islet cells (not shown). (*A*) During acinar development expression of Ptf1a represses Nkx6, thereby suppressing alternative duct and endocrine cell fates. The commitment of early acinar cells requires the formation of a Ptf1a-Rbpj trimeric complex (PTF1-J). Acinar maturation requires formation of a Ptf1a-Rbpjl trimeric complex (PTF1-L) and is dependent on muscle, intestine, and stomach expression 1 (*Mist1*) and recombination signal-binding protein for immunoglobulin kappa J region-like (*Rbpjl*) and possibly other unknown factors. Maturation produces pyramidal polarized acinar cells with specialized organelles and high-secretory capacity. Cholecystokinin (CCK) and NFAT signaling influence adaptive growth of acinar cells. (*B*) Duct cells derive from apolar progenitors that become polarized through unknown mechanisms and form primary cilia, an organelle whose development requires both hepatocyte nuclear factor 6 (*Hnf6*) and *Hnf1β*. Duct cell heterogeneity within pancreatic branches is depicted (see text).

islet cell differentiation. For additional information on extrinsic factors that regulate acinar development see Puri and Hebrok (2010).

3.2 Acinar Cell Maturation and Function

Acinar cells are polarized pyramidal-shaped cells with high secretory capacity. To accommodate their secretory burden, acini have numerous mitochondria, unusually extensive Golgi and endoplasmic reticulum, and prominent electron-dense zymogen granules (Fig. 2A) (Pictet et al. 1972). *Mist1*, a bHLH transcription factor, has been implicated in regulating acinar cell polarity and exocytosis (Pin et al. 2001). *Mist1* is postulated to regulate RAB26 and RAB3D to control acinar exocrine granule maturation, similar to its role in stomach chief cells (Tian et al. 2010). Global profiling of adult pancreatic gene expression has recently enhanced our understanding of regulatory mechanisms controlling secretory functions in acinar cells (MacDonald et al. 2010; Dorrell et al. 2011b). This work suggests that the protein–protein interactions of PTF1 may be critical in driving acinar maturation and function. During the primary transition, PTF1 forms a trimeric complex with TCF12 and Rbpj (a complex called PTF1-J) (Beres et al. 2006; Masui et al. 2007) that is required for acinar cell development and function. As pancreatic progenitors commit to an acinar fate, Rbpj is replaced by Rbpjl within the PTF1 complex (termed PTF1-L) (Fig. 2A). Evidence suggests that this switch from PTF1-J to PTF1-L is crucial for the high-level expression of genes encoding hydrolytic enzymes, mitochondrial components, and exocytosis machinery—the hallmark components of functionally mature acini (Beres et al. 2006; Masui et al. 2007, 2010). Autoregulation of Ptf1a and Rbpjl may ensure maximal secretory protein synthesis and mitochondrial metabolism, a feature that has also been postulated to reinforce or maintain acinar cell identity (Masui et al. 2008). In the future, identification of PTF1-L and Mist1 targets should reveal how these factors coordinately regulate acinar development. Conditional inactivation of Rbpjl and Rbpj during acinar

maturation, as well as identification of additional PTF1-J and PTF1-L complex components, should further delineate the dynamic genetic and biochemical mechanisms governing establishment of acinar cell fate and function.

3.3 Acinar Cell Growth, Regeneration, and Plasticity

Acinar cells continue to differentiate, mature, and proliferate until weaning (Desai et al. 2007). Adult acinar cells, however, show a low basal proliferative index and appear to have poor regenerative capacity (Desai et al. 2007; Scott Swenson et al. 2009). *Ptf1a* and *Mist1* are expressed in adult acini and recent studies show that both factors induce expression of the cell-cycle inhibitor *Cdkn1a* (p21) (Jia et al. 2008). Thus, expression of these transcription factors may contribute to the low proliferative index of adult acinar cells. In both experimental and physiological settings, however, acinar cells can adaptively expand. For example, growth occurs in response to a high protein diet, hyperphagia, pregnancy, and lactation. Recent studies reveal that one regulator of this postnatal facultative expansion is the hormone cholecystokinin (CCK) (Fig. 2A). An increase of endogenous CCK secretion can be induced by systemic administration of protease inhibitors, leading to pancreatic exocrine growth (Gurda et al. 2008). This acinar growth response to CCK stimulation may be dependent on calcineurin/NFAT signaling (Gurda et al. 2008, 2010). It will be interesting to assess if CCK affects other intrinsic growth regulators like Ptf1a, Mist1, and p21. Genetic inactivation of β-catenin and c-Myc also results in acinar hypoplasia (Murtaugh et al. 2005; Murtaugh 2008; Nakhai et al. 2008a,b), suggesting that both β-catenin and c-Myc enhance acinar cell proliferation and/or survival. Creation of new methods for conditional genetic gain- or loss-of-function studies in mouse acinar cells should help to dissect the signaling connections between growth factors and intrinsic factors like β-catenin and c-Myc.

Although the regenerative potential of acinar cells appears limited, recent studies suggest that acinar cells have the potential to produce nonacinar cell types in the pancreas. Blaine et al. (2010) showed that on overexpression of TGFα, pancreatic acinar cells have the ability to transdifferentiate into hyperplastic duct cells, which are characteristic of both chronic pancreatitis and pancreatic adenocarcinoma in humans. In addition, Zhou et al. (2008) showed that acinar cells are responsive to cellular reprogramming. By expressing three key transcription factors in β-cell development (*Ngn3*, *Pdx1*, and *MafA*), they were able to reprogram acinar cells into cells that resembled β cells. Whether all acinar cells are equally responsive to cellular reprogramming remains to be seen. Dorrell et al. (2011a) have identified different subsets of acinar cells based on cell surface marker expression. Gene-expression profiling of acinar cell subsets at various stages and under different injury conditions may further elucidate the molecular programs that influence acinar cell plasticity, regeneration, and function.

3.4 Duct Cell Development and Biology

Duct cells are ciliated, polarized epithelial cells that secrete bicarbonate, mucins, and form extensive networks of tubules that begin as centroacinar cells (thought by some to be modified ductal cells contiguous with acinar cells) (Fig. 2B). Although the potential of duct cells in regeneration of endocrine cells has sparked interest in ductal biology, the transcriptional programs that influence duct cell development and function are not well understood. Morphological and functional heterogeneity reflecting duct cell location has likely complicated attempts to understand duct cell development (Kopp et al. 2011). Columnar epithelia form the main ducts, whereas stratified squamous and simple squamous epithelia form the interlobular and intralobular ducts, respectively (Fig. 2B) (Githens 1988). The biological significance of this heterogeneity has not been determined and may be relevant to the understanding of duct cell-dependent diseases such as pancreatic adenocarcinoma, cystic fibrosis, and pancreatitis (Sharer et al. 1998; Morris et al. 2010; Braganza et al. 2011).

Advances in the biology of pancreatic ducts would be accelerated by identification of molecular markers of duct cell subsets at specific developmental stages. For example, unlike the marker Ptf1a for acinar cell progenitors and Ngn3 for islet cell progenitors (see Sect. 4 below), markers (or regulators) of a ductal cell progenitor have not been identified. Thus, multiple fundamental questions about duct cell specification and allocation are ripe for study. For example, what distinguishes a bipotent trunk progenitor from a differentiating duct cell? What signaling pathways promote or inhibit ductal specification and differentiation? Recent studies suggest that formation of primary cilia, a subcellular signaling organelle in duct cells, requires the transcription factors *Hnf1β* and *Hnf6/Onecut1* (Haumaitre et al. 2005; Zhang et al. 2009). In the absence of Hnf6, ductal cysts form and ductal cells lack a primary cilium (Pierreux et al. 2006; Zhang et al. 2009). Recently, the transcription factors *Meis1/2* were shown to regulate ductal Krt19 expression (von Burstin et al. 2010). Although these studies suggest that Hnf6/Onecut1, Hnf1β, and Meis factors regulate specific aspects of duct development, many genetic determinants of duct formation and maturation remain unknown. It seems likely that flow cytometry-based purification like that performed by Dorrell et al. (2011b) on

subsets of human duct cells, or methods like laser-capture microdissection may shed light on embryonic pancreas duct development.

3.5 Duct Cell Regeneration and Plasticity

Compared to embryonic ductal cells, even less is known about the mechanisms regulating renewal, regeneration, and fate in adult ductal cells. Subsets of researchers have investigated the regenerative potential of ductal cells, with specific emphasis on ductal cell transdifferentiation into endocrine cells (Desgraz et al. 2011). Some investigations have reported regeneration of β cells and other pancreatic cell types from duct or duct-associated cells (Inada et al. 2008; Xu et al. 2008; Furuyama et al. 2011), but some workers have questioned the principal conclusions from these studies, based on lineage tracing evidence (Solar et al. 2009). For example, some studies have suggested the possibility of adult human ductal cell conversion toward endocrine fates (Bonner-Weir et al. 2000), whereas other studies by Furuyama et al. (2011) suggest that adult duct cells can physiologically replenish acinar and duct cells but not endocrine cells during adulthood. The potential of adult duct cells to replenish nonduct cells is even more limited according to lineage tracing studies by Ferrer, Sander, and colleagues (Solar et al. 2009; Kopp et al. 2011). By lineage tracing the progeny of Hnf1β$^+$ and Sox9$^+$ adult duct cells, respectively, both groups concluded duct cells can produce endocrine and duct cells during embryogenesis but not after birth (Solar et al. 2009; Kopp et al. 2011). These discrepancies may reflect duct cell heterogeneity and differences in the cell labeling strategies used. Again, additional rigorous lineage tracing or cell purification are needed to verify these findings. For further information on some of the controversies surrounding duct cell regeneration and plasticity see Xia et al. (2009) and Kawaguchi et al. (2011).

It seems clear that the potential of duct cells to contribute to the neogenesis of endocrine cells may be stage dependent, but the question of adult duct cell plasticity in the setting of pancreatic injury deserves further investigation, especially because most pancreatic neoplasms are likely of ductal lineage. Further analysis of duct cell markers and molecular understanding of duct cell biology may yield diagnostic markers of this disease.

4 ENDOCRINE PANCREAS DEVELOPMENT

Mature pancreatic endocrine cells derive from a subset of epithelial cells that transiently express *Neurogenin3* (*Ngn3*), delaminate from the epithelia, and aggregate in clusters called Islets of Langerhans (Fig. 3). Lineage tracing studies suggest that Ngn3$^+$ endocrine progenitors are unipotent, postmitotic cells that engender separately five endocrine cell types: α, β, δ, PP, and ε cells (Fig. 4A) (Gu et al. 2002; Desgraz and Herrera 2009; Miyatsuka et al. 2011). However, the persistence of ε cells in adult islets is not established. The birth of each endocrine cell type from Ngn3$^+$ progenitors may be temporally regulated: in genetic complementation studies permitting controlled *Ngn3* activation, Grapin-Botton and coworkers found evidence for sequential "competence" states in the pancreas that led to the birth of α cells first, then β cells and δ cells, followed by PP cells (Johansson et al. 2007). The birth of ε cells was not reported. Although these findings help frame our understanding of islet cell allocation, the basis of these postulated cell-intrinsic periods of competence has not yet been elucidated.

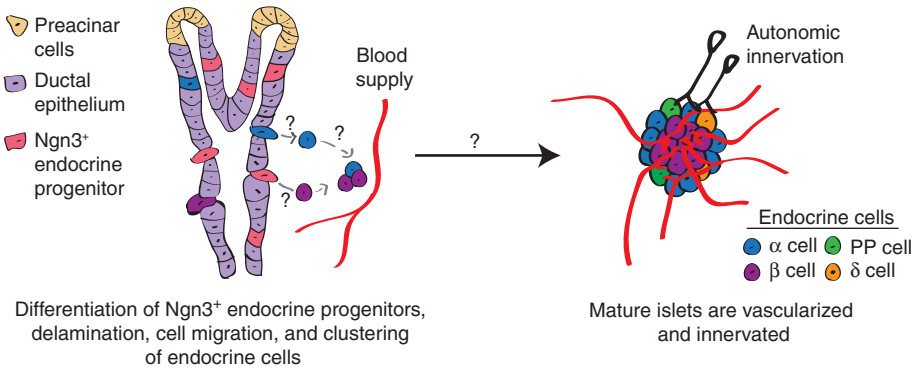

Figure 3. Events culminating in islet morphogenesis. Endocrine cells derive from unipotent *Neurogenin3* (Ngn3$^+$) endocrine progenitors (pink). On differentiation, endocrine cells delaminate from the ductal epithelia (light purple), migrate toward the mesenchyme (not shown), and aggregate into clusters called islets. For simplicity only α cells (blue) and β cells (purple) are depicted delaminating from the ductal epithelia and migrating toward blood vessels (red). Coincident with islet morphogenesis, vascularization, and innervation of islets by the autonomic nervous system occurs (black).

Recent work suggests that the threshold of *Ngn3* expression may be important in committing pancreatic progenitors to the endocrine lineage (Wang et al. 2010). According to Wang et al. (2010), epithelial progenitors that fail to attain a specific threshold of Ngn3 expression default to a ductal or acinar fate. The ability of these failed endocrine progenitors to adopt a ductal or acinar fate may be stage dependent (Beucher et al. 2011). By lineage tracing failed endocrine progenitors at various stages, Gradwohl and colleagues determined that before E12.5, failed endocrine progenitors can differentiate into acinar or duct cells but after E12.5 they differentiate into duct cells and not acinar cells. This study supports previous findings by Zhou et al. (2007) suggesting that after the secondary transition endocrine progenitors arise from bipotent "trunk" progenitors committed to ductal and endocrine lineages, whereas early endocrine progenitors arise from multipotent progenitors (panel 3 of Fig. 1).

Although the levels of Ngn3 are important in triggering endocrine fate, how this occurs is not known. Some *Ngn3* downstream factors that are critical for endocrine differentiation and development have been identified and include *NeuroD1*, *IA2*, *Pax4*, *Arx*, *Rfx6* (Smith et al. 2010; Soyer et al. 2010), and others (summarized in Fig. 4A and reviewed by Rukstalis and Habener 2009; Seymour and Sander 2011). How these transcription factors might specify changes that contribute to allocation of specific islet cell subsets remains unclear. Moreover, it is not yet known if Ngn3 activity may also induce repressors of exocrine differentiation.

Based on loss-of-function analysis in mice, Ngn3 likely also regulates other crucial features of islet development, including epithelial delamination, cell migration, and cell-cycle exit (Rukstalis and Habener 2007; Miyatsuka et al 2011). Elucidation of the basis for $Ngn3^+$ progeny delamination and migration will likely benefit from development of videomicroscopy methods at a single cell resolution. Possible mechanisms regulating delamination include (1) asymmetric cell division or (2) epithelial-to-mesenchymal transition (EMT). Prior studies suggest EMT may be regulated in pancreatic cells by Snail2 (Rukstalis and Habener 2007), a zinc-finger protein known to regulate EMT in other tissues. Recently, German and colleagues have implicated *Cdkn1a* as a possible Ngn3 target that stimulates cell-cycle exit to restrict expansion of islet progenitors (Miyatsuka et al. 2011). As described below, β-cell progeny of $Ngn3^+$ cells recover their ability to expand after the secondary transition, suggesting that *Cdkn1a* restriction of endocrine cell proliferation may be transient.

In mice, the stereotyped architecture of islets with insulin-expressing β cells contiguous with non-β cells is detected late in gestation by E18 (Fig. 3) (Herrera et al. 1991). Other evolutionarily conserved features of islet maturation include vascularization and innervation by the autonomic nervous system (ANS) (Fig. 3). Advances in our understanding of the basis of islet cell developmental interactions with vascular endothelial elements have been recently reviewed (Brissova et al. 2006; Eberhard and Lammert 2009). Here we focus on specific aspects of the development of islet α cells and β cells.

5 ISLET α-CELL DEVELOPMENT

Because of the primacy of insulin in mammals, the developmental biology of glucagon-producing α cells has received comparatively far less attention. However, several factors have led to intensified interest and investigations of α-cell development, including discoveries about the role of α cells and glucagon in type 2 diabetes (reviewed in Edgerton and Cherrington 2011), the realization that a broader understanding of islet cell biology is needed to control development of replacement islets from multipotent cell sources, and the beguiling possibility of converting α cells into functional β cells (Collombat et al. 2009; Thorel et al. 2010). Here we review aspects of α-cell specification, differentiation, and maturation. For a more detailed review on α-cell physiology and glucagon regulation please see Gromada et al. (2007) and Gosmain et al. (2011).

Genetic studies of α-cell development have discovered various transcription factors that influence α-cell development and maturation such as *Pax6, MafB, Arx*, and *Foxa2* (Fig. 4A) (Gosmain et al. 2011). *Pax6* mutants lack α cells, and have reduced glucagon expression and *MafB* expression, another regulator of glucagon expression that is selectively expressed in adult α cells in mice (Artner et al. 2006; Nishimura et al. 2006, 2008; Gosmain et al. 2010). Regulation of glucagon processing may also be regulated by *Foxa2*. Conditional knockout studies show that mutant α cells lacked the enzyme that processes proglucagon, and consequently had a 90% reduction of glucagon expression (Lee et al. 2005). Although these transcription factors affect some aspect of α-cell fate and maturation, much like the β cell (discussed below), we do not know the molecular mechanisms governing the maturation of α cells toward glucagon-secreting α cells.

Recently, there has been increased progress in our understanding of the mechanisms regulating α-cell development and fate (reviewed by Bramswig and Kaestner 2011). *Arx*, a transcription factor expressed in α cells and PP cells, is required for α-cell formation and maintenance of adult α cells. *Arx* null mutants reveal an early loss of mature α cells with a concomitant increase in β cells and δ cells, whereas misexpression of *Arx* in β cells promotes development of α cells and PP cells (Collombat et al. 2007). In mice with pancreas-specific loss of *Arx*, there is complete α-cell

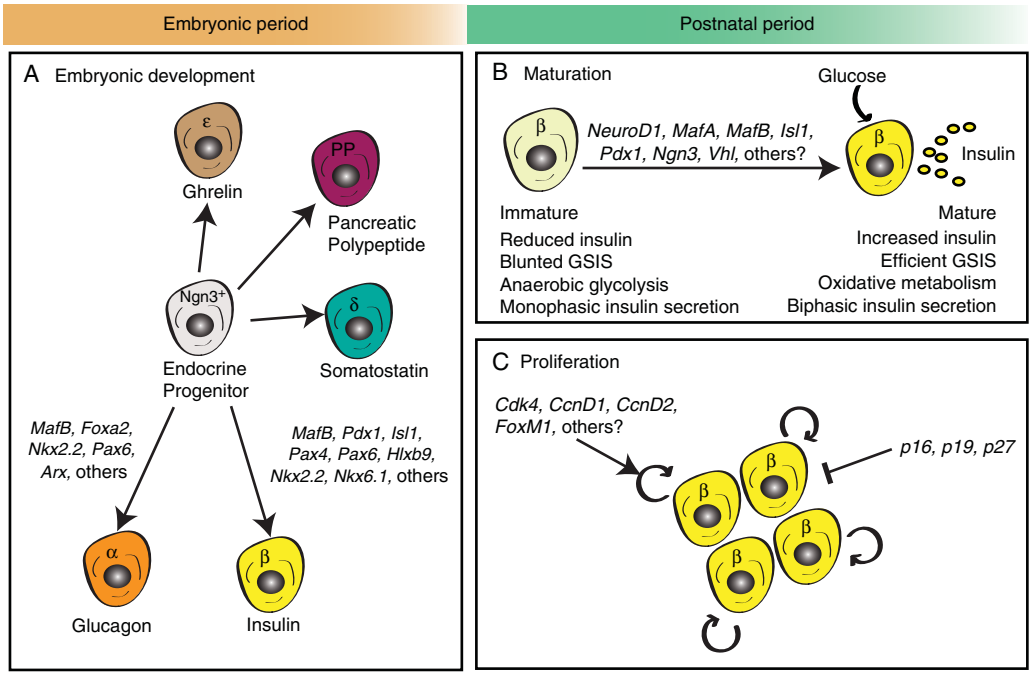

Figure 4. Embryonic and postnatal development of pancreatic endocrine cells. (*A*) During embryonic development, pancreatic endocrine cells are formed by differentiation from progenitor cells expressing the bHLH transcription factor *Neurogenin3* (*Ngn3*). Differentiation into distinct lineages requires the expression of a cascade of different transcriptional factors (TFs). Key α-cell TFs include *Forkhead box A2* (*Foxa2*), *NK2 homeobox 2* (*Nkx2.2*), *Paired box 6* (*Pax6*), and *aristaless* (*Arx*), whereas β-cell differentiation requires expression of *musculoaponeurotic fibrosarcoma oncogene homolog B* (*MafB*), *Pancreatic and duodenal homeobox 1* (*Pdx1*), *Homeobox protein HB9* (*Hlxb9*), *Pax4, Pax6, Islet1* (*Isl1*), *Nkx2.2*, and *Nkx6.1* among others (*B*,*C*). During the "postnatal period" (loosely defined here as "birth until weaning" in mice and "birth until adolescence" in humans), β cells undergo two critical events that enable the establishment of a normal, functional β-cell mass. (*B*) First, β cells undergo functional *maturation* by increasing insulin production and enhancing glucose-stimulated insulin secretion (GSIS). Known transcriptional regulators of the maturation process include *Neurogenic differentiation 1* (*NeuroD1*), *MafA, MafB, Isl1, Pdx1, Ngn3*, and *Von Hippel–Lindau* (*Vhl*). (*C*) Second, β cells undergo a transient burst of β-cell *proliferation* that coincides with a significant increase in β-cell mass expansion. Cell-cycle regulators of this process include cyclin-dependent kinases (*Cdk4*), D-type cyclins (*CcnD1* and *CcnD2*), CDK inhibitors (CKIs—$p16^{INK4a}$, $p19^{Arf}$, and $p27^{Kip1}$), and the transcription factor *FoxM1*. Refer to text, Table 1, and cited references for further information.

aplasia (Hancock et al. 2010). Thus, *Arx* is required both for α-cell fate acquisition and repression of non-α-cell fate commitment. In contrast, *Pax4* is a homeodomain factor required for β-cell and δ-cell development. In *Pax4* mutant mice α-cell hyperplasia develops (Sosa-Pineda et al. 1997). Consistent with these phenotypes, recent studies provide evidence for mutual cross-repression of Arx and Pax4 (Collombat et al. 2003, 2009). In β cells, *Arx* silencing has been found to be reinforced by the Dnmt1 and Dnmt3a DNA methyltransferase. Conditional inactivation of Dnmt1 or Dnmt3a in β cells led to derepression of *Arx* and glucagon, leading to their conversion toward an α-cell phenotype (Dhawan et al. 2011; Papizan et al 2011, respectively). It seems self-evident that investigating the transcriptional and epigenetic regulatory mechanisms that establish and stabilize α-cell fate will be essential for understanding the development of all pancreatic islet cells.

5.1 Regulation of Islet α-Cell Mass

Unlike recent growth in our understanding of the mechanisms regulating β-cell proliferation and growth in embryos and adult mice (Heit et al. 2006b), we know little about regulation of α-cell proliferation and expansion. α-Cell hyperplasia was observed in mice with targeted loss of glucagon receptor, preproglucagon, or the proglucagon-processing endopeptidase called prohormone convertase 2 (Wang et al. 2001; Gelling et al. 2003; Hayashi et al. 2009); however, it was unclear from these studies if hypoglycemia, hypoglucagonemia, or some other factor served as the stimulus for this α-cell hyperplasia, and the molecular basis of α-cell expansion in these studies was not detailed. In a model of α-cell to β-cell conversion resulting from *Pax4* misexpression, Collombat et al. (2009) suggested that hypoglucagonemia stimulated α-cell production, based on

their finding that glucagon replacement suppressed α-cell replenishment in this polygenic model. Systems that permit conditional inactivation of glucagon receptor in adult α cells (Thorel et al. 2010) should prove useful for investigating the role of glucagon deficiency in driving α-cell hyperplasia in these distinct models.

Prior studies from *Pax4* misexpression in the α-cell lineage suggested the possibility that α-cell neogenesis could be redirected to produce β cells (Collombat et al. 2009). However, this investigation also provided evidence for the possibility that postnatal α cells might be converted into functional β cells. To assess this possibility directly, Herrera and colleagues have recently used targeted conditional β-cell destruction combined with lineage tracing in mice (Thorel et al. 2010). These workers concluded that nearly complete β-cell destruction stimulated native programs to permit some α-cell to β-cell conversion, although the overall levels of this conversion were low. What are the molecular mechanisms regulating this conversion? If identified and harnessed, such knowledge might prove useful for developing both β-cell and non-β-cell sources of replacement islets for diabetes.

It seems likely that silencing of *Arx* combined with expression of *Pax4* will be one observation made in settings of adult α-cell to β-cell conversion. Studies from Lu et al. (2010) suggest that epigenetic regulation of α-cell fate will likely also underlie such conversion. Remarkably, these workers recently showed that conditional α-cell inactivation of *Men1*, which encodes the protein menin, a tumor suppressor and component of histone methyltransferase complexes in multiple endocrine cell types (Hughes et al. 2004; Yokoyama et al. 2004) leads to development of tumors resembling β-cell insulinomas. These workers concluded from genetic lineage-tracing studies that insulinomas derived directly from glucagon-producing *Men1*-deficient cells. In contrast, similar findings in a separate study by Shen et al. (2010) were interpreted as evidence for cell nonautonomous regulation of β-cell growth control, not conversion. Collectively, these and other reports (see Dhawan et al. 2011; Yang et al 2011) provide evidence that manipulation of genetic or epigenetic regulators may be used to redirect islet cell fates.

6 ISLET β-CELL DEVELOPMENT

β cells differentiate from pancreatic endocrine progenitor cells expressing *Ngn3* (Gu et al. 2002). Formation of functional glucose-sensing, insulin-secreting β cells requires the expression of a cascade of additional transcription factors (summarized in Fig. 4A and expertly reviewed by others: Gittes 2009; Puri and Hebrok 2010; Seymour and Sander 2011;). On differentiation, most fetal β cells remain postmitotic until late in gestation (Bouwens and Rooman 2005) and are considered to be functionally "immature" (Asplund et al. 1969; Rozzo et al. 2009). Here we focus on the postnatal development of β cells that culminates in their functional maturation and proliferation to establish an appropriate functional β-cell mass.

6.1 β-Cell Maturation

Mature β cells are capable of sensing glucose and other secretagogues, and, in turn, secreting appropriate levels of insulin to match these physiological signals. Fetal insulin$^+$ β cells originating from the wave of β-cell development in the secondary transition appear on E13.5, but these immature β cells have a reduced ability to both synthesize and secrete insulin in response to secretagogues (Asplund et al. 1969; Boschero et al. 1990; Rozzo et al. 2009). From birth, confronted with new host energy sources and requirements, the newborn β cell rapidly matures. Although this facultative development has been recognized for >40 years, the mechanisms underlying this postnatal "maturation" remain unclear. Immature fetal and neonatal β cells are defined by several physiological hallmarks, including decreased low K_{ATP} resting conductance and high voltage-gated Ca^{2+} conductance (summarized in Table 1). Compared to adult β cells, these neonatal β-cell properties manifest as a relatively depolarized resting membrane potential accompanied by increased basal insulin secretion (Rozzo et al. 2009). *Lactate dehydrogenase A* (*LDHA*), although normally low in mature β cells (Sekine et al. 1994; Schuit et al. 1997), is high in immature β cells (Boschero et al. 1990; Gu et al. 2010). Thus, glycolysis, not oxidative metabolism, predominates in neonatal islets, resulting in higher levels of lactate production, reduced relative ATP generation, and blunted glucose-stimulated insulin secretion (GSIS) (Asplund and Hellerström 1972; Ishihara et al. 1999; Rozzo et al. 2009). Finally, although immature β cells do show increased basal rates of O_2 consumption, they fail to increase their oxidative metabolism in response to high glucose exposure as compared to mature β cells, and therefore insulin secretion is lower and monophasic, rather than biphasic as in adult islets (Freinkel et al. 1984; Hole et al. 1988).

6.2 Physiological and Molecular Markers of β-Cell Maturity

Physiological β-cell maturation is accompanied by changes in expression of several markers. In addition to LDHA, fetal and neonatal β cells have increased expression of NPY, an inhibitor of adenylyl cyclase (Motulsky and Michel 1988) that enhances β-cell GSIS (Imai et al. 2007; Myrsén-Axcrona et al. 1997a,b; Whim 2011). Microarray studies have begun to unveil the gene-expression landscape of maturing islets and revealed altered expression of β-cell surface

Table 1. "Immature" β-cell physiological and molecular characteristics, and known transcriptional regulators of β-cell maturation

Physiology

Blunted secretion in response to glucose and other secretagogues (Asplund et al. 1969; Grill et al. 1981; Boschero et al. 1990; Rozzo et al. 2009)
Increased anaerobic glycolysis (Asplund and Hellerstrom 1972; Boschero et al. 1990; Jermendy et al. 2011)
Increased baseline oxygen consumption (Asplund and Hellerstrom 1972; Freinkel et al. 1984; Boschero et al. 1990; Hughes 1994)
Poor increase in oxidative metabolism in response to increased glucose exposure (Hole et al. 1988; Boschero et al. 1990; Rozzo et al. 2009; Gu et al. 2010)
Decreased KATP resting conductance (Rozzo et al. 2009)
Increased voltage-gate Ca^{2+} conductance (Rozzo et al. 2009)
Increased basal insulin secretion and resting membrane potential (Rozzo et al. 2009)
Monophasic insulin secretion (Freinkel et al. 1984; Hole et al. 1988; Hughes 1994)
Decreased cAMP response to glucose (Grill et al. 1975)

Associated gene-expression changes

Increased
- *Ldha* (Boschero et al. 1990; Sekine et al. 1994; Schuit et al. 1997; Gu et al. 2010)
- *Npy* (Myrsen-Axcrona et al. 1997a,b; Imai et al. 2007; Gu et al. 2010; Whim 2011)
- *Mmp-2*, *Spd* (Aye et al. 2010)
- *Ck-19* (Aye et al. 2010; Gu et al. 2010)

Decreased
- *Ins2* (Rozzo et al. 2009; Aguayo-Mazzucoto et al. 2011)
- *Glut2* (Aguayo-Mazzucoto et al. 2011; Jermendy et al. 2011)
- *Gck*, *Glp1r*, *Pcsk 1/3* (Aguayo-Mazzucoto et al. 2011)
- Oxidative metabolism genes (*Pyruvate carboxylase,* mitochondrial shuttles, etc.) (Jermendy et al. 2011)

Transcriptional regulators

NeuroD1 (Gu et al. 2010)
MafA (Zhang et al. 2005; Wang et al. 2007; Artner et al. 2010; Aguayo-Mazzucoto et al. 2011)
MafB (Artner et al. 2007, 2010)
Islet1 (Du et al. 2009)
Ngn3 (Wang et al. 2009)
Pdx1 (Ahlgren et al. 1995, 1998; Jonsson et al. 1995; Offield et al. 1996)
Vhl (Zehetner et al. 2008; Cheng et al. 2010)

markers and genes encoding regulators of oxidative metabolism (Table 1) (Aye et al. 2010; Gu et al. 2010; Jermendy et al. 2011). Recently, Aguayo-Mazzucato et al. (2011) showed that in newborn rat islets, β-cell mRNAs encoding *Insulin2, Glut2, Glucokinase*, and *Pcsk1/3*, were expressed at roughly 10% of levels in adult β cells. Over the neonatal period, expression levels of these genes increased to adult levels (Aguayo-Mazzucato et al. 2011; C Benitez and SK Kim, unpubl.). It is likely that similar changes accompany maturation of islet β cells in mice and humans. If so, flow cytometry-based methods to isolate β cells at specific postnatal stages should refine our understanding of β-cell maturation.

6.3 Regulators of β-Cell Maturation

Transcriptional regulators of β-cell maturation include *Pdx1* and *NeuroD1* (Fig. 4B), both showing transiently increased expression between birth and weaning (Aguayo-Mazzucato et al. 2011). Pdx1 is known to regulate the expression of key β-cell genes including *Ins2, Glut2, Gck*, and *IAPP*, and *Pdx1* inactivation in mice resulted in reduced β-cell function and early-onset diabetes (Ahlgren et al. 1998). Additionally, human *PDX1* haploinsufficiency is associated with a rare monogenic form of type 2 diabetes called maturity onset diabetes of the young (MODY4), indicating conservation of Pdx1 activity in β-cell identity and function (Stoffers et al. 1997; reviewed by Murphy et al. 2008). MafA has also been shown to regulate the expression of genes involved in insulin biosynthesis and secretion (Zhang et al. 2005; Wang et al. 2007) and β cells lacking *MafA* are defective in their ability to secrete insulin in response to glucose or arginine (Zhang et al. 2005; Artner et al. 2010). Conversely, overexpression of *MafA* in neonatal rat islets was sufficient to increase expression of key β-cell function genes and up-regulate GSIS to levels approaching those of adult β cells (Aguayo-Mazzucato et al. 2011). A recent elegant study by Gu et al. (2010) showed roles for *NeuroD1*

in establishing and maintaining β-cell maturity. β cells lacking *NeuroD1* had defective insulin secretion and resembled immature β cells, with increased glycolysis, basal oxygen consumption, and expression of LDHA and NPY. Recent studies have also implicated other possible regulatory factors including MafB (Artner et al. 2007), Islet1 (Du et al. 2009), Ngn3 (Wang et al. 2009), von Hippel Lindau protein (Zehetner et al. 2008), and calcineurin signaling (Fig. 4B) (WR Goodyer and SK Kim, unpubl.). Future studies of regulators of β-cell maturation will be facilitated by the use of genetically modified mouse strains that permit conditional gene inactivation or expression specifically in adult β cells (Wicksteed et al. 2010).

6.4 Neonatal β-Cell Proliferation

In concert with their maturation, β cells replicate with relative vigor in neonatal mice and humans, a stage of significant β-cell expansion thought to modulate diabetes susceptibility (Butler et al. 2007). Studies in mice have identified regulators required for neonatal β-cell replication and establishment of β-cell mass, including cyclin-dependent kinases, D-type cyclins (Rane et al. 1999; Georgia and Bhushan 2004; Kushner et al. 2005), CDK inhibitors (CKIs) (Uchida et al. 2005; Chen et al. 2009), the transcription factor FoxM1 (Zhang et al. 2006), and other factors (Fig. 4C). Islet CcnD2 and FoxM1 mRNA levels are highest in neonatal mice and decline in adults, whereas the opposite is true for $p16^{INK4a}$ and $p19^{Arf}$ indicating that their transcription may regulate and limit β-cell proliferation. Moreover, it is unknown if these or other factors regulate neonatal β-cell expansion in humans (Heit et al. 2006a,b). Recently several factors have emerged as transcriptional regulators of these target genes including *Islet1* (Du et al. 2009), *Survivin* (Wu et al. 2009), and *Ezh2* (Chen et al. 2009). Further studies will need to validate these factors within human islet neonatal development, and determine if and how β-cell maturation and proliferation are coordinated during this crucial developmental window.

7 β-CELL DENSE CORE GRANULE BIOGENESIS

Although transcriptional regulation has dominated the landscape of β-cell developmental biology, other fruitful frameworks for organizing investigation exist, including studies of organelle biology and biogenesis. β cells have hallmark organelle features including a rich complement of mitochondria, zinc transport systems, and morphologically distinct secretory vesicles, called dense core granules (DCGs). Prior studies have reviewed mitochondrial biogenesis in β-cell development (see Maechler et al. 2010, and references therein) and here we focus on developmental regulation of DCGs.

DCGs are complex, membrane-bound organelles that originate from the *trans*-Golgi network (TGN) and reduced DCG number within β cells has been associated with decreased insulin secretion in several mouse models of diabetes (Like and Rossini 1976; Bruin et al. 2008; Pechhold et al. 2009). As an inactive prohormone, proinsulin is initially sorted into immature secretory granules (ISGs) within the TGN. Following budding, ISGs undergo a critical maturation process during which several events occur including (1) acidification of the granule lumen, (2) proteolytic processing of proinsulin by proconvertase 1/3 (Pcsk1/3), Pcsk2, and carboxypeptidase E, (3) budding off of missorted proteins, excess membranes, and clathrin coat in constitutivelike vesicles, and (4) acidification and removal of water resulting in the condensation of the granule (Kim et al. 2006; Hou et al. 2009; Suckale and Solimena 2010). The maturation process allows for the crystallization of insulin into a "dense core" of tightly packed hexamers. For more information on the storage, mobilization, and exocytosis of DCGs, we refer to other recent reviews (Kim et al. 2006; Hou et al. 2009; Suckale and Solimena 2010).

In addition to insulin, DCGs harbor several principal protein components including islet amyloid polypeptide (IAPP), endo- and exopeptidases, SNARE complex components and other exocytosis regulators, granins (including *chromogranin A* [*ChgA*] and *ChgB*), and transmembrane proteins like IA2 (also called ICA152/PTPRN) (Suckale and Solimena 2010). ChgA and ChgB are of particular interest as they have been shown to be both necessary and sufficient for the biogenesis of DCGs by providing a driving force for DCG budding at the TGN (Kim et al. 2001; Day and Gorr 2003; Huh et al. 2003; Mahapatra et al. 2005), although this conclusion has been contested by more recent work (Hendy et al. 2006; Obermüller et al. 2010).

Following β-cell depolarization, a process mediated by Ca^{2+} influx and signaling, there is a transient increase in DCG biogenesis to replenish the depleted pools (Kim et al. 2006). Maintenance of DCG stores reflects both transcriptional and posttranscriptional mechanisms. On glucose stimulation, the RNA-binding protein polypyrimidine-tract binding protein 1 (Ptbp1) translocates from the nucleus into the cytoplasm where it binds and stabilizes mRNAs encoding DCG components, thereby promoting biogenesis (Knoch et al. 2004, 2006). The transmembrane protein IA2 has also been implicated as another regulator of DCG formation and insulin secretion, via linked transcriptional and posttranscriptional mechanisms in response to β-cell depolarization (Saeki et al. 2002; Harashima et al. 2005;

Kubosaki et al. 2005; Mziaut et al. 2006). These studies, mostly in vitro, suggest how activity-dependent regulation maintains DCGs in adult β cells, but it remains unclear how Ca^{2+}-dependent pathways might regulate transcription of hallmark DCG components like insulin, granins, and IAPP in postnatal β cells, or how β-cell DCG formation is regulated in vivo. Diabetes in humans and experimental animals exposed to calcineurin inhibitors like tacrolimus (FK506) and cyclosporin A, and studies of conditional *Cnb1* inactivation in mice suggest that calcineurin pathways may regulate β-cell functions (reviewed in Heit 2006b), but it remains unclear if these factors regulate transcription or assembly of β-cell DCG components. Identification of the mechanisms regulating hallmark features of β-cell maturation like DCG formation should enhance efforts to create replacement islet cells from multipotent stem cell sources (reviewed by van Hoof et al. 2009; McKnight et al. 2010).

8 CONCLUDING REMARKS

Efforts to improve knowledge about mechanisms governing development and growth of the pancreas are rooted in the conviction that such knowledge will prove relevant to diagnosing, prognosing, or treating diverse, common diseases like diabetes mellitus and pancreatic adenocarcinoma. Based on its emergence as a paradigm of solid organ development, these efforts may also help to transform our conception of development and maintenance of cell fate and physiological function in other epithelial organs. To achieve specific goals of regenerative therapy, like β-cell regeneration in diabetes, a focused effort on deciphering the genetic and epigenetic mechanisms underlying pancreas development is essential. However, aspects of pancreas cell biology and development, including epithelial morphogenesis, organelle biogenesis, and physiological maturation deserve increased attention. A major "roadblock" to progress in these areas will be the accessibility of human tissues and the development of tools and assays to study human pancreas cell biology.

A more integrative approach to pancreas development may prove useful in treating various pancreatic diseases. Pancreas cancers that present at late stages are rapidly fatal; in contrast, discovery and surgical excision of the rarer subset of local tumors is often curative. We foresee that a combinatorial approach to pancreas developmental biology may permit prospective modeling of the first stages of human pancreatic intraepithelial neoplasia (PanIN). Combined with epigenetic and high-throughput sequencing approaches (Ting et al. 2011), these approaches could accelerate development of diagnostic strategies for detecting pancreatic adenocarcinoma at earlier, resectable stages. Likewise, development of similar approaches may permit detection of β-cell destruction, presaging type 1 diabetes mellitus, in "preclinical" stages (Akirav et al. 2011). If so, further destruction might be prevented by judicious use of immunosuppression; preservation of β-cell mass might then expand subsequent regenerative "treatment" options. For type 2 diabetes mellitus, whose origins reflect a multiplicity of pathogenetic mechanisms, induced pluripotent stem cell-based modeling of islet cell growth and adaptation should permit molecular studies to investigate the basis of β-cell failure risk in this disease, including investigations focused on the multiple candidate loci implicated by GWAS (reviewed by Billings and Florez 2010; Imamura and Maeda 2011). Thus, we envision that developmental and molecular studies of pancreas biology may specifically accelerate early diagnostic regimes that could significantly impact disease progression and outcomes. These studies should also help to frame and define the acceptable "endpoints," including quality and safety benchmarks, for producing surrogate or replacement functional β cells from multipotent stem cell sources.

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