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Review

Developmental biology of the pancreas: A comprehensive review

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ABSTRACT

Pancreatic development represents a fascinating process in which two morphologically distinct tissue types must derive from one simple epithelium. These two tissue types, exocrine (including acinar cells, centro-acinar cells, and ducts) and endocrine cells serve disparate functions, and have entirely different morphology. In addition, the endocrine tissue must become disconnected from the epithelial lining during its development. The pancreatic development field has exploded in recent years, and numerous published reviews have dealt specifically with only recent findings, or specifically with certain aspects of pancreatic development. Here I wish to present a more comprehensive review of all aspects of pancreatic development, though still there is not a room for discussion of stem cell differentiation to pancreas, nor for discussion of post-natal regeneration phenomena, two important fields closely related to pancreatic development.

Basic pancreatic embryology

In order to form the mature architecture of the pancreas (see Fig. 1), there is a carefully orchestrated series of embryologic events that must take place. The first morphologic evidence of the pancreas is a condensation of mesenchyme overlying the dorsal aspect of the endodermal gut tube in the duodenal anlage of the foregut, just distal to the stomach. Shortly after this condensation, on the 26th day of gestation in humans, and at approximately 9.5 days gestation in mice (E9.5, where E0.5 is defined as noon of the day of discovering a vaginal plug) with significant variation among strains (personal observation) at approximately the 25th somite stage, the endoderm evaginates into the overlying mesenchyme. (Kallman and Grobstein, 1964; Pictet and Rutter, 1972; Pictet et al., 1972; Munger, 1958). Over the ensuing few hours in the mouse, the dorsal bud evagination continues to elongate, with a wide opening and an apparent lack of cell division (Wessels and Cohen, 1967). This initial dorsal bud arises just before loss of contact of the dorsal gut tube with the notochord due to fusion of the paired dorsal aortas in the midline that intervenes between the notochord and dorsal gut (Pictet and Rutter, 1972) (see Fig. 2). Also, the coelomic epithelium, which at this time makes up the only mesenchyme around the pancreas, moves dorsally and begins to compartmentalize the pancreas and gut away from dorsal aorta and from other dorsal non-gut structures. It is also during this time that we see proliferation of the mesenchymal cells in between the coelomic epithelium and pancreatic epithelium (Pictet and Rutter, 1972). Approximately 12 h after dorsal bud evagination in the mouse, and 6 days after dorsal bud evagination in humans, the ventral bud begins to arise from the caudal aspect of the hepatic/biliary bud evagination, through a process that morphologically is similar to the dorsal bud, but the molecular control of ventral pancreas development is markedly different from that of the dorsal pancreas. Once evaginated, the pancreatic buds undergo elongation of a stalk region and branching morphogenesis of the more apical region of the bud. This branching morphogenesis appears to follow a unique pattern. Unlike the more typical 90° outgrowth pattern of branching morphogenesis seen in developing lung, kidney and salivary glands, the pancreas undergoes acute angle branching which, due to the proximity to one another of new adjacent branches, tends to exclude intervening mesenchyme. This exclusion of mesenchyme may in turn influence epithelial–mesenchymal interactions and lineage selection (see Fig. 3).

During this early developmental period, little cellular ultrastructural differentiation is seen, except that some endocrine granules become visible. Also during this early phase, as a result of both gut rotation and elongation of the dorsal and, especially, the ventral stalk (these stalks will give rise to the main pancreatic ducts), ventral and dorsal buds come into contact with one another within the forming C-loop of the duodenal anlage. This contact and then fusion of the buds occurs around E12 to 13 in the mouse, and E37 to 42 in humans. Coalescence of the two buds leads to fusion of the ventral bud duct with the distal portion of the dorsal bud duct. This fused duct runs the entire length of the pancreas, and is termed the duct of Wirsung. The proximal portion of the dorsal bud duct persists as the smaller (accessory) duct of Santorini. Thus, the future duct of Wirsung originates from both the ventral bud epithelium and the distal dorsal bud epithelium, whereas the future duct of Santorini originates only from the proximal portion of the dorsal bud epithelium.

At approximately E13 to 14 in the mouse, dramatic changes occur in the cellular architecture of the pancreas. Beyond the early, predominantly glucagon-positive endocrine cells, there is then a major amplification of endocrine cell numbers, particularly β-cells, during what has been termed the "secondary transition." Similarly, rapid branching morphogenesis and acinar cell differentiation occurs,

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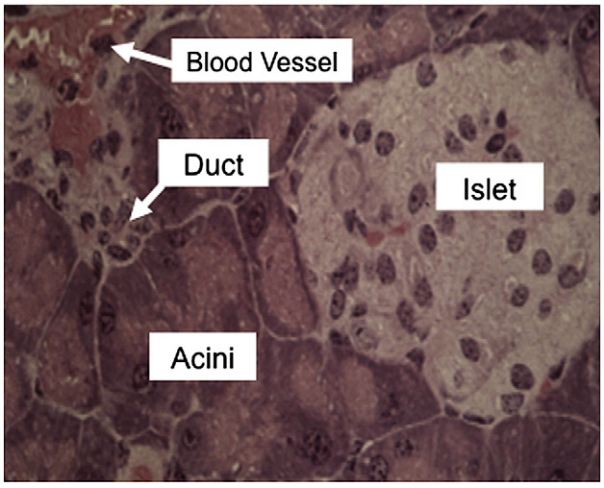

Fig. 1. The elegant architecture of the mature pancreas consists of the endocrine islets of Langerhans, the digestive enzyme-secreting acinar cells contained in clusters of acini, and the acinar-draining ducts with accompanying blood vessels and lymphatics. Other than the vessels, the pancreas has very little mesenchyme-derived tissue. Interestingly, the complex epithelium-derived structures are all formed from a single-cell thick sheet of foregut endoderm.

accompanied by exponential increases in acinar enzyme gene expression, development of large amounts of rough endoplasmic reticulum, and formation of zymogen granules. It is at this time that the pancreas becomes opaque to the naked eye due to the large amount of zymogen granules. Similarly, insulin levels undergo a 300,000-fold increase between E12 and 20 in the rat, compared with only a 1000-fold increase in the number of insulin-positive cells present (Clark and Rutter, 1972). Early in gestation, measured glucagon levels are 1000-fold higher than insulin, but relative glucagon levels diminish rapidly as the pancreas grows (Pictet and Rutter, 1972).

Pancreatic endocrine cells

Glucagon-containing α-granules are first seen at E9 in the mouse (Pictet et al., 1972). Insulin-containing β-granules are not typically seen until the secondary transition period. Morphologically, the earliest endocrine cells are integrated in amongst the exocrine/epithelial cells, and maintain contact with the lumen. Interestingly, the lumen at these early times can be difficult to appreciate, and only by careful topographical image analysis does it become clear that what seems to be a thick, multi-layered epithelium is actually a single sheet of cells with a highly convoluted, imbricated lumen. These epithelial cells maintain critical tight junction connections with adjacent epithelial cells and retain E-cadherin positivity. It is at this time that a key morphologic transition occurs. Endocrine cells, typically glucagon-positive, convert to non-epithelial cells and lose connection with the lumen and tight junctions. This conversion to non-epithelial location of endocrine cells has been postulated to entail a change in cell division polarity, from perpendicular to the basement membrane to parallel to the basement membrane (Pictet and Rutter, 1972). There appears also to be down regulation of pdx1 (a key marker of early pancreatic progenitor cells) in these endocrine progenitor cells as they become non-epithelial. This conversion process has been postulated to parallel epithelium-to-mesenchyme transformation.

Over the next several days (E14 to E18 in the mouse pancreas) the demarginating new endocrine cells begin to accumulate along the ducts and blood vessels in a cord-like linear pattern (see Fig. 4). Over the next few days, and then continuing after birth, these linear endocrine collections coalesce into aggregates that represent the first islets of Langerhans, which consist of insulin-producing β-cells, glucagon-producing α-cells, somatostatin-producing δ-cells, pancreatic polypeptide-producing PP cells, and ghrelin-producing ε-cells.

Early tissue interactions

The exceedingly complex repertoire of morphologic events that occur during pancreatic development, together with the similarly complex pattern of cellular differentiation and lineage selection, appear to all be mediated in great part by tissue interactions. Pancreas development has classically been described as an epithelial–mesenchymal process. However, prior to the presence of pancreatic mesenchyme other key tissue interactions are known to occur.

During gastrulation in chicken and zebrafish, FGF4 signals from the mesectoderm to the endoderm to induce the dorsal foregut to become competent to receive permissive pro-pancreatic notochord signals (Wells and Melton, 2000). In zebrafish, subsequent instructive gastrulation signals for the anterior foregut to become competent to receive pro-pancreatic notochord signals come in the form of retinoic acid and/or BMPs (Stafford and Prince, 2002; Tiso et al., 2002). Early after gastrulation, as the foregut endoderm curls up and closes ventrally, the dorsal aspect of the endoderm in the prepancreatic region is in contact with the notochord, and the ventral pancreas is in contact with the lateral plate mesoderm (Kumar et al., 2003).

Notochord

Once the gut tube is established, the development of the dorsal pancreas is then controlled by the notochord, through permissive interactions. In mice, the notochord is in contact with the dorsal prepancreatic endoderm from the time of notochord formation up until E8 (somite 13), at which time the paired dorsal aortas fuse in the midline to intervene between the notochord and the dorsal foregut (see Fig. 5). Kim et al., showed elegantly that notochord removal from early chicken embryos *in vitro* (9 somite stage, which is far in advance

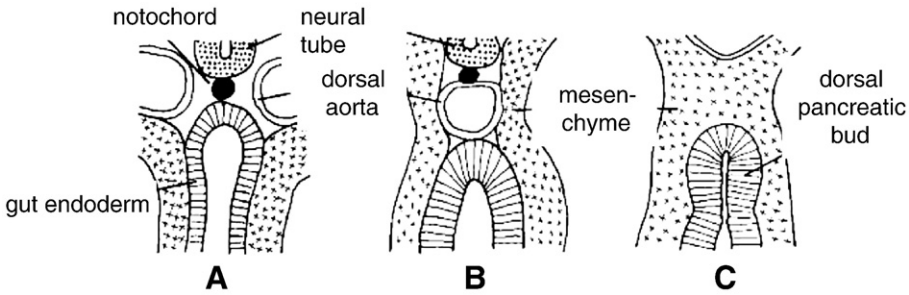

Fig. 2. (A) The close relationship between the dorsal aspect of the gut endoderm and the notochord at E8 is shown. (B) The two paired dorsal aortae have fused in the midline by E8.5–9.0 to intervene between the gut epithelium and notochord. (C) The mesenchyme has proliferated to create a distance between the dorsal epithelium and the dorsal aorta by E9.0–9.5 (with permission, Slack, 1995).

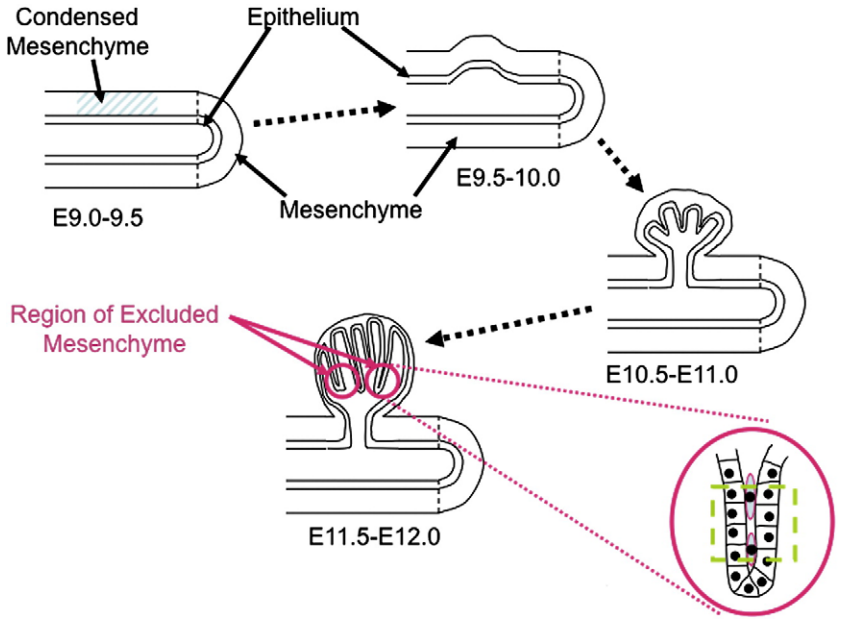

Fig. 3. Schematic representation of epithelial–mesenchymal interactions. Early, at E9–9.5, there is a condensation of mesenchyme that subsequently, within the next 12 to 24 h, leads to an evagination of the epithelium. This evagination is followed shortly by the onset of branching morphogenesis, but unlike most branching organs, the pancreas has a more acute angle to its branches (rather than the 90^0 branching seen in lung and kidney), resulting in subsequent exclusion of most of the mesenchyme from within the clefts of the branched epithelium (see insert). This relative exclusion of mesenchyme may predispose to endocrine differentiation, since the absence of contact with mesenchyme is thought to lead to endocrine differentiation (putative endocrine progenitor region shown in the green dotted-line box in the insert).

of the loss of contact between the notochord and the endoderm at 22 somites) prevented proper dorsal pancreas formation and inhibited expression of pancreas-specific genes (Kim et al., 1997). Neither the global endoderm marker HNF3β nor ventral pancreas development was affected. Interestingly, there were still *forme fruste* dorsal bud/branching events in the absence of notochord. In a subsequent study from the same laboratory, Hebrok et al., followed up on an incidental observation that sonic hedgehog (Shh, which they had used as a marker of notochord) was specifically absent in the prepancreatic endoderm (Hebrok et al., 1998). Grafting experiments with chicken endoderm showed that notochord proximity to the endoderm could suppress Shh expression. Similarly, deleting notochord in explanted chick embryo cultures resulted in Shh expression ectopically in the pancreatic region, with failure of pancreatic development (see Fig. 5). Also, implantation of an ectopic additional notochord could cause suppression of endodermal Shh, with ectopic induction of cell-shape changes similar to early pancreas formation. The role of hedgehog signaling in pancreatic development is discussed in detail the "Hedgehog signaling" section. Using a candidate approach of known notochord-produced morphogens, activin βB and FGF2 at physiologic concentrations were found to be able to replace the notochord effect. Based on the observation that exogenous Shh could override the pancreas-inducing effect of activin βB, and that the notochord makes Shh, it seems that Shh itself must be a key anti-pancreatic factor, rather than merely being a marker of non-pancreatic endoderm.

The ventral pancreas derives from ventral endoderm (which has no contact with the notochord) under the control of signals from the overlying cardiogenic mesenchyme. Pro-hepatic signals such as FGF's induce liver formation from the ventral endoderm at the expense of ventral pancreas formation. Absence of FGF's and cardiogenic mesenchyme, however, will lead to "default" differentiation into ventral pancreas (Deutsch et al., 2001b).

Endothelium

As mentioned above, the notochord loses contact with the dorsal pre-pancreatic endoderm due to fusion of the paired aortas in the midline in between notochord and endoderm. The endothelium has

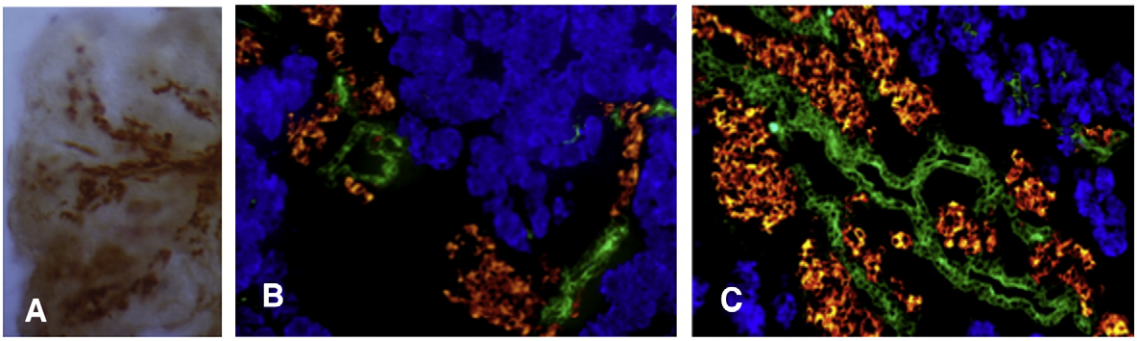

Fig. 4. Mid-gestation endocrine cells develop as periductal accumulations. (A) Whole-mount insulin-staining of E15 mouse pancreas showing the cord-like arrangement of the insulin cells (with permission, Slack, 1995). (B) Histologic sections with insulin in yellow, ducts in green, amylase in blue showing the cord-like distribution of the ducts and endocrine cells. (C) A TGF-β receptor II mutant mouse with amplification of these periductal cord-like structures is shown for emphasis (Tulachan et al., 2007).

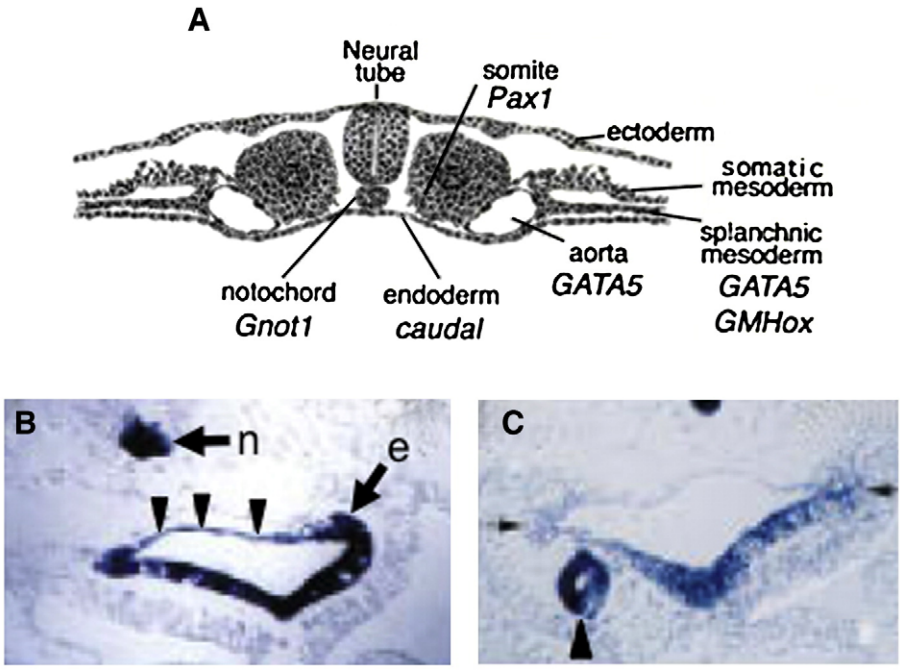

Fig. 5. The role of sonic hedgehog signaling between the notochord and the foregut. (A) The notochord is shown in proximity to the median part of the endoderm prior to the endoderm forming the gut tube. (B) As the gut tube forms, Sonic hedgehog is expressed throughout the gut epithelium (e), as noted here with *in situ* hybridization. The notochord (n), which also stains strongly for sonic hedgehog, is distant from the gut epithelium in this non-pancreatic region of the foregut, so sonic hedgehog is expressed in the foregut at this level (arrowheads). (C) An ectopic notochord (arrowhead) placed adjacent to non-pancreatic foregut epithelium leads to suppression of sonic hedgehog and subsequent ectopic pancreas formation (with permission, Hebrok et al., 1998).

been found to strongly influence pancreatic development. Lammert et al. showed an inductive role for the early aortic endothelium (Lammert et al., 2001) (see Fig. 6) in inducing pancreatic differentiation, and specifically endocrine differentiation, from the early endoderm. These effects of endothelium are described in detail in the "Blood vessel and endothelial-derived factors" section.

Mesenchyme

After this early dependence on the dorsal aorta, pancreatic epithelium then quickly becomes enveloped in pancreatic mesenchyme, thus separating the pancreatic epithelium from the dorsal aorta. This early enveloping dorsal mesenchyme is thought to harbor key

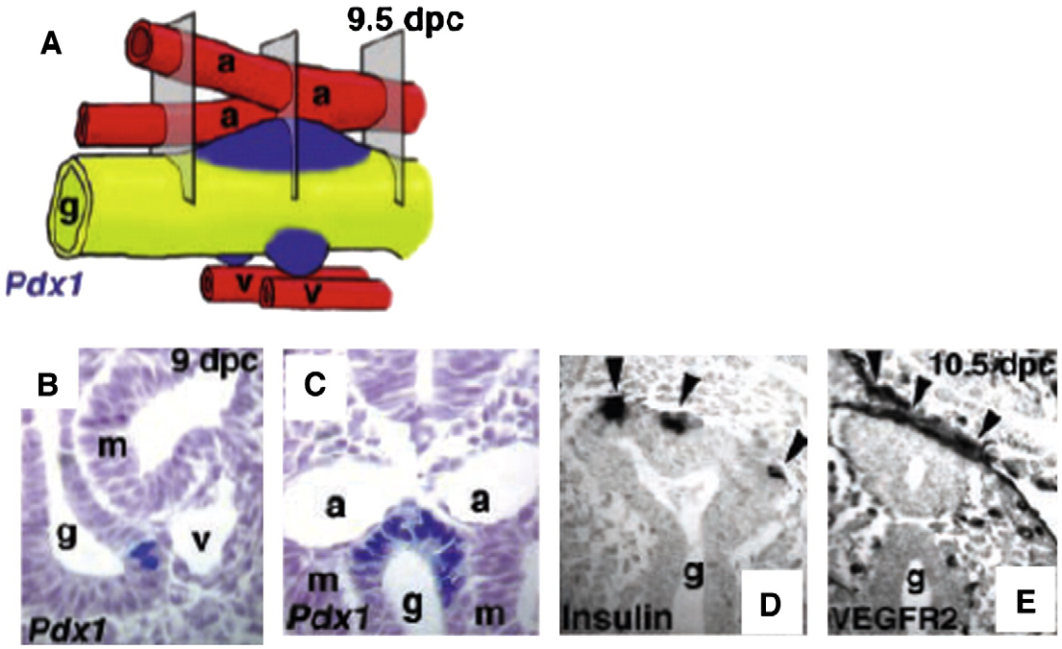

Fig. 6. Blood vessels are thought to be inductive of pancreatic differentiation. (A) Schematic for the induction of the dorsal (above) and ventral (below) pdx1-positive epithelial evagination from the endoderm. (B) The pdx1-positive cells (blue) can be seen ventrally in a cross-section of the gut in proximity to the vitelline vein (v). (C) Dorsally the pdx1-positive cells are in proximity to the dorsal aortae (a). In addition to pdx1 expression, insulin expression is shown by arrowheads in (D), and these insulin-positive cells are in proximity to the large blood vessel which stains positive for VEGF R2 in (E) (with permission, Lammert et al., 2001).

permissive and instructive signals for the generation of differentiated pancreatic cell types, and for proper pancreatic morphogenesis. Golosow and Grobstein reported the first definitive evidence that the early pancreatic epithelium is dependent on inductive influences from the mesenchyme (Golosow and Grobstein, 1962). They noted that mesenchyme was necessary for cytodifferentiation and morphogenesis of the pancreas, and that non-pancreatic sources of mesenchyme could replace the pancreatic mesenchymal effect. Interestingly, the specific source of the non-pancreatic mesenchyme could influence the type of pancreatic differentiation. Salivary mesenchyme, for example, induced almost exclusively acinar differentiation, with little or no endocrine differentiation. Many of these studies suggested that the mesenchymal effects were due to diffusible factors since the mesenchymal effects could occur across a filter barrier. These studies may have been confounded by the use of a transfilter-system where the filter pore sizes were large enough to admit small cellular processes (Kallman and Grobstein, 1964). A second confounding factor was that the cultured epithelia were embedded in clotted rooster plasma and chicken embryo extract, likely rich in active growth factors. Nevertheless, the basic observations have passed the test of time.

These early studies showing an inductive role for mesenchyme led to an extensive search for a presumed "mesenchymal factor" by Rutter and colleagues. Beyond the initial protein biochemical characterization (Ronzio and Rutter, 1973), and the finding that a mesenchymal factor was able to induce differentiation when in physical contact with the pancreatic epithelial cells (Levine et al., 1973), further pursuit of this "mesenchymal factor" seemed to reach a dead end. Heterologous tissue recombination experiments showed that pancreatic mesenchyme could induce endocrine cells to form from non-pancreatic embryonic foregut epithelial cells (Kramer et al., 1987) or from allantoic cells (Stein and Andrew, 1989). Mesenchyme was found to be critical for acinar development, and in the presence of basement membrane, in lieu of mesenchyme, ducts would develop (Gittes et al., 1996). Furthermore, the absence or depletion of mesenchyme revealed that there was a "default" differentiation of pancreatic epithelium toward islets (Gittes et al., 1996); (Miralles et al., 1998b). Further studies revealed that the age and location of the mesenchyme had a primary role in determining pancreatic epithelial fate (Rose et al., 1999) (see Fig. 7). Li et al. then showed that there were several separable components to the mesenchymal effect (Li et al., 2004). Proximity or contact of epithelial cells to mesenchyme led to exclusively acinar/exocrine differentiation. Absence of proximity/contact of mesenchyme with the pancreatic epithelium led to exclusively endocrine differentiation. Interestingly, the presence of mesenchyme, but in the absence of proximity or contact, led to enhanced insulin differentiation over glucagon or other non-insulin cell differentiation. Together these results suggested that there was a pro-exocrine factor in mesenchyme that was cell contact-mediated, and then an additional, diffusible pro-endocrine/pro-insulin factor was secreted by the mesenchyme.

Along similar lines, Raphael Scharfmann's group recently showed that proteoglycans (glypicans and syndecan) and the proteoglycan-producing enzyme heparan sulfate α-sulfotransferase were elevated in the E13.5 rat pancreas, and were localized to the epithelial–mesenchymal interface. Inhibition of the sulfation of these proteoglycans blocked mesenchyme-induced exocrine differentiation (Zertal-Zidani et al., 2007). Other studies from Scharfmann's laboratory have further delved into the multiple effects of mesenchyme on lineage selection. It appears that contact of mesenchyme with epithelium may both enhance notch signaling-induced hairy enhancer of split 1 (hes1) expression and inhibit neurogenin 3 expression (see below in the "Notch signaling" section) to suppress endocrine differentiation (Duvillie et al., 2006). They found a diffusible factor in mesenchyme that prevented maturation of β-cells from endocrine-committed progenitor cells. This latter result would seem to be at odds with the initial observations above by Rose and by Li. Interestingly, in the follow-up paper from Scharfmann's group, they found that the observed enhancement of endocrine differentiation in other studies was likely explained by the ability of mesenchyme to expand the pool of pdx1-

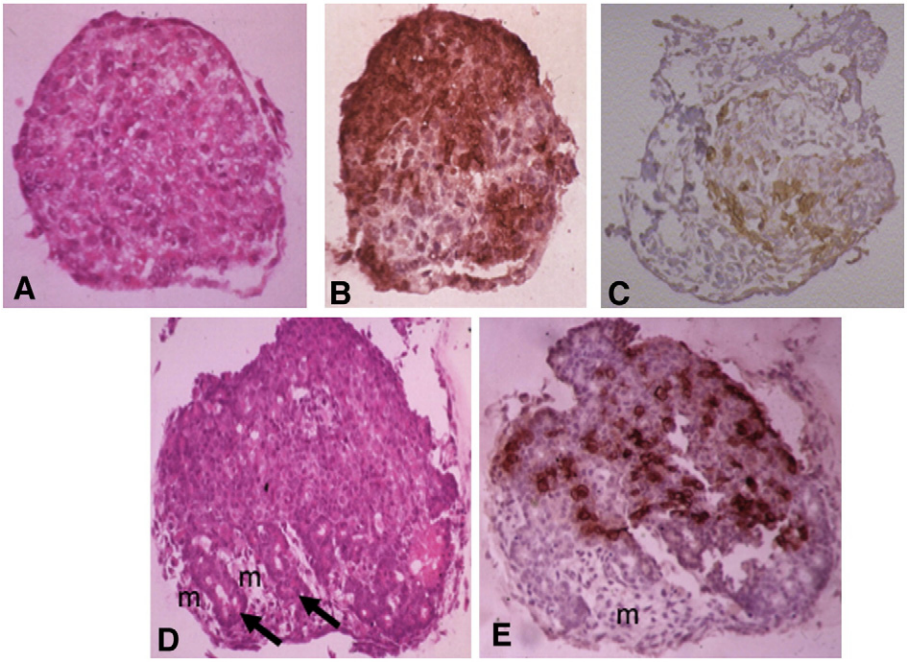

Fig. 7. The pancreatic mesenchyme is an important regulator of the differentiation of the pancreatic epithelium, and its effects depend on contact with the epithelium and on the age of the mesenchyme. (A) H&E staining of a co-culture of E10 mesenchyme with E11 epithelium, showing a lack of exocrine architecture. (B) Glucagon staining and (C) insulin staining of the sample in (A), here the younger mesenchyme induces a more immature epithelial phenotype with abundant glucagon and little insulin. In contrast, when older, E12 mesenchyme is recombined with the same E11 epithelium, then instead there is evidence of exocrine structures (arrows) in the region of contact between the epithelium and mesenchyme (m). In addition, in (E) there is an abundance of insulin-positive staining, suggesting a more mature endocrine phenotype of the epithelium, and interestingly there is endocrine development only in the region of the epithelium that is not in contact with the mesenchyme (m) (with permission, Rose et al., 1999).

positive progenitor cells, and therefore extend the period during which ngn3-positive cells could form from these progenitor cells (Attali et al., 2007).

A new and interesting role for pancreatic mesenchyme has recently been suggested by studies of bapx1, a member of the Nkx family of transcription factors. This factor is expressed in pancreatic mesenchyme, and bapx1 null mutant mice had failure of separation of pancreatic mesenchyme and spleen. The spleen is mesenchyme-derived and normally arises in immediate proximity to the pancreatic mesenchyme, possibly from the same early pool of mesenchymal cells. The failure of separation of pancreatic mesenchyme and spleen in bapx1 mutant mice interestingly led to formation of gut-like evaginations from the prepancreatic foregut. Similarly, splenic mesenchyme placed adjacent to prepancreatic foregut endoderm also induced gut structures. These results support an instructive role for pancreatic mesenchyme in diverting foregut epithelium away from the intestinal lineage, possibly mediated by PTF1a (PTF1a, described in the "PTF1a" section later, is a transcription factor marker of early pancreas-committed cells within the foregut) (Asayesh et al., 2006). In addition to specification, the bapx1-expressing mesenchyme, through secretion of FGF's, is instructive for the leftward lateral growth of the dorsal pancreatic bud that occurs beginning around E11–12 in mice (Hecksher-Sorensen et al., 2004).

Mesenchymal FGF

Beyond the initial biochemical characterization of a mesenchymal factor by Rutter and colleagues, more sophisticated molecular technology available in the last two decades has led to a greatly improved understanding of the molecules within the mesenchyme that may play an inductive role. Several families of signaling molecules have been implicated in regulating mesenchymal–epithelial interactions.

Fibroblast Growth Factors (FGF's), for example, are well-known to mediate multiple developmental processes, and have been shown to be expressed in many epithelial–mesenchymal interface regions, with a particularly important role in regulating branching morphogenesis (Reviewed in (Hogan, 1999)). They are a large family of ligands (greater than 20) that signal through multiple different tyrosine kinase FGF receptors. Scharfmann's group studied the role of FGF's in pancreatic mesenchyme-to-epithelium signaling in detail. They found that FGF's 1, 7, and 10 were expressed in the pancreatic mesenchyme, whereas FGF receptor 2B (FGFR2B), a specific receptor isoform that binds all three of those FGF ligands, was expressed in pancreatic epithelium. FGF ligand signaling to FGFR2B induces pancreatic epithelial proliferation, both *in vitro* and *in vivo*, but at the apparent expense of cellular differentiation (Elghazi et al., 2002; Celli et al., 1998; Miralles et al., 1999; LeBras et al., 1998; Le Bras et al., 1998). Similarly, FGF7 and 10 signaling has been implicated in mesenchyme-to-epithelium signaling in the developing human pancreas (Ye et al., 2005). Mesenchymal FGF signaling to the epithelium may also specifically favor exocrine differentiation. Scharfmann's group found that exogenous FGF's could enhance the presence of amylase-positive cells by 20-fold (here it is not clear whether recruitment of progenitor cells or proliferation of existing progenitor cells is responsible for the increase). A pro-acinar/exocrine role for FGF was also suggested by Dichmann et al., wherein a pdx1-FGF4 transgenic mouse (with FGF4 expressed in all early pancreatic epithelial cells) had cystic/ductal degeneration of the pancreas with persistent acini, but severe destruction of the endocrine tissue (Dichmann et al., 2003). Here, there also was an inappropriate proliferation of pancreatic mesenchyme, which could confound the interpretation of lineage effects since mesenchyme enhances exocrine differentiation and suppresses endocrine differentiation. Similarly, two studies showed that pdx1 promoter driving expression of FGF10 led to enhanced proliferation of undifferentiated pancreatic epithelial cells, but suppressed endocrine differentiation (Norgaard et al., 2003; Hart et al., 2003). Furthermore, Elghazi et al., added FGF7 to E13.5 pancreatic explant cultures and found enhanced epithelial growth with suppressed endocrine differentiation (Elghazi et al., 2002). This effect of exogenous FGF7 was thought to be indicative of an existing active endogenous FGF signaling pathway, which the authors felt was probably normally mediated endogenously by FGF10 rather than FGF7. In addition to the transgenic overexpression data above, Bhushan et al. showed that FGF10 null mutant mice had blunted branching pancreatic morphogenesis and a paucity of pancreatic endocrine cells, possibly due to inadequate proliferation of endocrine progenitors (Bhushan et al., 2001). Pulkkinin et al., showed that FGFR2B null mutant mice lacked the normal early proliferation and branching morphogenesis of the pancreas (Pulkkinen et al., 2003). However, unlike the FGF10 null mutants, the FGFR2B null mutant pancreas had normal subsequent exocrine and endocrine development despite the blunted earlier epithelial development. Thus, the role of FGF 10 in signaling through FGFR2b may be to induce proliferation of epithelial cells and to prevent endocrine differentiation.

The observed effects of FGF's are suggestive of a role for notch signaling (see "Notch signaling" section). FGF signaling early in development, likely mediated at least in part by notch signaling (Hart et al., 2003; Norgaard et al., 2003) and PTF1a expression (Jacquemin et al., 2006) is necessary to maintain a progenitor epithelial cell population. Miralles et al. further showed that notch signaling was a critical mediator of FGF10-induced embryonic pancreatic epithelial proliferation and suppression of differentiation (Miralles et al., 2006). Further differentiation of these expanded progenitor cells into exocrine or endocrine cells may be additionally modulated by FGF signaling. In FGFR3 null mutant mouse experiments, for example, different than the role of FGFR2B above, FGFR3 was found to be necessary for normal suppression of islet progenitor cell growth and post-natal islet growth, and FGF9 may be the key ligand here (Arnaud-Dabernat et al., 2007). More recent studies have suggested that FGF signaling may be important to specification of the pancreatic mesenchyme itself (Manfroid et al., 2007). Together, all of these studies reflect FGFs' role in dorsal mesenchyme signaling to dorsal pancreatic epithelium. However, FGF's seem to play an opposite role in ventral pancreas formation, since FGF's from the cardiogenic mesenchyme on the ventral side of the foregut actually inhibit ventral pancreas development in favor of liver development (Deutsch et al., 2001a).

TGF-β signaling

In addition to FGF, many other extracellular signaling pathways have been implicated in pancreatic development. The TGF-β superfamily represents a large signaling family with roles in nearly every biological process known, particularly developmental processes. The superfamily can be divided into four major categories: 1) TGF-β isoforms proper (including TGF-β 1, 2, and 3 in mammals); 2) activins; 3) BMPs; and 4) other types, including MIS and GDF. These molecules signal through a large family of typically heterodimeric receptors to activate smads and other intracellular pathways to initiate specific cell responses. The level of signaling modulation is achieved through various extracellular binding proteins that affect ligand-receptor interactions.

TGF-β isoforms

The mammalian TGF-β isoforms (TGF-β1, -β2, and -β3) are present in the embryonic pancreas at least as early as E12.5, localized to the epithelium. Over mouse gestation, the three TGF-β ligands, which all seem to co-localize throughout gestation, become progressively focused to acinar cells (Crisera et al., 1999, 2000b). A key receptor for these ligands, TGF-β receptor type II (TBR-II) localizes to the

epithelium and mesenchyme early (E12.5) in gestation, but then later is specifically only in the pancreatic ducts, with focal and intense staining of the ducts by E18.5. Some TBR-II was also seen in developing blood vessels at this time (Tulachan et al., 2007). TGF-β signaling to the epithelium early in pancreatic development seems to be important, but then later signaling to the ducts may be more important. This signaling occurs through cooperation between TBR-II and TGF-β receptor type I (TBR-I or Alk5). TBR-I showed a similar expression pattern to TBR-II in early pancreatic epithelium, and then later specifically in ducts. This parallel expression pattern suggests that TBR-II and TBR-I/Alk5 together mediate TGF-β isoform signaling in the developing pancreas (Tulachan et al., 2007).

A role for TGF-β signaling in pancreatic lineage selection was suggested by Sanvito et al., wherein exogenous TGF-β was thought to enhance endocrine differentiation in the early pancreatic epithelium (Sanvito et al., 1994). Here, prolonged organ explant cultures were used, exhibiting acinar autolysis, perhaps due to acinar zymogen maturation and release, with enzyme activation. Thus, the observed endocrine enhancement could have reflected sparing of the endocrine cells from autolysis. In an effort to better analyze a possible role for TGF-β's, two groups created transgenic mice expressing TGF-β1 under the rat insulin promoter (Sanvito et al., 1995; Lee et al., 1995b). In both cases the only potential developmental effect was islet disruption into smaller clusters, with no effect on overall islet mass. This disorganized islet phenotype is consistent with subsequent studies by Miralles et al., showing that TGF-β isoforms were important regulators of matrix metalloproteases that, in turn, appear to control the ability of endocrine progenitor cells to migrate and coalesce into islets in the late-gestation embryonic mouse pancreas (Miralles et al., 1998a).

Since TGF-β1/2/3 triple null mutant mice and the TBR-II null mutant mice are early embryonic lethal, a dominant-negative form of TBR-II was used to study inhibition of TGF-β isoform signaling. The adult mice expressing the dominant-negative receptor have ductal hyperplasia and acinar atypia (Bottinger et al., 1997). Despite the fact that the MT-1 promoter driving this dominant-negative receptor is expressed in all organs, the predominant phenotype in the adult animals is in the pancreas, suggesting that TGF-β isoform signaling is particularly important in the pancreas. More recent studies have shown that expression of the dominant-negative form of the TBR-II receptor in the embryonic pancreas results in an enhanced proliferation and accumulation of periductal endocrine cells at mid-to-late gestation (Tulachan et al., 2007). These data, together with the pattern of expression of TGF-β ligands and receptors described above, suggest that TGF-β signaling to ductal progenitors normally serves to restrict the recruitment of ductal or periductal cells into the endocrine lineage.

Activins and BMP's

Activin ligands. In addition to the TGF-β isoform sub-family signaling discussed above, activins and BMP's are two other key sub-families within the TGF-β superfamily. Activins and BMP's share many binding partners, receptors, and inhibitors. They participate in signaling in a large number of developmental processes. Activins have been found to be expressed in early gut endoderm (Verschueren et al., 1995; Manova et al., 1995), and are expressed in the early pancreatic rudiment. Activin A and B localize to the developing pancreatic endocrine cells, particularly in glucagon-positive cells (Furukawa et al., 1995; Maldonado et al., 2000). Exogenous activin added to growing embryonic pancreas explant cultures inhibited branching morphogenesis, a process associated with ductal and acinar differentiation (Ritvos et al., 1995). Inhibition of branching by activin is consistent with later studies by Miralles et al., in which follistatin, a known inhibitor of activins that is present in early pancreatic mesenchyme, was able to replace the pro-exocrine/anti-endocrine effect of mesenchyme (Miralles et al., 1998b). Demeterco et al., added exogenous activin A to cultured human fetal pancreas and found a modest increase in insulin content and insulin-positive cell numbers (Demeterco et al., 2000). These collective observations support a role for activin in the early pancreas to enhance endocrine differentiation and suppress exocrine/acinar differentiation. Slightly later in mouse development (E12.5), an unusual and unexplained effect of exogenous activin A was to induce pancreas explants to form intestine. This effect was mediated through increased Shh expression and was blocked by either the hedgehog inhibitor cyclopamine or by the activin inhibitor follistatin (van Eyll et al., 2004). It remains unknown whether follistatin has any effect on pancreas development independent of its ability to inhibit key endogenous TGF-β ligands (follistatin can bind and inhibit activins and BMP's, and perhaps other TGF-β superfamily members (Keah and Hearn, 2005)).

There have been some investigations into the mechanisms by which activin may induce insulin-positive differentiation. Activin can specifically decrease expression of Arx (a transcription factor critical for α-cell differentiation) and pre-proglucagon in a pancreatic duct-like progenitor cell line (AR42J cells), a glucagon-expressing α-cell line (α-TC cells), and in human islets (Mamin and Philippe, 2007). Also, activin seems to induce the neuroendocrine phenotype in AR42J cells directly through induced expression of neurogenin3 (ngn3), a key determinant of pancreatic endocrine lineage selection, growth, and differentiation (see "Neurogenin3 (ngn3) and its targets" section) (Zhang et al., 2001). This activin-induced ngn3 expression occurs through an activin A/HGF response element in the *ngn3* gene. This response element is not influenced by canonical activin signaling since overexpression of smad7, an inhibitor of canonical activin downstream signaling (smads 2 and 3), had no effect on ngn3 expression. Instead, ngn3 expression here was induced by the non-canonical target of activin, p38 MAPK (Ogihara et al., 2003). Earlier studies had suggested that activin A-induced smad2 was necessary for apoptosis and phenotypic changes in AR42J cells, so clarification of the exact downstream activin A mechanisms in these cell lines is still lacking (Zhang et al., 1999).

Activin receptors. Several studies have addressed the role of endogenous activin signaling in pancreas development. As mentioned above, the complexity of activin ligand and receptor signaling makes this area difficult to study. Loss-of-function activin studies using transgenic expression of a dominant-negative activin receptor II under either a global β-actin promoter or the β-cell-specific human insulin promoter, both led to islet hypoplasia (Shiozaki et al., 1999; Yamaoka et al., 1998). If, instead of a dominant-negative activin receptor, a constitutively-active activin RII was inserted under the same human insulin promoter, surprisingly a similar islet hypoplasia phenotype was seen. These results suggest that a specific window of activin receptor II signal dosing is necessary for proper islet development.

Since the dominant-negative activin receptor type II can bind many potential TGF-β superfamily ligands, a key subsequent study analyzed activin-receptor type IIA and/or activin receptor type IIB null mutant mice (Kim et al., 2000). Both receptors are expressed in the pancreatic anlage, but the activin receptor type IIA null mutant mouse had no pancreatic abnormalities (nor did the activin receptor type IIB heterozygous mutants). Activin receptor type IIB null mutants, especially if there was an additional activin receptor type IIA heterozygous mutation, were born with a small pancreas that tended to partially wrap around the intestine, perhaps as a *forme fruste* of annular pancreas. This annular pancreas phenotype may relate to altered Indian hedgehog signaling in these mice (Hebrok et al., 1998) (see "Hedgehog signaling" section). Interestingly, notochord-derived activin signaling inhibits sonic hedgehog expression in the pre-pancreatic endoderm (see "Notochord" section), and in these activin receptor type II mutants, inappropriate sonic hedgehog overexpression was seen.

Further studies of the activin receptor type IIB homozygous null mutants with the additional activin receptor type IIA heterozygous mutation (double-null mutants die prior to gastrulation) revealed mainly a reduction in endocrine cells, not exocrine cells at E13.5. Later in gestation, the islets were found to be hypoplastic. Thus, regardless of the ligand that may be involved, the activin receptor type II family seems to be important for pancreatic morphogenesis and specifically endocrine and islet development.

GDF 11. In an effort to demonstrate the key ligands in the developing pancreas that may normally induce the activin receptor type II-mediated effects, Growth Differentiation Factor 11 (GDF11, also called BMP11 and a TGF-β superfamily member that binds activin receptor type IIA and B) was identified as a possible ligand that may promote β-cell differentiation by endocrine progenitors. Two separate studies showed slightly different results with GDF11 null mutant mice (Harmon et al., 2004; Dichmann et al., 2006) (see Fig. 8). Harmon et al. showed that GDF11 is expressed in embryonic pancreatic epithelium diffusely from E11.5 to E13.5, and then becomes focused to acinar cells by E18.5. Here the GDF11 null mutant mouse developmental phenotype was thought to be similar to that of the activin receptor type IIB mutants described above, supporting GDF11 as the ligand acting through activin-receptor type IIB. The pancreas of GDF11 null mutant mice developed with more ngn3-positive endocrine progenitor cells, less mature insulin-positive β-cells, and more glucagon-positive α-cells. Thus, GDF11 may be a key TGF-β superfamily member regulating formation of the ngn3-positive cells, possibly by affecting HNF6 expression, a known regulator of ngn3 expression (Jacquemin et al., 2000). In addition, GDF11 may then promote ngn3-positive cells to form β-cells rather than α-cells, possibly through enhanced mafA expression in insulin-lineage-committed Nkx6.1-positive cells (see "Transcription factors" section) (Smart et al., 2006). GDF11 has been shown to work through the intracellular mediator smad2, and consistent with the phenotype of the GDF11 mutants, heterozygous smad2 mutants showed increased ngn3-positive cells with a subsequent decrease in β-cell mass. These authors made the important conclusion that smad2 is a critical GDF11/activin receptor type II intracellular target for β-cell recruitment.

Dichmann et al., found differing results in their GDF11 null mutant mouse, with an overall 43% reduction in the size of the late-gestation pancreas, entirely due to loss of acinar cells, with a much greater acinar loss than in the Harmon study. Numbers of ngn3-positive cells were still increased, though only doubled, and only late in gestation. There was no difference in the total number of mature endocrine cells. Additional experiments were performed to test the dependency of ngn3-positive cells on GDF11 and activin receptor type IIA and IIB signaling to form mature endocrine cells. These authors expressed a dominant-negative Alk-4 (a smad2 activator) in ngn3-expressing chicken endoderm cells, but again in contrast to the smad2 heterozygous mutant mice in the Harmon study, no effect on endocrine differentiation was seen. The reason for one study showing a major role for GDF11 in endocrine development and one not could be due to differences in genetic strain background, or perhaps due to the slight differences in the experimental approach.

Consistent with the finding that smad2 heterozygous mutant pancreases had a similar phenotype to GDF11 null mutant pancreases, and that GDF11 is known to signal through the activin receptors, which signal through smad2 intracellularly, Goto et al. found that activin receptor IIB signaling through smad2 was important in promoting endocrine development (Goto et al., 2007).

BMP signaling. Despite the extensive work implicating TGF-β superfamily signaling in pancreatic development, strikingly little direct analysis of BMP-specific pathways in pancreatic development has occurred. Some role for BMP signaling in pancreatic differentiation is suggested by a few *in vitro* studies. Exogenous BMP's 4, 5, and 6 are all able to induce dispersed E15.5 mouse pancreatic cells to form insulin-positive epithelial colonies when grown in the presence of laminin (Jiang et al., 2001). BMP's are known to be expressed in the developing pancreas (Dichmann et al., 2003; Jiang and Harrison, 2005; Goulley et al., 2007; Hogan, 1996). In AR42J cells, BMP signaling was found to be necessary for glucagon-like peptide 1-induced insulin-positive differentiation (Yew et al., 2005), and also BMP's promote proliferation of AR42J cells (Hua et al., 2006).

Results from transgenic mice are confusing with regard to roles for BMP signaling. BMP4 overexpression enhances duct-cell proliferation in a transgenic model of islet neogenesis (Hua et al., 2006). However, a pdx1-BMP6 transgenic mouse developed with complete pancreatic agenesis (Dichmann et al., 2003), a phenotype the authors attributed to an epiphenomenon of overinduction of intestinal smooth muscle in

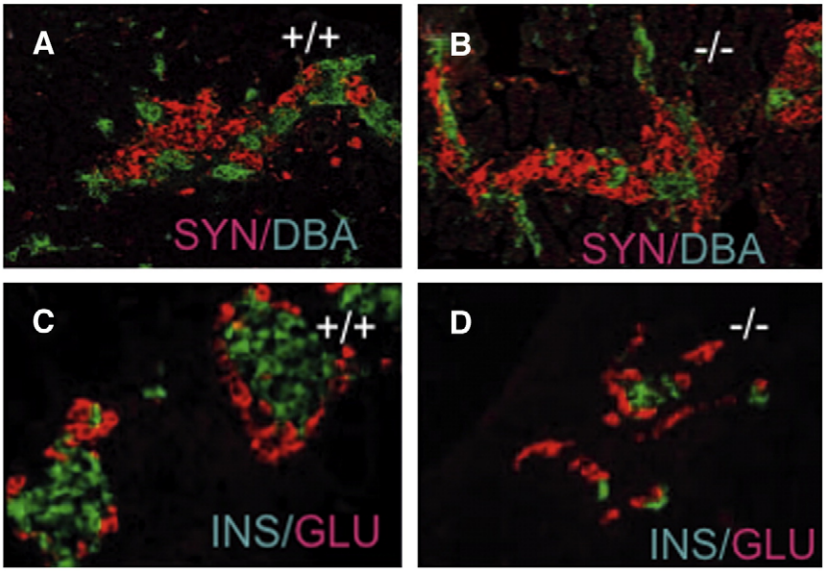

Fig. 8. GDF11 null mutant mice develop with an abundance of endocrine progenitor cells. (A, B) synaptophysin-positive (SYN) endocrine progenitor cells in greater abundance in the GDF11 null mutant (B) compared with littermate controls (A) (ductal staining here is identified by DBA). (C, D) the development of glucagon cells (red) is favored over insulin cells (green) in the mutant mice (D) compared with littermate controls (C) (with permission, Harmon et al., 2004).

the duodenal anlage, which disrupted pancreatic development. Conditional deletion of the BMP receptor 1A in insulin-positive cells did not have an effect on development, though did lead to altered glucose homeostasis (Goulley et al., 2007). Also, in contrast to the pdx1-BMP6 study, pdx1-BMP4 mice were born with a normal pancreas. Transgenically driven BMP inhibitors, noggin or smad6, under the pdx1 promoter had no embryonic pancreatic phenotype. Thus, clearly BMP signaling has a potential role in pancreatic development, but a more precise understanding of that role awaits further characterization.

Smads

As discussed above, smad molecules appear to play a role in pancreatic development. They are expressed in neonatal islets (Brorson et al., 2001), are necessary for both insulin-positive differentiation by AR42J cells (Zhang et al., 1999; Yew et al., 2005), and for proper regulation of the endocrine progenitor cell compartment *in vivo* (Harmon et al., 2004; Goto et al., 2007). There has been a general interest in the role of TGF-β superfamily members and smads in pancreatic development and differentiation because smad4 was specifically identified as mutated in 50% of pancreatic cancers (Hahn et al., 1996). Studies of smad4 function have not uncovered a key role in pancreatic development (Simeone et al., 2006; Bardeesy et al., 2006), though smad4 may play a role in maintaining normal differentiation of ductal or islet cells. Smad6 is a specific inhibitor of smads1, 5, and 8; the canonical downstream mediators of BMP signaling. Smad7 inhibits all smads including both BMP-induced smads (smads1, 5, and 8) and TGF-β isoform/activin-mediated smads (smads2 and 3). Interestingly, when smad7 was expressed under the pdx1 promoter in transgenic mice, there was a dramatic (85–90%) reduction in the number of β-cells present at birth (Smart et al., 2006). As mentioned above, a pdx1-smad6 transgenic mouse did not have a developmental phenotype. Thus, together these results suggest that smad2 and 3 may be the key smad mediators of β-cell differentiation. Additionally, the β-cell loss with smad7 expression seems to be replaced by the formation of glucagon cells, suggesting a possible role for smads2 and 3 in regulating the balance between the formation of β-cells and α-cells.

Notch signaling

Because of the well-established parallels between pancreatic endocrine development and neuronal development, the notch-signaling pathway, a regulator of neuronal differentiation in *Drosophila*, was studied in pancreatic development. Notch is a cell membrane-bound receptor that serves to maintain cells in an undifferentiated state when bound by notch ligands such as jagged, serrate, or delta-like molecules. A landmark paper from the Edlund laboratory demonstrated that notch signaling was a key mediator of fate decision in pancreatic development (Apelqvist et al., 1999) (see Fig. 9). Null mutant mice for either a key notch ligand present in the developing pancreas, delta-like 1, or for the transcription factor RBP-Jk (a target of notch signaling), both resulted in an accelerated and over-abundant commitment of the early embryonic pancreatic epithelium to the endocrine lineage, suggesting that notch signaling was necessary to prevent endocrine differentiation by these progenitor cells. This study also showed that forced overexpression of ngn3 (which normally is suppressed by notch signaling and is an important pancreatic pro-endocrine transcription factor, see "Neurogenin3 (ngn3) and its targets" section for more details), led to a similar "endocrine-only" phenotype. No clear "lateral inhibition" parallel has been established in the pancreas with respect to notch ligand/receptor signaling, as was demonstrated in *Drosophila*. Subsequent studies by Schwitzgebel et al. confirmed that pdx1-ngn3 forced expression led to the development of mainly glucagon cells, with very few insulin-

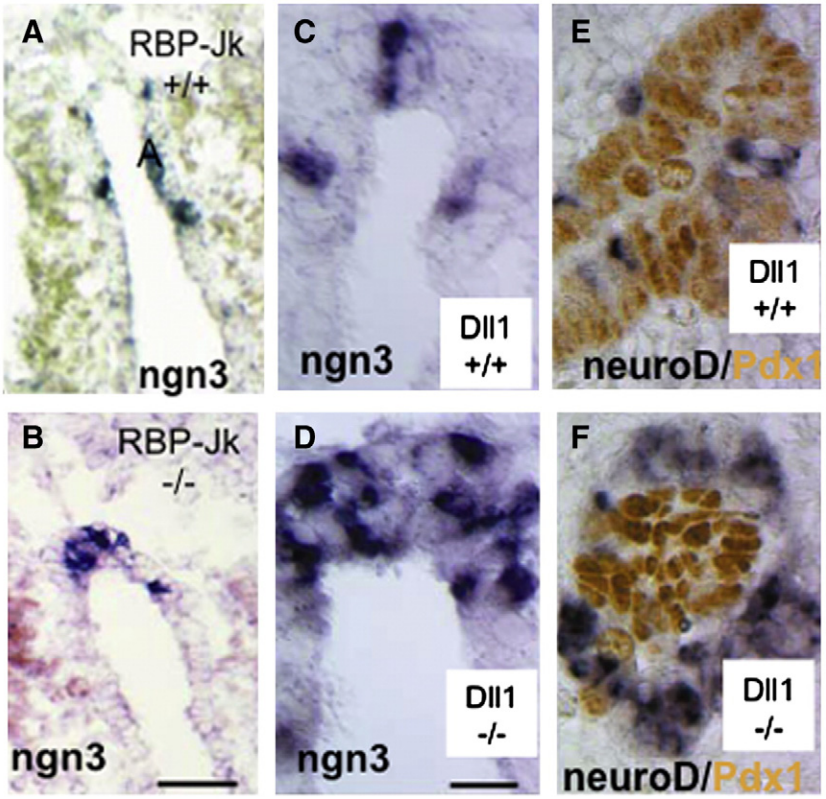

Fig. 9. Notch signaling activation leads to premature commitment to the endocrine lineage. (A, B) In RBP-Jκ mutants (B) ngn3 is expressed early and in more cells than in wild-type littermates (A). In delta-like 1 null mutants (D and F), there is premature and higher expression of ngn3 and neuroD in cells committed to the endocrine lineage, compared with wild-type littermates (C and E) (with permission, Apelqvist et al., 1999).

positive cells present (Schwitzgebel et al., 2000). Those results suggest that additional signals beyond simple notch-versus-ngn3 are necessary to determine insulin-cell development. Such additional pro-β-cell signals may rest with the recently reported pro-insulin effects of mesenchyme (Li et al., 2004; Duvillie et al., 2006; Attali et al., 2007), and perhaps with GDF11 signaling (see GDF11 section). Beyond the initial endocrine-versus-exocrine lineage decision, Notch signaling was found to also play a role in growth and branching of the undifferentiated pancreatic epithelium.

Further studies have confirmed a key role in pancreatic development for other members of the notch signaling pathway. Hairy enhancer of split 1 (hes1) is upregulated by notch and responsible for ngn3 suppression. Hes1 null mutant mice have severe pancreatic hypoplasia (Jensen et al., 2000c); this hypoplasia was not apoptotic, but was instead due to an inappropriate early commitment of precursor cells to becoming endocrine cells. Notch signaling was further implicated in pancreatic specification of endoderm, since the hepatic/biliary field and intestinal field of the endoderm had ectopic pancreas in notch signaling mutants (see below). Notch receptors and ligands are expressed in the developing pancreas well beyond the initial lineage selection period (Lammert et al., 2003; Yoshida et al., 2003). Acinar cell formation, which occurs through branching morphogenesis of the early epithelium followed by acinar cell differentiation, has been shown to be regulated by notch signaling (Hald et al., 2003). Overdriving notch signaling using transgenic expression of an active intracellular domain of notch under the pdx1 promoter led to the absence of significant numbers of mature endocrine or acinar cells (similar to the FGF overexpression mice described above), suggesting that active notch signaling, perhaps acting downstream of FGF's, may select for a progenitor epithelial cell population (Murtaugh et al., 2003). Importantly, using tamoxifen-regulatable promoters, delayed activation of notch signaling until E13 to E15 still led to the absence of acinar cells, with persistence of epithelial progenitor cells, implying that even if notch was induced in pdx1-positive epithelial cells later in gestation, perhaps even after the epithelial cells had initiated a differentiation program, those cells could still be reverted to progenitor-like cells (Murtaugh et al., 2003). The mechanism for this role of notch signaling in preventing acinar differentiation may be through inhibition of PTF1a, a transcription factor that can activate an acinar cell repertoire of genes, and that is associated with acinar differentiation (Esni et al., 2004). Ngn3-positive cells, which represent immature and still proliferative endocrine progenitor cells, could be reverted to a duct-like progenitor-cell phenotype (Murtaugh et al., 2003). However, driving notch in mature pancreatic endocrine cells could not revert them to a progenitor state (Murtaugh et al., 2003; Fujikura et al., 2006), so notch effects seem to require a relatively undifferentiated cell in order to induce effects. Along similar lines, regeneration of islet cells from pre-existing differentiated cells after partial pancreatectomy does not involve an ngn3-protein-positive state and, therefore, may represent a notch-independent way of forming new endocrine cells (Lee et al., 2006). Recent evidence suggests that post-pancreatectomy islets have high levels of ngn3 mRNA, but mRNA translation to protein is suppressed by micro RNA's (see "Micro RNA" section) (Joglekar et al., 2007).

The mechanism by which notch maintains the proliferation of a stem/progenitor pool in the pancreatic epithelium most likely involves mesenchymal FGF signaling. As discussed earlier, mesenchymal FGF's appear to play an important role in mediating mesenchyme-to-epithelium signaling. FGF signaling to promote expansion of an epithelial progenitor pool requires active notch signaling through Hes1 (Miralles et al., 2006; Norgaard et al., 2003). Furthermore, Hes1 normally downregulates p57, a key cyclin kinase inhibitor, to allow expansion of these pancreatic progenitor cells (Georgia et al., 2006).

The exact role of notch signaling in pancreatic lineage selection remains complex. Clearly, early notch signaling favors non-endocrine lineages over endocrine lineages, and is critically mediated by Hes1 inhibition of ngn3. The control point for notch receptor function may be enzymes that regulate sugar residues on the notch receptor. In zebrafish, the enzyme *manic fringe* can alter notch receptor function and drive premature ngn3-positive expression and endocrine differentiation (Xu et al., 2006). Thus, in order for a progenitor cell to become an ngn3-positive endocrine-committed progenitor, notch receptor modification may be necessary. Further zebrafish analyses showed that later in gestation notch signaling can affect lineage selection within the endocrine compartment. For example, deltaA mutants (a notch ligand) showed a shift away from α-cells and toward β-cells, and jagged 1B mutants had more α-cells, with no change in β-cell numbers (Zecchin et al., 2007).

One reason for the complexity of notch signaling in pancreatic development lies in the key downstream transcription factor, RBP-J, which mediates signaling by all of the notch receptors. Recently, another key pancreatic regulatory transcription factor, PTF1a, has been found to have complex interactions with RBP-J. PTF1a is part of a heterotrimeric transcriptional complex, PTF1 that was originally described as a ubiquitous acinar enzyme gene regulator (Petrucco et al., 1990). Subsequently, the basic helix–loop–helix moiety of PTF1, called PTF1a or p48 was found to be present early in embryonic pancreas. p48/PTF1a null mutant mice developed with no pancreatic bud, but had the fascinating phenotype of islets developing in the spleen, derived from prepancreatic endodermal cells that had migrated out of the epithelium and into the overlying pre-splenic mesoderm (see Fig. 12 and "PTF1a" section). These two separate roles for PTF1a, in the early embryonic pancreas and then later in gestation, corresponds with to two different functions of RBP-J. RBP-J has two paralogs, RBP-Jκ and RBP-Jl. Both molecules can bind PTF1a, but only the "κ" form can mediate notch signaling (Beres et al., 2006). PTF1a determines pancreatic development in the endoderm, and in Hes1 null mutant mice the resulting PTF1a overexpression induces pancreas formation in ectopic sites, such as intestine and liver (Sumazaki et al., 2004; Fukuda et al., 2006). These PTF1a-integrated functions of notch seem to require RBP-Jκ interaction with PTF1a (Masui et al., 2007). As pancreatic development progresses, and acinar cells begin to form, which is presumably dependent on PTF1a (Esni et al., 2004) and on notch repression, the PTF1a-RBP-Jκ complex begins to activate expression of the "l" form. RBP-Jl expression then becomes amplified, and competes for PTF1a binding with RBP-Jκ, resulting in a displacement of RBP-Jκ by RBP-Jl. Consistent with the fact that RBP-Jl is present in maturing acinar cells, RBP-Jl is not notch-dependent, and RBP-Jl is the paralog that participates with PTF1a in the PTF1 heterotrimer complex that regulates mature acinar enzyme gene expression. Thus, PTF1a and notch signaling create a complex interactive program to regulate pancreatic field commitment, endocrine-versus-endocrine lineage commitment, and acinar cell maturation and function.

Lastly, notch appears to play a potential positive role in duct formation (Greenwood et al., 2007). Such a role was suggested by the fact that notch mutants lack cells positive for duct markers (Lorent et al., 2004; Yee et al., 2005). Pax4 is a marker of committed endocrine cells downstream of ngn3. Driving notch activity through transgenic expression of the notch intracellular domain peptide in pax4-positive cell lineages led to more pancreatic ducts, representing the only clear positive data for a role for notch signaling in duct formation (Greenwood et al., 2007).

Hedgehog signaling

Like notch signaling, the hedgehog signaling pathway regulates differentiation in many developing tissues. The three hedgehog ligands, Sonic, Desert, and Indian, all bind to a patched (ptc) receptor. Ligand binding relieves ptc-induced repression of membrane-bound smoothened, which then in turn regulates the Gli family of transcription factors. Hedgehog signaling is controlled through: 1) the

regulated release of ligands from cells; 2) inhibiting extracellular binding of ligand by hedgehog interacting protein (Hhip) in a dominant-negative receptor fashion; and 3) sequestering ligand on the ptc receptor.

In the early embryo Sonic hedgehog (Shh) is expressed in almost the entire gut epithelium. However, Shh expression is inhibited in the pancreatic domain of the foregut as a prerequisite to pancreas formation. Nearby notochord has a Shh-suppressive effect on the endoderm in the region of the pancreas, and notochord co-cultured with endoderm rescued pancreas differentiation, as did Shh inhibitory antibodies (see "Notochord" section) (Hebrok et al., 1998). Similarly cyclopamine, which inhibits Shh signaling in target tissues at the level of the smoothened receptor, could induce heterotopic pancreas formation, presumably by expanding the pancreatic field in the gut, but only into pdx1-positive fields (stomach and duodenum). The notochord effect may be through the release of activins, which are Shh inhibitors (Hebrok et al., 1998). Such a role for activins would explain the expanded Shh expression and decreased pancreas formation seen in activin receptor mutants (Kim et al., 2000). Simple deletion of Shh does not actually lead to an expanded pancreatic field, but the relative size of the pancreatic field is increased since the size of the pancreas is preserved, despite the embryo being smaller (Hebrok et al., 2000).

While Shh inhibition by the notochord controls formation of the dorsal pancreatic field in the endoderm, the notochord does not control formation of the ventral pancreas. The inductive counterpart to the notochord for the ventral pancreas is the cardiogenic mesenchyme, which is known to regulate pancreatic development through FGFs (Deutsch et al., 2001a), which in turn regulate Shh expression. One of Shh's roles in the non-pancreatic endoderm is to induce the overlying mesenchyme (which expresses the ptc receptor) to undergo intestinal smooth muscle differentiation (Apelqvist et al., 1997). In the pancreatic field of the endoderm, however, where Shh is normally absent, the mesenchyme begins to involute by E15. This mesenchymal involution explains why, unlike the intestine, the mature pancreas has no overlying smooth muscle, and no external coverage at all.

The contradiction of a lack of an expanded pancreatic field in Shh null mutants, but the presence of an expanded pancreatic field (ectopic pancreas) in cyclopamine-treated (Shh-inhibited) endoderm could be explained by cyclopamine blocking other (non-Sonic) hedgehogs. Indian hedgehog (Ihh) and Desert hedgehog, and the receptor ptc are expressed in the foregut and pancreas (Hebrok et al., 2000; Thomas et al., 2000), and Ihh null mutants are born with a small pancreas (Hebrok et al., 2000), suggesting a direct pro-pancreatic role for Ihh. Further support for a role of Ihh, particularly in ventral pancreas formation, is that when a Shh null mutation is combined with a heterozygous Ihh mutation an annular pancreas develops (annular pancreas is a malformation in which the ventral pancreas-derived "head" of the pancreas encircles the duodenum).

Ptc null mutant embryos, which have constitutive hedgehog signaling due to the loss of ptc as a smoothened repressor, lack pdx1 and glucagon expression in the pancreas at E9.5, further supporting the need for Shh suppression to allow proper early pancreas formation (Hebrok et al., 2000). A similar hedgehog signaling overactivity is seen in Hhip mutants (Hhip is the decoy receptor that acts to inhibit hedgehog signaling). These mice develop with a smaller pancreas and fewer islets. Mice with a combined Hhip null mutation and a heterozygous ptc mutation, which would presumably lead to even higher levels of hedgehog signaling, had severe pancreatic hypoplasia (Kawahira et al., 2003). Little data currently exists regarding the potential role of hedgehog signaling in later gestation pancreas. Transgenic expression of Shh or Ihh under the pax4 promoter, with over-expression in the epithelium beginning at E12.5 (Smith et al., 2000), led to a dramatic loss of endocrine and exocrine tissue, but with a large amount of mesenchyme/stroma. These results suggest a role for hedgehog suppression in maintaining proper pancreatic development beyond early development (Kawahira et al., 2005). Later, in mature β-cells, which express ptc, Shh upregulates pdx1 expression and function and, therefore, insulin expression (Thomas et al., 2001b, 2000). The true complexity of hedgehog signaling and pancreatic development is further underscored by the fact that in zebrafish embryos, instead of inhibiting pancreatic development, hedgehog signaling is actually *necessary* for pancreatic endocrine cells to form (Roy et al., 2001; diIorio et al., 2002, 2007).

Retinoids

Retinoid signaling plays an important role in numerous developmental processes. Several studies have suggested a role for retinoid signaling in the developing pancreas. In support of a role for retinoid signaling in endocrine differentiation, retinoid binding proteins and retinoic acid receptors have been found in developing pancreatic islets and in insulinoma cell lines (Tulachan et al., 2003; Martin et al., 2005; Kato et al., 1985; Chertow et al., 1979, 1983; Kobayashi et al., 2002; Stafford et al., 2006), and exogenous retinoids are able to enhance the proportion of insulin-positive cells in isolated chick embryo endoderm (compared with the proportion of glucagon-positive cells). Beyond these effects of retinoids on endocrine cells, retinoids can induce the dorsal lip cells of *Xenopus* gastrulae to form endocrine and acinar pancreatic elements (Moriya et al., 2000b, 2000a). In the embryonic mouse pancreas, retinoids were found to induce endocrine and ductal differentiation (Tulachan et al., 2003; Shen et al., 2007), as well as influence the later decision between ductal and acinar/exocrine differentiation (Kobayashi et al., 2002). These latter effects appear to be mediated through epithelial–mesenchymal interactions.

Much of the recent work on the role of retinoid signaling in pancreatic development has come from zebrafish studies. RALDH2 is an enzyme that produces retinoic acid and is present in the developing pancreas, specifically in the mesenchyme (Tulachan et al., 2003; Martin et al., 2005; Molotkov et al., 2005; Stafford et al., 2006). In zebrafish, mesoderm-derived retinoic acid signals to the endoderm (Martin et al., 2005; Stafford et al., 2006) to induce pancreatic differentiation. Using RALDH2 mutant strains and injection of dominant-negative retinoic acid receptor constructs, endogenous retinoic acid signaling was found to be necessary for endocrine pancreatic formation in zebrafish, and for the formation of the entire pancreas in *Xenopus* and quail (Stafford et al., 2004; Chen et al., 2004; Kumar et al., 2003; Stafford and Prince, 2002). These effects on pancreatic lineages appear to be due to loss of pancreatic field specification within the endoderm. Conversely, exogenous retinoic acid could actually expand the pancreatic field (Stafford and Prince, 2002; Stafford et al., 2004; Chen et al., 2004). Beyond defining the pancreatic field within endoderm, mesenchymal retinoic acid in zebrafish specifically signals to endoderm to induce insulin-positive differentiation, and constitutive retinoic acid receptor activity in endoderm cells was instructive for insulin-positive differentiation (Stafford et al., 2006). Thus, a clear and important role for retinoid signaling has been established for multiple aspects of zebrafish pancreatic development.

These non-mammalian animal studies correlate fairly well with mouse data. RALDH2 null mutant mice lack a dorsal pancreas, but the role of RALDH2 production of retinoic acid in mammalian ventral pancreas is less clear (Martin et al., 2005; Molotkov et al., 2005). RALDH2 is present in both dorsal mesenchyme and lateral plate mesoderm, but by using a retinoic acid-responsive reporter to demonstrate cells with active retinoid signaling, most retinoic acid responding cells were found in the dorsal pancreatic bud (including some insulin and glucagon-positive cells), but not in ventral pancreas. Also, in contrast to the non-mammalian studies, a RALDH2 mutation may not affect the pancreatic field within the endoderm since Sonic hedgehog suppression still occurs properly (although little pdx1 and PTF1a was seen in this RALDH2 deficient prepancreatic endoderm).

RALDH2 is absent in the developing normal mouse pancreas after E12.5, suggesting a diminished role for retinoids in later stages of pancreatic development. Thus, while retinoid signaling clearly plays an important role in pancreatic development, the varied findings between different species and at different points in gestation underscore the likely complexity of the mechanisms by which retinoids act.

Epidermal growth factor (EGF) family

The EGF family of growth factors consists of at least 30 member-ligands that signal through at least 4 ErbB tyrosine kinase receptors. Two paired articles in 1990 showed that transgenic mice with diffuse overexpression of TGF-α, an EGF-family member, under a metallothionein promoter had severe pancreatic fibrosis (Jhappan et al., 1990; Sandgren et al., 1990). The overall complexity of EGF-family signaling has made understanding its role in pancreatic development difficult. Numerous studies of the spatiotemporal expression of EGF-family ligands and receptors have been performed. HB-EGF is a membrane-bound ligand that can be cleaved by metalloproteases into a diffusible active molecule. It is expressed in early embryonic pancreatic ducts and later in neonatal islets (Kaneto et al., 1997). HB-EGF was of interest since it was predominantly co-localized with pdx1, and its promoter elements were bound and activated by pdx1. However, a pdx1-HB-EGF transgenic mouse had minimal developmental changes, despite the use of a 4.6 kb pdx1 promoter element, which confers early embryonic pancreatic expression. These mice did have islets with glucagon-expressing cells dispersed throughout the islet instead of the normal peripheral glucagon-cell location (Means et al., 2003), suggesting a role for HB-EGF in islet architecture. Many other EGF ligands are present in the embryonic pancreas (Huotari et al., 2002).

Many studies have used exogenous EGF ligands to manipulate pancreatic development and to induce β-cell formation. Betacellulin is an EGF family member that was initially isolated from a β-cell tumor (Shing et al., 1993). At E11.5 it is expressed in the pancreatic epithelium (Thowfeequ et al., 2007). When betacellulin and activin A were added to AR42J cells 100% of the cells became insulin-positive (Mashima et al., 1996a). Betacellulin was also able to induce proliferation of undifferentiated epithelial cells in human fetal pancreas, and when used together with activin A, islet-like cell clusters formed (Demeterco et al., 2000). Similarly, in E11.5 mouse pancreas cultures, betacellulin was able to expand a pdx1-positive population of epithelial cells and induce insulin-positive differentiation at the expense of acinar differentiation (Thowfeequ et al., 2007). Thus betacellulin appears to be an important potential inducer of β-cell formation. This important insulinogenic effect is specifically mediated by the receptor ErbB1, since ErbB1 null mutant embryonic pancreas was resistant to any betacellulin-induced insulinogenic effect. However, a true endogenous role for betacellulin in pancreatic development remains elusive since betacellulin null mutant mice develop with a normal pancreas (Jackson et al., 2003), and betacellulin neutralizing antibody has no effect on insulin-positive differentiation in cultured pancreas (Huotari et al., 2002).

EGF itself is known to be an important regulator of many epithelial–mesenchymal interactions (Warburton et al., 1992), and early studies showed that exogenous EGF could induce duct development in cultured embryonic pancreas (Sanvito et al., 1994). Similarly, Scharfmann's group showed that exogenous EGF could enhance proliferation of undifferentiated epithelial cells. Interestingly, these expanded undifferentiated cells could subsequently be channeled to endocrine differentiation by removal of the EGF (Cras-Meneur et al., 2001). Whether these effects of EGF represent true endogenous mesenchyme-to-epithelium signaling remains to be determined.

EGF receptors

Beyond the complexity of the EGF ligands, most of the signals are then transduced by one of a family of four tyrosine kinase-containing ErbB receptors (ErbB1 through 4). The importance of ErbB1 (the original EGF receptor, which is expressed throughout the embryonic mouse pancreas) was shown in a null mutant mouse that had diminished development of endocrine cells, with an overall smaller pancreas, perhaps due to decreased branching morphogenesis (Miettinen et al., 2000). More recently, a transgenic mouse with pancreas specific (pdx1 promoter) expression of a dominant-negative form of the ErbB1 receptor was able to survive beyond the neonatal period, but had loss of post-natal β-cell proliferation, supporting an endogenous role for EGF receptors in not only prenatal, but also post-natal β-cell growth (Miettinen et al., 2000, 2006). In addition to ErbB1, ErbB4 is expressed throughout the pancreatic epithelium at E12.5, and then becomes restricted to ducts by E16.5 (Kritzik et al., 2000). ErbB4 was specifically important in δ-cell development since neutralizing antibodies against NRG4, an EGF family ligand that only binds ErbB4, specifically blocked δ-cell development (Huotari et al., 2002). Further analysis of ErbB2–4 function awaits conditional mutants, since all three null mutants are early embryonic lethal (Gassmann et al., 1995; Lee et al., 1995a; Erickson et al., 1997).

Hepatocyte growth factor (HGF)

Significant evidence has accumulated supporting an endogenous role for HGF in inducing β-cell formation. HGF and its receptor c-met were found to be expressed in E13 mouse pancreatic mesenchyme and epithelium, respectively (Sonnenberg et al., 1993). Also, human pancreatic fetal fibroblasts, which develop from early embryonic mesenchyme, express high levels of HGF, and c-met is expressed in developing human fetal β-cells. Interestingly, conditioned medium from human pancreatic fetal fibroblasts was able to induce β-cell proliferation and the formation of islet-like cell clusters. This effect was specifically blocked by HGF neutralizing antibodies (Otonkoski et al., 1994, 1996). Similarly, exogenous HGF had a pro-β-cell effect on human fetal pancreas in culture, more so than FGF or TGF-β (Otonkoski et al., 1994). HGF is similar to FGF in being an extracellular matrix-bound growth factor. Consistent with its interaction with extracellular matrix, HGF's pro-β-cell effect in cultured human pancreas was greatly enhanced by the presence by matrix molecules and by cell–cell contact (Beattie et al., 1996).

In an effort to harness the pro-β-cell effects of HGF *in vivo*, rat insulin promoter (RIP)-HGF transgenic mice were created, resulting in an increased number of islets with enhanced insulin content (Garcia-Ocana et al., 2000). Since β-cells express c-met, two groups created β-cell specific c-met ablation using RIP-cre with a floxed c-met. The islet size was reduced in one of the two strains, but no other major developmental effects on islets were seen (Dai et al., 2005; Roccisana et al., 2005).

Interestingly, a role for HGF signaling to c-met in the regulation of pancreatic endocrine differentiation was supported by observations in cell lines. HGF could prevent dexamethasone-induced acinar differentiation of AR42J cells and, when combined with activin A, HGF could induce insulin-positive differentiation (Mashima et al., 1996b). Similarly, using a pancreatic cell line resembling ducts (ARIP cells), HGF alone could stimulate insulin-positive differentiation after 72 h. Interestingly, these cells recapitulated the canonical pancreatic endocrine developmental pathway, with early (6-hour onset) expression of ngn3 and later (24 h) expression of neuroD (Anastasi et al., 2005). As evidence of the importance of HGF signaling to endocrine development *in vivo*, neonatal pancreas was dispersed and c-met-positive cells sorted using anti-c-met antibodies. These c-met positive cells behaved like multi-potent pancreatic progenitors, forming cell clusters that, when transplanted *in vivo* formed pancreatic exocrine and endocrine elements (Suzuki et al., 2004). Demonstrating a role for HGH/c-met in pancreatic differentiation will require the functional inactivation of c-met in pancreatic multipotent progenitors or in pancreatic endocrine progenitors using conditional knock-out approaches.

Wnt signaling

Wnts are a highly complex family of signaling molecules that have been shown to play a role in multiple aspects of pancreatic development. The Wnt ligands typically signal through the frizzled (frz) family of seven-pass transmembrane receptors, together with the co-receptor LRP 5/6 (lipoprotein-related peptide) to stabilize a key intracellular factor, β-catenin. Normally, in the absence of Wnt signaling, β-catenin is phosphorylated by a protein complex containing axin, APC, and Gsk3β, which leads to ubiquitination and destruction of β-catenin. β-catenin, independent of Wnt signaling, can stabilize cadherin interactions with cytoskeletal actin through the adaptor protein α-catenin. Wnt ligand signaling can be inhibited by the presence of dickkopf, a secreted frizzled-related peptide (sFrp) that can bind LRP's and inhibit their activity. Wnt signals are thought to mediate epithelial–mesenchymal interactions in many developing organs (Nusse and Varmus, 1992).

A very early role for Wnt signaling in pancreatic specification within the foregut was recently established in *Xenopus* (McLin et al., 2007). Absence of Wnt8 in the mesoderm prevented foregut formation by the endoderm. Ectopic Wnt signaling prevented the normal formation of foregut from the anterior endoderm, resulting in the absence of liver and pancreas. This Wnt-induced foregut inhibition appears to be mediated by β-catenin-induced vent2 expression, a homeodomain-containing transcription factor that represses Hhex, a key patterning gene for foregut development. Thus, in normal foregut development the loss of Wnt signaling leads to destruction of β-catenin, with resulting unrepressed Hhex expression and formation of liver and pancreas.

Beyond early endoderm patterning, the role of Wnt/β-catenin signaling in pancreatic development is complex and dependent on the time and place of Wnt signaling. A detailed analysis of Wnt expression patterns revealed that many Wnts, frz's, LRP5/6, and sFrp's are expressed in the developing pancreas (Heller et al., 2002). In general, Wnt ligands tend to be localized to the pancreatic mesenchyme during early pancreatic development, whereas frz's and sFRP's are in both the mesenchyme and the epithelium, with some of them localizing specifically to endocrine cells. Consistent with the role of Wnts in the foregut and pancreas specification discussed above, pdx1-Wnt1 and pdx1-Wnt5a transgenic mice had pancreatic agenesis and severe hypoplasia, respectively, confirming a specific role of Wnts in suppressing pancreatic development. Transgenic expression of wnts2, 4, 6, or 7a had no phenotype.

Beyond these early findings by Heller et al., numerous recent reports attempted to dissect the role of Wnt/β-catenin signaling in pancreatic development (see Fig. 10). Five separate studies have used various forms of the pdx1 promoter to conditionally regulate Wnt-β-catenin signaling in the pancreas. Murtaugh et al., showed that β-catenin ablation in pdx1-positive pancreatic progenitors specifically prevented exocrine development, and slightly reduced proliferation of pdx1-positive progenitor cells (Murtaugh et al., 2005). Using a similar strategy, Wells et al., showed a more severe reduction in the proliferation of pdx1-positive progenitors and a striking loss of exocrine/acinar tissue (Wells et al., 2007). In this study by Wells et al., the mice were born with essentially a complete absence of acinar tissue, but with a normal complement of fully functional islets. In both of these studies, the loss of exocrine/acinar tissue seemed to be due to loss of canonical Wnt signaling, rather than loss of the membrane stabilization

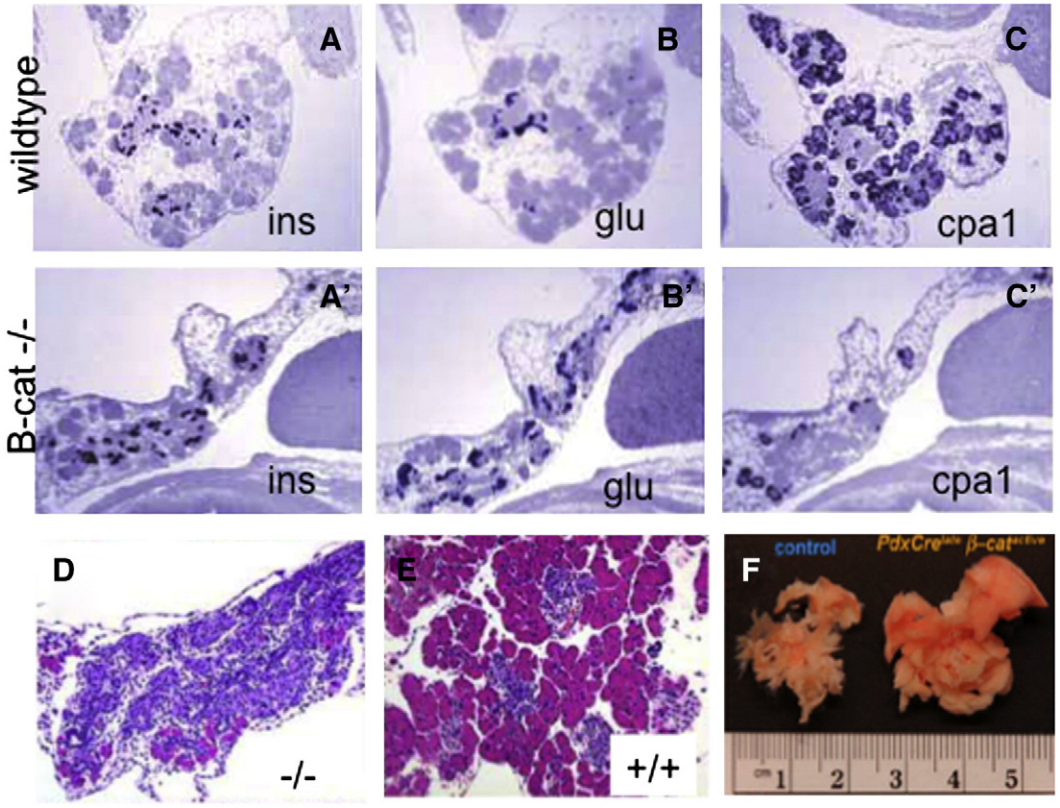

Fig. 10. Multiple studies have shown alterations in pancreatic development based on changes in Wnt signaling. In a pdx1-specific knock-out of β-catenin, Murtaugh et al., showed relative preservation of insulin and glucagon cells in the knock-out pancreas (A and B vs. A′ and B′), whereas there was a dramatic loss specifically of acinar cells (carboxypeptidase1-positive, cpa1) (C′ compared with C) (with permission, Murtaugh et al., 2005). Similarly, Wells et al., showed a dramatic loss of acinar tissue in pdx1-specific β-catenin knock-outs (D) compared with wild-type littermates (E) (with permission, Wells et al., 2007). Heiser et al., showed that hyperfunctioning β-catenin specifically in pdx1-positive cell progeny led to a massive 4.6-fold enlargement of the pancreas (F) (with permission, Heiser et al., 2006).

(cadherin–actin interaction) function of β-catenin. The general localization of Wnt's to the mesenchyme (Heller et al., 2002), together with the exocrine/acinar-specific effect of β-catenin ablation are in line with the known mesenchyme dependence of exocrine/acinar growth and differentiation. Some of these conditional β-catenin mutant mice still developed acinar tissue, but the acinar cells were all β-catenin-positive, suggesting a mosaic cre expression allowing growth of some acinar cells through "escape" β-catenin expression.

The complexity of pdx1 promoter-driven studies of Wnt signaling is best illustrated by studies from the Hebrok laboratory in which an early-expressing pdx1-cre ($pdx1^{early}$), a late-expressing pdx1-cre, and a tamoxifen-regulatable pdx1-cre were all used to conditionally express a constitutively active stabilized β-catenin (stabilized due to excision of the exon 3 phosphorylation site). Early production of the constitutively active β-catenin, in either $pdx1^{early}$-cre transgenics, or tamoxifen treatment of the pdx-cre-ER embryos at E11.5, led to near-total pancreatic hypoplasia due to overactive Wnt signaling (Heiser et al., 2006). These results are consistent with the above findings by Heller et al. with the pdx1-Wnt1a/5a transgenic animals in which premature Wnt signaling blocks pancreatic development. Hebrok's group further found an absence of FGF10 and high levels of Shh at E10.5 in the $pdx1^{early}$-cre promoter mice. This absent FGF10/high Shh could explain the pancreatic hypoplasia since FGF10 null mutant mice or pdx1-Shh transgenic mice both have pancreatic agenesis (Bhushan et al., 2001; Ahlgren et al., 1997).

While overactive Wnt signaling may give rise to pancreatic agenesis or hypoplasia affecting both endocrine and exocrine development (Heiser et al., 2006; Heller et al., 2002), Papadopoulou and Edlund found that a soluble frz protein, which broadly inhibits Wnt ligands, when expressed under a pdx1 promoter in transgenic mice, led to severely decreased numbers of proliferating epithelial progenitor cells, and eventual pancreatic hypoplasia affecting both exocrine and endocrine development (Papadopoulou and Edlund, 2005). These results suggest that some Wnt signaling may affect exocrine development, whereas other Wnt signaling may affect endocrine growth and development. With either the "late"-expressing pdx1-cre, or else late tamoxifen treatment of tamoxifen-inducible pdx1-cre-ER embryos, no effect on embryonic pancreatic development was seen.

A fifth study, by Dessimoz et al. yielded results that did not agree with the above four studies (Dessimoz et al., 2005). Using a pdx1-cre promoter to conditionally inactivate β-catenin, instead there were no acinar abnormalities, and only a mild suppression of endocrine cell number, with a 60% decrease in the number of islets. The lack of an acinar phenotype here may be due to a mosaic expression of pdx1-cre since all of the acinar tissue appeared to have escaped deletion and was thus β-catenin-positive. The endocrine differences in this study are harder to understand, but could be due to the fact that the β-catenin conditional mutant mice had one β-catenin null mutant allele, so that the effectiveness of β-catenin deletion in individual cells may have been more effective.

A role for Wnt signaling in later-gestation endocrine development has also been found. In zebrafish, either frz2 or Wnt5 inhibition with morpholinos led to failed migration of insulin-positive cells and improper formation of islets (Kim et al., 2005). This phenotype was non-cell autonomous, likely related to migration guidance, since frz2 was only expressed in cells adjacent to insulin-positive cells. Wnt5a null mutant zebrafish had grossly normal pancreases, but had disrupted islet architecture, suggesting a role specifically in migration and islet aggregation.

Lastly, there appears to be a role for Wnt signaling in promoting post-natal pancreatic growth. Late pdx1-cre-induced β-catenin stabilization led to a striking 4.6-fold increase in pancreatic size in adult mice (Heiser et al., 2006) (see Fig. 10). When a similar conditional approach was used to stabilize β-catenin in insulin-expressing cells, only large islets and insulin hypersecretion were seen, confirming an overall growth promoting effect of β-catenin on multiple pancreatic cell types. Along similar lines, conditional overexpression of axin, which promotes β-catenin destruction, led to small islets with less insulin-positive cells (Rulifson et al., 2007).

Thus, these studies of Wnt signaling reflect multiple important roles for Wnts, particularly the canonical Wnt-β-catenin pathways, in all aspects of pancreatic development. The complex and varied nature of the different reports is reflective of the extreme complexity of Wnt signaling, and further studies will likely continue to delineate the exact mechanisms involved.

Blood vessels and endothelial-derived factors

Recently there has been increased interest in the role of endothelial cells and possibly blood flow in pancreatic development, especially endocrine cells. Teleologically, the known dependence of endocrine cells on an intimate relationship with capillaries for proper function would predict a carefully orchestrated, interdependent developmental process between endothelial cells and endocrine cells. At E8 the dorsal aortae fuse between the notochord and the dorsal foregut. Here, Lammert et al., used *in vitro* tissue interaction studies to show that the aortic endothelial cells could induce pancreatic bud-like structures and pdx1 expression in the adjacent endoderm, as well as subsequent insulin expression (Lammert et al., 2001) (see Fig. 6), and insulin-positive cell proximity to endothelium was observed *in vivo* in normal development. Removal of the dorsal aorta from *Xenopus* embryos led to the absence of pancreatic endocrine development. Interestingly, transgenic ectopic overexpression of VEGF-A under a pdx1 promoter led to more vessels and more islets (in exchange for much less acinar tissue), and ectopic insulin-positive cells in the stomach (Lammert et al., 2001). These ectopic islets suggest a threshold effect of blood vessel signaling since endothelium is normally present in the stomach, albeit at a lower level than in the VEGF overexpression system, but islets do not normally form in the stomach. Thus, endothelial cells appear to be a key instructive regulator of pancreatic endocrine commitment and differentiation. In a follow-up study, Lammert et al., showed that mice engineered to have VEGF-A deleted specifically from pdx1-expressing cells specifically lack islet capillaries (Lammert et al., 2003). A more recent study by Yoshitomi and Zaret showed that endothelial cells are necessary for PTF1a expression specifically in the dorsal foregut, and for evagination of the dorsal foregut. Interestingly, in contrast to the dorsal pancreas, even though the ventral foregut and ventral pancreatic bud evagination are in proximity to the vitelline veins, ventral pancreas formation is not dependent on endothelium (Yoshitomi and Zaret, 2004).

Beyond the role of endothelial cells, it appears that factors specifically carried in the bloodstream may have some key regulatory roles in pancreatic development. N-cadherin null mutant animals were found to develop with absence of the dorsal mesenchyme and no dorsal pancreas (Esni et al., 2001). However, a subsequent study found that if cardiac function was rescued in these null mutant animals (the global mutant animals' heart fails to form properly) through specific transgenic expression of N-cadherin only in the heart, then pancreas formation was also rescued, presumably due to maintaining blood flow to the pancreatic anlage (Edsbagge et al., 2005). Here, sphingosine-1-phosphate was found to be a blood-borne agent that acts through the S1P receptor to recruit and induce proliferation of pancreatic mesenchymal and endothelial cells. They proposed that immature embryonic vessels are leaky, and that S1P leaks out of the aorta from the embryonic blood to then attract dorsal mesenchyme to grow and intervene between the notochord and the dorsal foregut endoderm. A recent study suggested that these results could reflect a role for blood flow in affecting endothelial cells in their inductive capacity (Jacquemin et al., 2006). VEGF receptor null mutant mouse embryos lacked early pancreatic dorsal mesenchyme due to the absence of stimuli from the dorsal aorta. This study showed that

beyond the early direct influence of the dorsal aorta on the endoderm, a later influence of dorsal aorta on the intervening dorsal pancreatic mesenchyme exists, with dorsal aorta inducing FGF10 expression in the dorsal pancreatic mesenchyme.

Later in development, as endocrine cells start to form, there is a carefully orchestrated interplay between endocrine cells and endothelial cells. Early E13.5 endocrine cells express angiogenic VEGF-A and angiopoietin, and are in immediate proximity to developing capillaries (Brissova et al., 2006). By E16.5 there is blood flowing to developing islet cells prior to their assembly into mature islets. Interestingly, the developing endocrine cells do not form their own basement membrane, and thus are dependent on endothelial cells to make the basement membrane for them (Nikolova et al., 2006). Furthermore, endocrine cells are stimulated by basement membrane-derived laminin, bound to β1-integrins on the endocrine cells, to proliferate and to increase insulin synthesis. Islet cell-derived VEGF-A appears to be necessary for proper formation of endothelial fenestrations, and therefore for proper glucose sensing (Brissova et al., 2006). Thus, throughout development there is a complex and ever-changing relationship between foregut endoderm, endocrine cells, endothelium, mesenchyme, and blood flow.

Glucagon-family (and other peptide hormones) signaling

The glucagon family of peptide hormones includes the glucagon-like peptide 1 (GLP-1) and GLP-2, and also includes glucose-dependent insulinotrophic peptide (GIP), secretin, VIP, and others. While the role of these peptides has generally been well-studied in endocrine physiology, roles in pancreatic development are only recently becoming apparent.

Glucagon

As early as 1973, cells in the early rat pancreas at the time of evagination from the foregut were found to have α-granules containing glucagon (Rall et al., 1973). Since the known metabolic functions of glucagon are not yet relevant to the embryo's overall physiology at that early embryonic stage, it seemed plausible that glucagon may have a specific developmental role. Several lines of evidence suggest that glucagon signaling is necessary for the early differentiation of insulin-expressing cells. First, *in vitro* studies of cultured pancreas show that glucagon is necessary for early formation of insulin cells (E11–E13), but not later in the E15 pancreas (Prasadan et al., 2002). Glucagon is specifically generated from pro-glucagon by the action of pro-hormone convertase2 (PC2), whereas PC1/3 acts on pro-glucagon to generate GLP-1 and other products. PC2 null mutant animals, which lack glucagon showed a similar loss of early insulin-expressing cells, with retention of the secondary wave of insulin-expressing cells (Vincent et al., 2003). PC2 is also responsible for generating other peptide hormones, but a specific role for glucagon itself was suggested when a glucagon receptor null mutant mouse was found to have a similar lack of early insulin-expressing cells (Vuguin et al., 2006). These studies together strongly implicate glucagon signaling, through the glucagon receptor, in the initiation of early insulin-positive differentiation. In *in vitro* studies, exogenous GLP-1 analogue (exendin-4) was able to rescue this early insulin differentiation in the absence of glucagon, though the signaling mechanism was not certain, and specific inhibition of GLP-1 signaling in the early pancreas had no effect on early insulin-positive differentiation (Prasadan et al., 2002).

GLP-1

A role for GLP-1 in insulin-positive differentiation has been aggressively pursued since GLP-1 is known to promote insulin synthesis and secretion in β cells, as well as promoting β-cell growth (Stoffers et al., 2000; Buteau et al., 1999, 2001). Also, the GLP-1 analogue exendin-4 can convert AR42J cells (Zhou et al., 1999; Yew et al., 2004) and ARIP cells (Hui et al., 2001) into insulin-expressing cells. A developmental role for GLP-1 is suggested, but not yet clear. GLP-1 receptor null mutant mice do not have an embryonic phenotype (Scrocchi et al., 1996). GLP-1 receptor is expressed at only low levels in the early embryonic pancreas, but then upregulates coincident with the secondary transition at E15 (Prasadan et al., 2002). GLP-1 exists in at least four forms, and the receptor signaling pathways for those different forms is not fully understood. Although mature α-cells have PC2 and make glucagon, they do not have PC1/3 and therefore do not make GLP-1. However, Wilson et al. found that immature glucagon-positive cells in the embryonic pancreas do in fact have PC1/3, and therefore presumably make GLP-1 (Wilson et al., 2002). Suzuki et al., showed that a less well-studied form of GLP-1 (GLP-1-37), which unlike other GLP-1 forms is present in α-cells, could stimulate formation of insulin/glucagon double-positive cells in the epithelium of the embryonic pancreas or in ducts (Suzuki et al., 2003). As will be discussed in the "Overview of endocrine pancreas lineage selection" section, the early insulin/glucagon double-positive cells may represent the first wave of endocrine cells during early pancreas development. Less well-studied forms of GLP-1 were strongly upregulated in glucagon-receptor null mutant mice, and compensation by these other forms may explain the relatively weak embryonic phenotype in those mice (Gelling et al., 2003; Parker et al., 2002). Further support for a possible compensation by GLP-1 comes from a streptozotocin model of β-cell regeneration, wherein α-cells produced more GLP-1 during β-cell regeneration, and GLP-1 receptor inhibition blunted this β-cell regeneration (Thyssen et al., 2006).

Besides GLP-1, the other incretin molecule is GIP, which actually makes up 80% of the normal "incretin effect" (Gault et al., 2003) ("incretin effect" is defined as enhanced insulin secretion after an intestine-derived glucose load). GIP has also been implicated in β-cell development. GIP receptor, like the glucagon receptor and GLP-1 receptor, is expressed on β-cells (Huypens et al., 2000). GIP signaling leads to altered expression of important pancreatic endocrine transcription factors, including GATA4, Isl1, and pdx1 (Jepeal et al., 2005), and a GIP analogue is able to enhance insulin-positive differentiation in embryonic stem cells (Marenah et al., 2006). However, GIP receptor null mutant mice and GLP-1 receptor/GIP receptor double null mutant mice have normal pancreatic development (Miyawaki et al., 1999; Hansotia et al., 2004), so the exact role of GIP in pancreatic development remains unclear.

Beyond the glucagon family, other peptide hormones have been studied for a possible direct signaling role in pancreatic development. Mice with a double mutation for insulin I and II have large islets, but have no developmental defects (Duvillie et al., 1997, 2002). Maternal insulin is unlikely to be rescuing development in these insulin-knockout animals since insulin does not readily cross the placenta (Widness et al., 1983). The pancreatic polypeptide family of peptides (PP, PYY, and NPY) has been studied mainly for a possible role as a marker of progenitor cells (see "Overview of endocrine pancreas lineage selection" section), but no specific signaling role in development has been found other than localization of a key PYY receptor, Y1, to non-endocrine pancreatic cells in the vicinity of potential early endocrine progenitor PYY-positive cells (Jackerott and Larsson, 1997).

Extracellular matrix and cell adhesion molecules

Beyond the clearly established role of mesenchyme in the induction of exocrine and endocrine pancreatic development, the extracellular matrix molecules in the mesenchyme, especially in the basement membrane, play multiple important roles. The pancreatic epithelium is contained within a continuous sheath of basement membrane that creates the epithelial–mesenchymal interface (Hisaoka et al., 1993). There are microscopic breaks in this sheath in the region where early endocrine cells are forming. In other organs, branching morphogenesis is tightly regulated by basement membrane

components. Fibronectin, laminin-1 and collagen-IV have all been localized to the basement membrane at the pancreatic epithelial–mesenchymal interface (Hisaoka et al., 1993) at early gestational times in rat embryos. Laminin-1 has been shown to play a role early in gestation where it is present in the mesenchyme, then becomes focused to the epithelial–mesenchymal interface and then, through interactions with epithelial α_6-containing integrins, mediates pancreatic duct formation (Crisera et al., 2000a). Matrigel, whose major constituent is laminin-1, was found to induce duct formation in isolated E11 mouse pancreatic epithelium (Gittes et al., 1996). Later, Li et al., showed that laminin-1 mediates the pro-exocrine effects of mesenchyme (Li et al., 2004). Laminin-1 has been shown to have a pro-β-cell role slightly later in gestation. Specifically, laminin-1 as a substrate enhanced β-cell differentiation in dispersed E13 pancreatic epithelial cells, through binding to α-dystroglycan (Jiang et al., 1999, 2001). Lastly netrin-1, a diffusible laminin-like molecule that is known to regulate neuron and axon guidance (De Breuck et al., 2003) is present adjacent to E15–E18 pancreatic exocrine and endocrine cells, along with the netrin receptor neogenin (Fitzgerald et al., 2006). The other netrin receptor, DCC, may serve a function later in development to guide neural crest migration into the pancreas to form pancreatic ganglia (Jiang et al., 2003). Cirulli's group reported that netrin is localized to epithelial cells at their basal surface and binds $\alpha_6\beta_4$ and $\alpha_3\beta_1$ integrin during pancreatic epithelial adhesion and migration (Yebra et al., 2003). A more general role for integrin–matrix interactions in the developing human pancreas was shown by Cirulli et al. wherein epithelial $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrins interacted with fibronectin, collagen-IV, and especially vitronectin to both maintain extracellular matrix anchorage ($\alpha_v\beta_5$) and to allow epithelial migration ($\alpha_v\beta_3$). Here, the expression pattern of these three matrix components was suggestive of their role in the migration of endocrine progenitor cells out of ducts to form islets. Further support for this role of matrix guidance of migration comes from human fetal explants that failed to form proper islets when treated with cyclic RGD inhibitors of the $\alpha_v\beta_3/_5$ integrins (Cirulli et al., 2000).

In addition to interactions with the extracellular matrix, cell–cell adhesion also appears to be very important in pancreatic development. Cadherins are calcium-dependent cell membrane-bound molecules that mediate cell–cell adhesion and the sorting of different cell populations. As described in the Wnt signaling section above, cadherin intracellular carboxyl tails interact with catenins to help form tight junctions between epithelial cells. E-cadherin and R-cadherin are localized to ducts, and then are down-regulated as cells move out of the ducts to start to form islets (Dahl et al., 1996; Sjodin et al., 1995). N-cadherin showed a different pattern than R- or E-cadherin, localizing to mesenchyme but not epithelium in the E9.5 pancreas. After E9.5, N-cadherin then became localized to the endoderm, and by E12.5 was only in the islets (Esni et al., 2001). As discussed earlier, the absence of N-cadherin in the pancreas has no effect on pancreatic development. However, forced dominant-negative E-cadherin expression in β-cells, which inhibits both E-cadherin and N-cadherin signaling, led to a specific loss of embryonic β-cell aggregation, with islets consisting only of α-cells (Dahl et al., 1996). Thus, collectively the cadherins appear to play important roles in migration and differentiation of pancreatic endocrine progenitor cells.

Another cell adhesion molecule, N-CAM, which is expressed in mature α- and PP-cells (Cirulli et al., 1994), is localized to epithelium and mesenchyme early in pancreatic development and, similar to N-cadherin, it becomes restricted to endocrine cells by E11.5 (Esni et al., 1999). N-CAM null mutant embryos had improperly aggregated endocrine cells within the islet.

A unique role was found for another matrix molecule, Ep-CAM in human fetal pancreas, where interestingly Ep-CAM was upregulated specifically in endocrine cells as they began to migrate out of the early embryonic ducts, but then was downregulated as the islet cells matured (Cirulli et al., 1998). Further support for a role for Ep-CAM in maintaining a ductal endocrine progenitor was found when inhibition of Ep-CAM *in vitro* with antibodies induced endocrine cell maturation and hormone production.

Other extracellular molecules

In addition to the now fairly extensive list of signaling molecules that have been studied in-depth in the developing pancreas, numerous other molecular pathways with potentially important influences on pancreatic development have been studied, though in less detail. The Scharfmann laboratory has initiated many of these studies. For example, vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) are both paracrine-acting proteins that can stimulate cAMP production and growth in E13 embryonic pancreatic epithelium, with enhanced numbers of amylase-positive and insulin-positive cells. The common receptor for these two peptides, VPAC2, colocalizes with pdx1 in E12 to E16 pancreatic rat epithelium, and turns off when the cells become insulin or glucagon-positive, suggesting a key role for VPAC2 signaling in controlling growth and lineage selection of uncommitted pancreatic progenitor cells (Rachdi et al., 2003). In zebrafish the same group found that calsenilin, a neuronal presenilin regulator, identified through an endocrine pancreas expression screen, was present in all endocrine cells. A role for calsenilin in promoting endocrine differentiation was suggested by the fact that calsenilin-inhibition with morpholino antisense led to markedly decreased numbers of endocrine cells, with failure of residual endocrine cells to aggregate into islets (Stetsyuk et al., 2007).

Recently the Scharfmann laboratory found the surprising result that glucose was specifically necessary for endocrine, but not exocrine development in *in vitro* cultured pancreas. In the absence of glucose (except possibly for some glucose in the added serum), exocrine pancreas developed normally, but endocrine progenitors were unable to progress past the ngn3-positive stage (Guillemain et al., 2007).

An interesting role for glucocorticoid receptor signaling in regulating β-cell mass has recently been shown by the Breant laboratory. Late-gestational malnutrition in pregnant rats led to decreased fetal β-cell mass due to a decreased numbers of islets (Garofano et al., 1997), which seemed to translate to poor proliferation and greater senescence of β-cells in adult mice (Garofano et al., 1998, 1999, 2000). These studies suggested that type II diabetes may stem from *in utero* and perinatal insults with enhanced glucocorticoid levels, which would then decrease β-cell mass and islet numbers (Blondeau et al., 2001). Interestingly, this *in utero* effect of glucocorticoids on β-cell mass was originally suggested by early studies of protein levels in E14 rat pancreas in which dexamethasone treatment enhanced amylase levels and suppressed insulin levels (Van Nest et al., 1983), and was then later supported by the observation that dexamethasone had a similar pro-exocrine and anti-insulin effect on AR42J cells (Shen et al., 2003). More recently, the Breant laboratory confirmed that dexamethasone could enhance acinar development and suppress β-cell development (Gesina et al., 2004). The glucocorticoid receptor was implicated here specifically in β-cell development, since pdx1-cre-mediated β-cell-specific ablation of the glucocorticoid receptor specifically caused a doubling of the number of β-cells, with no effect on other cell types. However, the effect of the glucocorticoid receptor was not due to a role in mature β-cell function since glucocorticoid receptor deletion under RIP-cre control, which would delete the glucocorticoid receptor later on in developing insulin-positive cells, resulted in no loss of β-cells (Gesina et al., 2004).

An observation in the early pancreatic development literature that is important to remember for developmental studies is a direct effect of BrdU on pancreatic differentiation. BrdU at 20 μM inhibited acinar differentiation in E14 rat pancreas, and the pancreas appeared to develop with more ducts (Githens et al., 1976). It was also shown in

these studies that acinar-associated protein levels were suppressed in exchange for elevated levels of possible ductal proteins.

Another potential signaling pathway affecting pancreatic development is through the stem cell factor receptor c-kit, (Li et al., 2007). Signaling through this receptor may lead to endocrine differentiation by cells lining the early pancreatic ducts, and c-kit is present in human fetal duct cells, and then later in endocrine cells. C-kit stimulation in cultured human fetal pancreas led to enhanced insulin expression and proliferation of insulin-positive cells. Furthermore, c-kit siRNA treatment of these pancreases inhibited insulin-positive differentiation.

Transcription factors

Pdx1 and pbx-1

Pancreatic development over the last 15 years has been dominated by studies of transcription factors and transcription factor hierarchies during development. A central and heavily studied transcription factor in pancreatic development is pancreatic duodenal homeobox 1 (pdx1). Pdx1 was originally identified based on its ability to bind the insulin and somatostatin genes, and has also been called stf1, idx1, ipf1, and the *Xenopus* ortholog, x1Hbox8 (Ohlsson et al., 1991; Leonard et al., 1993; Ohlsson et al., 1993; Miller et al., 1994; Peshavaria et al., 1994). Pdx1 is only expressed in the foregut and its derivates, and is first expressed at E8.5 (10 somites) in the pre-pancreatic region of the mouse foregut, and then expands to be expressed in the distal stomach, common bile duct, and duodenum by E10 to E11.5 (Offield et al., 1996; Guz et al., 1995; Jonsson et al., 1995; Wessels and Cohen, 1967). This 10-somite onset of pdx1 expression correlates with the earliest point at which ex vivo foregut can form pancreas, and suggests that pdx1 is a key component of pancreatic specification. In the early pancreas, pdx1 is expressed throughout the epithelium, but then is suppressed in cells as they commit to the endocrine lineage (as cells move out of the epithelium and express glucagon) (Jensen et al., 2000b), or commit to ducts (Jonsson et al., 1995; Gu et al., 2002) (see Summary and overview of endocrine pancreas lineage section for more details). As endocrine cells begin to differentiate toward the insulin-positive β-cell lineage, pdx1 reappears, and is known to be necessary for proper glucose-responsive regulation of insulin synthesis in β-cells (MacFarlane et al., 1994; Marshak et al., 1996). Low pdx1 expression persists in other endocrine cell types, acinar cells and ductal cells (Guz et al., 1995; Wu et al., 1997), though without a clear developmental role.

In 1994 the Edlund laboratory in Umea, Sweden published a landmark paper in which pdx1 (then called ipf1) null mutant mice were created, and found to have pancreatic agenesis (Jonsson et al., 1994). In a follow-up study, an early pancreatic dorsal bud was detected in these mice, although no ventral bud was ever seen. This dorsal bud persisted, forming a few insulin and glucagon-expressing cells, but without expansion of those cells (Ahlgren et al., 1996) (see Fig. 11). A similar exon-2 deleted pdx1 null mutant mouse was described two years later in which a similar early abortive dorsal bud formed, with only glucagon-expressing cells, no insulin-expressing cells, and metaplasia of the duodenal mucosa into a bile duct phenotype associated with duodenal obstruction (Offield et al., 1996). Clearly, pdx1 is not necessary for early budding of the dorsal pancreas, nor for early endocrine cells to form, but is necessary for later steps in pancreatic development. Following these null mutant mouse studies, pdx1 mutations in humans were found to be associated with pancreatic agenesis in homozygous null mutants (Stoffers et al., 1997b), and with insulin insufficiency in heterozygous mutants (Dutta et al., 1998; Stoffers et al., 1997a). Zebrafish with pdx1 null mutations are also apancreatic (Yee et al., 2001).

Because of its obviously central role in pancreatic and β-cell development and function, an unprecedented number of mutant mouse strains designed to study pdx1 regulation have been generated.

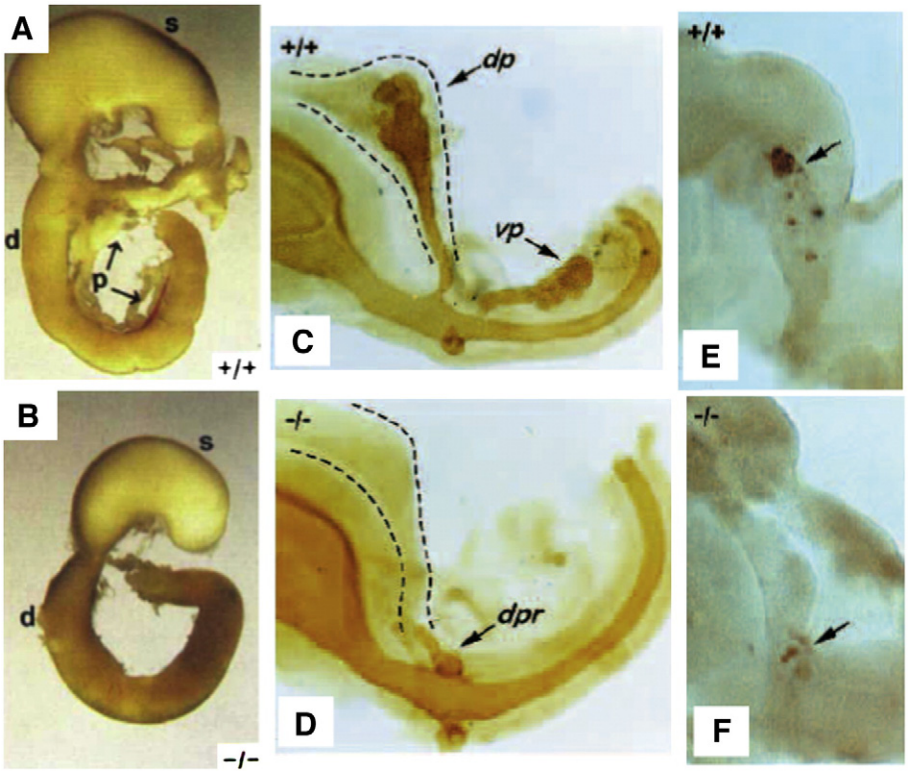

Fig. 11. Null mutant mice for pdx1 (also called ipf1 or idx1) were born with agenesis of the pancreas (p), with occasional duodenal (d) obstruction (A, B) and a dilated stomach (s) (with permission (Jonsson et al., 1994). Further investigations showed that these mice had an attenuated dorsal pancreatic rudiment (dpr) compared with wild-type dorsal pancreas (dp) (C and D). Similarly, insulin cells are present (arrow), but in greatly diminished numbers in null mutant animals (E and F) (with permission, Ahlgren et al., 1996).

Key studies from the MacDonald laboratory used a tetracycline-regulatable transgenic knock-in system to study the role of pdx1 *after* initial budding of the dorsal pancreas. With this delayed inhibition of pdx1 expression until after budding, there was severe blunting of pancreatic development, with only small ductal structures present, and complete absence of acini or β-cells. These results confirmed an ongoing important and necessary role for pdx1 in early acinar and endocrine development (Holland et al., 2002) subsequent to the initial formation of a pancreatic bud. Beyond this initial acinar development, subsequent maturation and growth of acini also seems to be pdx1-dependent. Later inhibition of pdx1 expression (E13.5 to E14) resulted in reduced numbers of acini, and the acini that were present were immature (Hale et al., 2005).

To further delineate the role of pdx1 in pancreatic development, the Wright laboratory has generated numerous important pdx1 mutant mouse strains. By deleting key 5′ regulatory elements, a hypomorphic pdx1 allele was created that led to delayed and diminished pdx1 expression. Using this hypomorphic allele (Δ) in a pdx1 null mutant background enabled the study of how different levels of pdx1 may affect pancreas development.(Fujitani et al., 2006). Mice with one hypomorphic allele and one null allele (Δ/−) were found to phenocopy the homozygous null mutant mice. With the next higher "dose" of pdx1 expression, Δ/Δ mice developed a pancreas with markedly diminished β-cells and no islets. Here, the distal half of the developing dorsal pancreas appeared atretic, with no acini, whereas the proximal half was clearly demarcated and had normal-appearing acini. Surprisingly, no ventral bud was seen due to a lack of ventral foregut PTF1a (see "PTF1a" section). Lastly, mice with one normal pdx1 allele and one hypomorphic pdx1 allele (Δ/+) had replacement of β-cells with α-cells and PP cells, with α-cells throughout the islet instead of only at the periphery. These mice were also glucose intolerant, suggesting deficient β-cell function. Thus, this study demonstrates a key role for proper *pdx1* gene regulation and dosing in multiple aspects of pancreatic endocrine and exocrine development, including early pancreatic commitment, exocrine/acinar differentiation, endocrine lineage selection, and β-cell function. The finding of glucose intolerance in the Δ/+ mice was not surprising given the wealth of data supporting that pdx1 heterozygous mutant β-cells are dysfunctional (Johnson et al., 2003; Brissova et al., 2002; Dutta et al., 1998; Boyer et al., 2006), and are unable to undergo compensatory hypertrophy (Kulkarni et al., 2004). The finding that Δ/+ β-cells were replaced by α-cells (and PP-cells) is consistent with studies in which pdx1 inhibition in β-cells (using either tetracycline-induced pdx1 inhibition (Lottmann et al., 2001; Thomas et al., 2001a; Holland et al., 2002) or insulin promoter-driven cre excision of pdx1 (Ahlgren et al., 1998; Gannon et al., 2008) led to β-cell depletion and a concomitant rise in the number of α-cells. This phenotypic change is not due to conversion of β-cells to α-cells, but rather seems to be due to the loss of normal β-cell inhibition of α-cell development (Gannon et al., 2008). Earlier deletion of β-cell pdx1 seems to lead to greater α-cell growth. Although pdx1 is co-expressed in early glucagon cells (Jensen et al., 2000b; Chiang and Melton, 2003), pdx1 in those cells may actually be acting to down-regulate glucagon expression and prevent α-cell differentiation, perhaps through competitively binding pax6 sites on the glucagon gene (Chakrabarti et al., 2002; Ritz-Laser et al., 2003; Flock et al., 2005).

Beyond the major pdx1 5′ prime regulatory mutations described in detail above (Fujitani et al., 2006), the Wright laboratory has delved deeply into the detailed developmental roles for several of the pdx1 regulatory elements, which overall are designated areas I through IV (in order from proximal to distal to the transcriptional start site). Areas I through III are highly conserved (Gerrish et al., 2000; Marshak et al., 2000) and were deleted in the hypomorphic Δ allele described above. This I–III region confers expression of pdx1 in the early pancreas and duodenum (Gannon et al., 2001; Stoffers et al., 1999; Wiebe et al., 2007). Areas I and II appear to confer β-cell specificity (Wiebe et al., 2007; Samaras et al., 2002; Van Velkinburgh et al., 2005), perhaps in cooperation with area IV (Gerrish et al., 2004). Area IV is involved in suppression of pdx1 in non-endocrine β-cells, in expression of pdx1 in the gut, and in development of the GIP-positive and gastrin-positive cells in the gut (Boyer et al., 2006). The overexpression of pdx1 under the PTF1a promoter resulted in no developmental changes (Miyatsuka et al., 2006).

Pdx1–pbx-1 interactions

Beyond the role of pdx1 as a monomer, its function also appears to be heavily dependent on interactions with other molecules present inside the cells. Pdx1 has a critical protein–protein interaction with pbx-1, a member of the TALE (three-amino-loop extension) class of homeodomain transcription factors (Dutta et al., 2001). Pdx1 and pbx1 have been found to interact with each other, and with other potential third partners to form a transcriptional regulatory complex that may play a role in pancreatic differentiation (Peers et al., 1995; Swift et al., 1998). Pdx1: pbx-1 heterodimers have a twenty-fold greater affinity for the insulin gene (Peers et al., 1995), but surprisingly a pdx1 mutant that cannot interact with pbx-1 is still able to rescue the diabetic phenotype of pdx1 heterozygous null mutant mice (Dutta et al., 2001). However, although the embryos of these pdx1 interaction mutants could specify pancreatic cells, and all cells types were present, cell types appear unable to amplify after initial commitment. This pbx-1 requirement for proper pdx1 function is also suggested by chick electroporation studies wherein ectopic pdx1 expression in the foregut (without concomitant pbx-1 expression) could induce pancreas-like evaginations to occur, with suppression of intestinal patterning factors, but with no further pancreatic differentiation or growth (Grapin-Botton et al., 2001).

Further evidence for a role for pbx-1, perhaps partly independent of its pdx-1 interactions, came from studies of a pbx-1 null mutant mouse (Kim et al., 2002). Pbx-1 was expressed in both epithelium and mesenchyme (pdx1 is not ever expressed in the mesenchyme) in the early embryo, and then became localized to ducts and islet cells later in development and postnatally. Pbx-1 null mutant mice lacked endocrine and exocrine differentiation. The lack of endocrine cells was due to the absence of pbx-1 in the epithelium, presumably in a cell-autonomous fashion, whereas the lack of exocrine cells was likely due to loss of a pbx-1-induced pro-exocrine mesenchymal factor.

PTF1a

PTF1a, or p48, was originally described as part of a large heterotrimeric transcriptional regulator complex called pancreas-specific transcription factor 1 (PTF1), which regulates acinar enzyme gene expression (Cockell et al., 1989; Petrucco et al., 1990). PTF1a or p48 is a basic helix–loop–helix protein that is the only cell-specific component of the PTF1 heterotrimer (Krapp et al., 1996). PTF1a appears to play an important role in early specification of pancreatic progenitor cells. PTF1a is first expressed slightly later than pdx1, at E9.5, specifically in cells of the foregut endoderm destined to give rise to dorsal and ventral pancreas (Burlison et al., 2008; Krapp et al., 1998). This early PTF1a expression in the dorsal pancreas is likely due to the influence of the nearby dorsal aortae (see "Blood vessels and endothelial-derived factors" section) (Yoshitomi and Zaret, 2004). Unlike pdx1, PTF1a is not expressed in other parts of the foregut. PTF1a expression continues mainly in epithelial and acinar cells, but expression is decreased in endocrine cells. Low level expression of PTF1a can be present in early endocrine progenitor cells (Kawaguchi et al., 2002; Chiang and Melton, 2003; Lin et al., 2004b; Zecchin et al., 2004; Zhou et al., 2007). By lineage tracing analysis, essentially all acinar cells, 95% of ductal cells, 75% of α-cells, and 100% of non-α endocrine cells are derived from PTF1a-positive progenitor cells.

PTF1a and pdx1 are co-expressed in pancreatic progenitor cells from E9.5 to E12.5. Pdx1 is expressed in a much broader domain than

PTF1a in the foregut, and therefore pdx1 expression is not dependent on PTF1a expression. However, Area III of the pdx1 promoter, which contributes to early embryonic pdx1 expression, can be bound and activated by PTF1a (Wiebe et al., 2007). Also, PTF1a expression is not dependent on pdx1 expression (Kawaguchi et al., 2002; Lin et al., 2004a). There is evidence that progenitor cells co-expressing pdx1 and PTF1a are instructed toward a pancreatic fate. Loss of hes1 expression (hes1 is a downstream target of notch, see "Notch signaling" section) in pdx1-expressing stomach, duodenum, and bile duct progenitors diverted those cells to a pancreatic fate (Fukuda et al., 2006; Sumazaki et al., 2004). Similarly, early transgenic expression of PTF1a in liver, stomach, and duodenum can divert those cells into forming pancreas (Jarikji et al., 2007; Afelik et al., 2006). When the pdx1/PTF1a co-expressing field in the foregut is expanded in *Xenopus*, the result is a "giant" pancreas, suggesting that more pancreatic progenitor cells were created as a result of the enhanced number of pdx1/PTF1a co-expressing cells (Afelik et al., 2006) (see Fig. 12). A direct correlation between pancreatic size and the number of initial progenitor cells has been clearly documented (Stanger et al., 2007).

An important global role for PTF1a in pancreas specification is confirmed by mouse null mutant and zebrafish knockdown studies. Like pdx1 null mutants, PTF1a null mutant mice develop with only an aborted dorsal pancreatic bud, and a minuscule ventral bud, which actually emanates from the bile duct (Krapp et al., 1998; Kawaguchi et al., 2002) (see Fig. 12). In PTF1a knockdown zebrafish and *Xenopus* embryos, the exocrine pancreas is absent (Afelik et al., 2006; Lin et al., 2004b). While the role of PTF1a in exocrine and acinar development is clear, a role in endocrine development is not. In PTF1a null mutant mice, acini and ducts do not form, but endocrine cells do develop and migrate out through the mesenchyme to populate the spleen (Krapp et al., 1998; Lin et al., 2004b). Similarly, humans with a PTF1a non-functioning mutation are born without any pancreas, and have neonatal diabetes (Sellick et al., 2004).

In addition to these known roles, PTF1a has a highly orchestrated and complex set of interactions with notch downstream intercellular mediators (RBP-J's) to regulate target patterning genes and acinar-specific genes (see "Notch signaling" section for details).

Neurogenin 3 (ngn3) and its targets

As described in the "Notch signaling" section earlier, ngn3 is a basic helix–loop–helix transcription factor whose expression is repressed by notch-mediated intracellular signaling (Apelqvist et al., 1999; Jensen et al., 2000c; Lee et al., 2001). Ngn3 has been a key target for study as it currently sits atop the pancreatic endocrine lineage transcription factor hierarchy. In mouse embryos ngn3 is first expressed in the early pancreatic epithelium at E9 (Gradwohl et al., 2000; Jensen et al., 2000b; Schwitzgebel et al., 2000), and then ngn3 expression escalates, it then peaks at around E15.5, but decreases substantially by E17.5 (Gradwohl et al., 2000; Schwitzgebel et al., 2000; Jensen et al., 2000b; Gu et al., 2002). Ngn3-positive cells are good candidates for endocrine progenitor cells. Ngn3-positive cells are proliferative and give rise to post-mitotic cells expressing the islet transcription factors neuroD, nkx6.1 and pax6 (Jensen et al., 2000a). Shortly after neuroD and pax6 are expressed, ngn3 is shut off, apparently due in part to auto-repression (Smith et al., 2004; Gu et al., 2002). Most importantly, favoring the progenitor cell status of ngn3-positive cells, all pancreatic endocrine cells derive from ngn3-positive cells (Gu et al., 2002).

Ngn3 appears to be a key driver of endocrine differentiation. In an effort to induce non-pancreatic tissues to become pancreatic islets, ngn3 has been expressed ectopically in multiple systems. In general, forced expression of ngn3 induces cells to commit to the endocrine lineage prematurely and exit from the cell cycle. When ngn3 is expressed at high levels and at an inappropriately early time in the developing mouse pancreas, the result is the that the entire pancreas

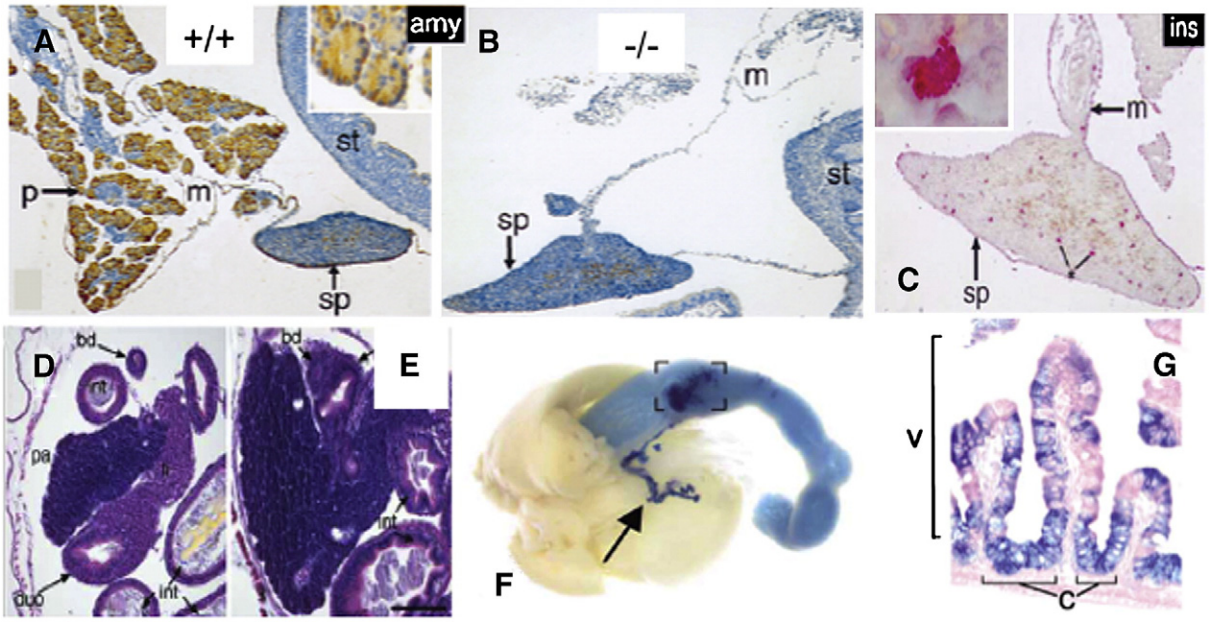

Fig. 12. PTF1a is critical for exocrine development, and marks the pancreatic lineage. PTF1a (also called p48) null mutant mice develop with no exocrine tissue (B) compared with wild-type littermates in (A), (amylase in brown) (p, pancreas; m, mesenchyme; st, stomach). In these animals, insulin-positive islets are interestingly localized to the spleen (sp), with the insert showing clusters of insulin-positive cells (C). Insulin-positive cells are also seen along the old pancreatic mesentery (m) (with permission, Krapp et al., 1998). (D, E) When the PTF1a/p48 field was expanded into the entire pdx1 domain in *Xenopus* embryos, a massive enlargement of the pancreas was seen, identified as the dark blue region in transgenic animals (E) compared with non-transgenics (D) (with permission, Afelik et al., 2006). Kawaguchi et al. showed that PTF1a diverts foregut cells away from the duodenal phenotype and toward the pancreatic phenotype. (F) lineage tagging of PTF1a promoter-positive cells in a PTF1a null mutant mouse shows an attenuated pancreatic duct (arrow) without proper formation of the pancreas, and histologic analysis of the duodenum shows that PTF1a lineage cells are diverted to becoming duodenal mucosal villus (v) and crypt (c) cells in the absence of PTF1a protein (G) (with permission, Kawaguchi et al., 2002).

consists of only small clusters of cells that are glucagon-positive (Apelqvist et al., 1999; Schwitzgebel et al., 2000; Johansson et al., 2007). When chick embryonic endoderm was electroporated with an ngn3 expression vector, the intestine was induced to express glucagon and somatostatin, but not insulin. Overexpression of ngn3 in human (Heremans et al., 2002) or mouse (Gasa et al., 2004; Mellitzer et al., 2006) pancreatic duct cells could induce an endocrine program.

Ngn3 appears to have the ability to initiate the full pancreatic endocrine program if expressed in the appropriate cells at the appropriate time. Johansson et al., using an elegant transgenic "add-back" system of ngn3 expression at specific times in an ngn3 null mutant background, showed that forced ngn3 expression later, at E11 to E12 was able to induce many pancreatic polypeptide- and insulin-expressing cells instead of the glucagon-only differentiation seen with earlier overexpression of ngn3 (Johansson et al., 2007). Interestingly, a few ngn3-positive cells have been found in mature islets (Gu et al., 2002), and ngn3 reporter mouse strains have been used to purify and study potential embryonic endocrine progenitor cells (Mellitzer et al., 2004; White et al., 2008). A recent landmark paper showed that ngn3 reporter mice could be used to sort cells from an E13.5 pancreas, or from a duct-ligated adult pancreas. Amazingly, both types of cells (adult or embryonic), when grafted into an explanted ngn3 null mutant foregut *in vitro* (which normally will not form any pancreatic endocrine cells), were able to form mature islets (Xu et al., 2008) (see Fig. 13). This study represents strong evidence of ngn3 as a marker of embryonic and adult-derived pancreatic endocrine progenitors.

NeuroD

Potential downstream mechanisms by which ngn3 may activate the endocrine program are of obvious interest. Ngn3 has neuroD as an immediate downstream target (Jensen et al., 2000b; Gradwohl et al., 2000; Jensen et al., 2000a; Gradwohl et al., 2000; Huang et al., 2000; Gasa et al., 2008; Gu et al., 2002) through binding and activating E-boxes of the neuroD regulatory sequence as an ngn3-E47 heterodimer (Huang et al., 2000). The close relationship between ngn3 and neuroD expression is highlighted by the fact that neuroD overexpression, either transgenically in mice, or in ductal cell lines, induces a similar phenotypic change to that seen with ngn3 overexpression (Schwitzgebel et al., 2000; Heremans et al., 2002; Gasa et al., 2008). NeuroD expression is dependent on ngn3 since ngn3 null mutant mice lack neuroD (Gradwohl et al., 2000). The onset of neuroD expression in endocrine cells of the embryonic pancreas represents a very important transition from proliferative ngn3-positive cells to post-mitotic cells (Jensen et al., 2000a; Gu et al., 2002). Unlike ngn3 null mutant mice, however, neuroD null mutant mice are still able to form all pancreatic endocrine cell types, but the number of cells is drastically reduced due to late-gestation apoptosis, depending on the background mouse strain (Naya et al., 1997; Huang et al., 2002). A role for neuroD in suppressing non-β-cell lineages has been suggested by the fact that the addition of a neuroD null mutation to Nkx2.2 null mutant mice (lacking β-, α- and PP-cells, see later section) will rescue α and PP-cells (Chao et al., 2007).

Other potentially important direct targets of ngn3 include Insulin-Associated 1 (IA1), a zinc finger protein that is activated by either ngn3 (Mellitzer et al., 2006) or by neuroD (Breslin et al., 2003). IA1 appears to be important in executing the endocrine differentiation process, since it is absent in ngn3 null mutant mice, and is normally expressed in the early pancreatic buds (Gierl et al., 2006) and in pancreatic AR42J cells that are becoming insulin-positive (Zhu et al., 2002). IA1 is still expressed in the pancreas of neuroD null mutant mice, perhaps participating in the small amount of endocrine differentiation that is known to occur in the pancreas of those mice (Mellitzer et al., 2006; Naya et al., 1997; Huang et al., 2002). Other ngn3 targets include Iroquois-type Homeobox proteins (Irx 1 and 2), which are expressed in early pancreatic endoderm and in α-cells (Petri et al., 2006), and neuroD2, which is present in embryonic pancreas and in α-cell lines (Gasa et al., 2008). Ngn3 also directly regulates expression of *pax* and *Nkx* genes, which are discussed later.

Regulation of ngn3 is obviously of high importance, and positive regulation by the HNF/Forkhead family of transcription factors appears to play an important role in this regulation (see "HNF cascade" section).

Pax6

Pax6 is a member of the pax family of transcription factors containing a paired box DNA binding domain. Pax6 also contains a second DNA binding domain, the homeodomain, and was found to be expressed throughout the early embryonic pancreas (E9.0), and in pancreatic endocrine cells (Turque et al., 1994). The onset of pax6 expression in the early pancreatic epithelium occurs within an endocrine committed population of epithelial cells that also expresses neuroD and isl1 (Jensen et al., 2000a). NeuroD, in cooperation with E47, can bind and activate the *pax6* gene (Marsich et al., 2003). These early pax6-positive cells then begin hormone expression. Pax6 expression is retained in cells committing to the endocrine lineage, either glucagon-positive, starting at E9.5, or insulin-positive, starting at E12.5 (Sander et al., 1997; St-Onge et al., 1997; Heller et al., 2004). Consistent with this hormone expression in pax6-positive cells, pax6 binding sites have been found in the A site of the G3 promoter region of the preproglucagon gene, and similar sites are present on the insulin and somatostatin genes (Sander et al., 1997). Consistent with pax6-positive cells giving rise to glucagon-positive cells, at E9.5 many epithelial cells are pax6-positive, and all of the glucagon-positive cells at that time are included in the pool of pax6-positive cells (Jensen et al., 2000a; Sander et al., 1997; Heller et al., 2004). Some of the early pax6-positive cells may give rise to duct cells, as shown by a lineage tagging study using 3.3 kb of the pax6 promoter to label cell lineages (Zhang et al., 2003).

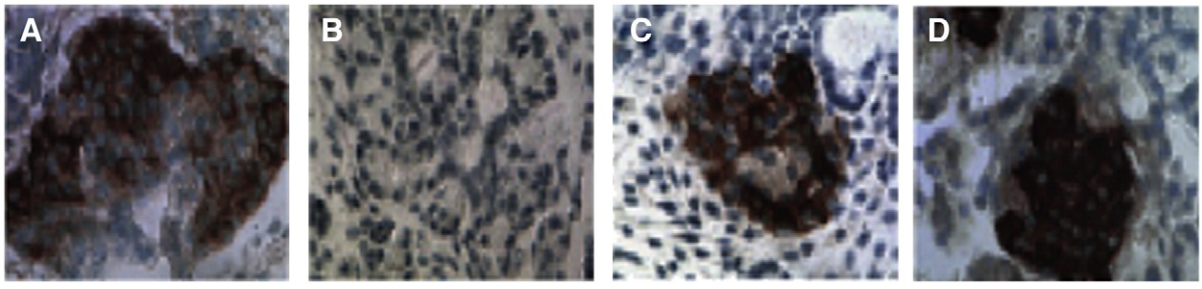

Fig. 13. Ngn3 can be used as a marker of endocrine progenitor cells in the embryonic pancreas, and in the pancreatic duct ligation model of pancreatitis in adult mice. Xu et al., showed that by purifying these ngn3-positive cells from E13.5 embryonic pancreas, and then injecting the cells into an ngn3 null mutant foregut *in vitro*, insulin cells could develop (C). No insulin cells are seen in ngn3 null mutant explants alone (B), and wild-type explants had robust insulin-positive differentiation (A). Similarly, sorting of adult ngn3-positive cells from the pancreatic duct ligation model also led to the formation of clusters of strongly insulin-positive cells in the ngn3 mutant explants (D) (with permission, Xu et al., 2008).

Pax6 is not absolutely necessary for pancreatic hormone gene expression since pax6 null mutant mice, or mice with a dysfunctional *small eye* mutation of the *pax6* gene, are both still able to form endocrine cells, though at a reduced rate (St-Onge et al., 1997; Sander et al., 1997). In these pax6 mutants early ("first wave") insulin-positive cells are scarce at E12.5. This absence of early insulin-positive cell development could be due directly to the absence of pax6, or instead due to the absence of glucagon which, as discussed earlier leads to failure of early insulin-positive cell differentiation (Prasadan et al., 2002; Vuguin et al., 2006). In addition to the paucity of endocrine cells forming in pax6 mutants, there is also a failure to amplify the endocrine cell population during the secondary transition, with an resulting 75–100% reduction in α-cells (St-Onge et al., 1997; Sander et al., 1997), and a 65% reduction in β-cells (Sander et al., 1997).

Heller et al. found that the more sensitive staining technique of immunoperoxidase, rather than immunofluorescence, allowed detection of hormones in more cells of the E19 pancreas of pax6 null mutant mice, suggesting that pax6 may play a role in hormone expression levels, rather than hormone gene activation (Heller et al., 2004). In addition, this group later showed a population of endocrine-like cells in the pax6 mutant pancreas at E19 that were negative for insulin, glucagon, somatostatin, and PP. These cells were ghrelin-positive ε-cells, and were five-fold more prevalent in these pax 6 mutant mice, suggesting that pax6 normally prevents the expression of ghrelin in pancreatic endocrine cells (Heller et al., 2005).

A role for pax6 in islet cell and possibly duct cell growth is further supported by mice in which transgenic expression of pax6 under the pdx1 promoter induced ductal and islet hyperplasia (Yamaoka et al., 2000). Specific transgenic expression of pax6 in β-cells under the insulin promoter, however, caused β-cell apoptosis and diabetes, suggesting that pax6 promotes proliferation only in pancreatic progenitors.

Pax4 and Arx

A second pax family member, pax4 shows similar sequence homology to pax6, including the presence of a homeodomain (Dahl et al., 1997). In the early embryo, at E9.5, pax4 is expressed almost exclusively in the pancreatic field of the foregut (Sosa-Pineda et al., 1997; Brink et al., 2001). The pax4-positive cell population then expands rapidly, peaking at E13–E15 (Wang et al., 2004), coinciding with the burst of new insulin-positive cells appearing in the secondary transition. Here, insulin-positive cells likely arise from pax4-positive cells since a fraction of the pax4-positive cells are insulin positive. As the endocrine cells mature, pax4 expression, which is predominantly in the developing β-cells, disappears (Smith et al., 1999). Thus, pax4 represents an endocrine progenitor marker that is fairly specific to the pancreas. Lineage-tagging studies show that although pax4-positive cells are mostly insulin cells, pax4-positive cells actually give rise equally to the four main endocrine lineages (Greenwood et al., 2007), suggesting that the reason that most pax4-positive cells are insulin-positive is that they are more proliferative after expressing insulin. Activation of pax4 in endocrine progenitor cells may be mediated by ngn3 and HNF1α (see "HNF cascade" section) since these factors bind the pax4 regulatory region and are necessary for pax4 expression in cell lines (Smith et al., 2003).

A key role for pax4 in β-cell development was demonstrated by a pax4 null mutant mouse in which there was failure to develop β-cells and δ-cells. These mice did have early embryonic insulin-positive cells, so pax4 seems specifically necessary only for formation of mature β-cells (Sosa-Pineda et al., 1997; Wang et al., 2004). In the absence of β-cells and δ-cells, the number of glucagon-positive cells was noted to be greatly increased, as if replacing the β-cells. These glucagon-positive cells were actually found to co-express glucagon and ghrelin (Heller et al., 2005; Wang et al., 2008). Mechanistically, this expansion of a glucagon/ghrelin double-positive cell populations likely reflects that pax4 can act as a transcriptional repressor, being especially effective at repressing ghrelin expression and pax6-mediated glucagon expression (Wang et al., 2008; Smith et al., 1999; Ritz-Laser et al., 2002).

Pax4 also directly inhibits expression of Arx, a homeobox-containing gene that enhances glucagon-positive cell differentiation (Collombat et al., 2003, 2005). Although pax4 null mutant mice lack somatostatin-positive δ-cells, a positive role for pax4 in δ-cell development remains unclear since double null mutant mice for Arx and Pax4 show large numbers of δ-cells, suggesting that persistent pax4 expression after the initial β/δ versus α/PP switch favors β-cells, but then the absence of pax4 secondarily leads to δ-cell formation (Collombat et al., 2005) (see Fig. 14).

Arx is downstream of ngn3 since it is absent from ngn3 null mutant mice. Arx null mutant mice have no α-cells, mainly due to loss of secondary transition glucagon-positive cells (Collombat et al., 2003), and the α-cell precursors seem to be shunted toward the β-cell and δ-cell lineages due to unopposed pax4 expression (Collombat et al., 2003, 2005). Conversely, overexpression of Arx in pdx1-positive progenitor cells was able to divert most β-cell and δ-cell precursors toward α-cells and PP lineages (Collombat et al., 2007), with no change in the total number of endocrine cells, again supporting opposing roles for pax4 and Arx in α/PP cells versus β/δ cells. Here it should be noted that Arx does not directly downregulate pax4 since Arx overexpressing cells had persistent pax4 expression. An additional role for Arx, in endocrine-versus-exocrine lineage selection, was suggested by the fact that the pdx1-Arx-transgenic mice had a loss of exocrine cells.

Lastly, although ngn3 expression is thought to be an irreversible commitment to the endocrine lineage, overexpression of a constitutively-active notch mediator in the pax4-positive subset of ngn3-lineage cells led to a surprising metaplasia of the cells into ducts (Greenwood et al., 2007). Thus, these pax4-positive cells may still represent an important multi-potent progenitor cell.

Nkx2.2

Nkx2.2 is a member of the NK family of homeodomain proteins that also contains engrailed-homologous repressor domains (Muhr et al., 2001). Nkx2.2 was identified as having a role in neuronal differentiation, and then was noted to also be expressed in α- and β-cells (Rudnick et al., 1994). Nkx2.2 is expressed in the early embryonic

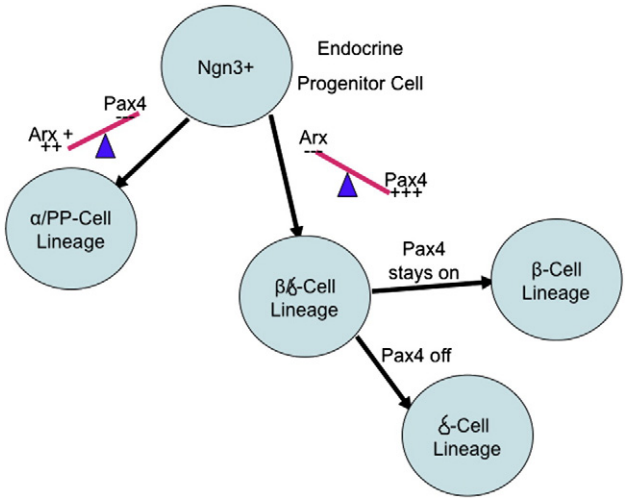

Fig. 14. Pax4 and Arx have competing roles in the determination first of ngn3-positive cells' commitment to either the α/PP-cell lineage or the β/δ-cell lineage. Subsequently, once some cells are committed to the β/δ-cell lineage, persistent pax4 seems to select for the β-cell lineage, whereas suppression of pax4 selects for the δ-cell lineage.

pancreatic epithelium by E9.5 (Sussel et al., 1998). By E10.5 about half of the epithelial cells are Nkx2.2-positive, and all of those Nkx2.2-positive cells co-express pdx1 (Chiang and Melton, 2003). As pancreatic development progresses, Nkx2.2 expression becomes focused to ngn3-positive cells, and persists in most endocrine cells, except not at all in the δ-cells (Sussel et al., 1998). Interestingly, expression of Nkx2.2 in these three different phases (i.e. early bud, ngn3-positive endocrine progenitors, and mature/maturing endocrine cells) appears to be under the control of three different first exons, each exon having different regulatory elements (Watada et al., 2003). The "A" first exon is expressed only in hormone-positive or hormone-negative/ngn3-positive cells at E10.5, and then accounts for most of the Nkx2.2-expression in the E15 pancreas. In mature islets, the A exon was only expressed in β-cells. This A exon is regulated by HNF3β/FoxA2 (see "HNF cascade" section) bound to either the *ngn3* gene (presumably for the embryonic expression) or neuroD (presumably for β-cells and other hormone-positive cells). The "B" first exon was expressed only in ngn3-positive cells, and then turned off when the cells became hormone-positive. "C" first exon-specific expression was not detected. The regulation of Nkx2.2 exons that leads to the specific Nkx2.2-expression in early pdx1-positive epithelium has not yet been determined.

Early expression of Nkx2.2 may play a role in pancreatic duct formation in zebrafish (Pauls et al., 2007), but in Nkx2.2 null mutant mice the only pancreatic defects are endocrine. Specifically, these mice develop with no detectable β-cells, an 80% reduction in α-cells, a "modest" reduction in PP-cells, and no effect on δ-cells (Sussel et al., 1998). There were a large number of cells that later were identified as ghrelin-positive ε-cells, with no glucagon co-expression, thus suggesting that Nkx2.2 normally may induce insulin-positive differentiation and repress ε-cell formation (Sussel et al., 1998; Prado et al., 2004). Consistent with its role in β-cell formation, Nkx2.2 can bind and activate mafA (see "MafA and mafB" section) (Raum et al., 2006) and insulin genes (Cissell et al., 2003). In addition, through creation of an Nkx2.2-engrailed fusion repressor construct expressed under the pdx1 promoter, the Sussel laboratory showed that the repressor function of Nkx2.2 was normally necessary for enhancing α-cell differentiation, and for suppressing ε-cell formation (Doyle et al., 2007). A double null mutant mouse for Nkx2.2 and neuroD had rescue of many α- and PP-cells, and suppression of ε-cells, suggesting that neuroD may mimic or replace the Nkx2.2 repressor function (Chao et al., 2007).

Nkx6.1 and Nkx6.2

Nkx6.1 and Nkx.6.2 are also members of the NK homeodomain protein family, which are very similar (Vallstedt et al., 2001) and both appear to play a central role in pancreatic, and specifically β-cell development. A role for Nkx6.1 and Nkx6.2 in pancreatic development was suggested by their expression in all early pancreatic endodermal cells (Sander et al., 2000; Oster et al., 1998a; Oster et al., 1998b; Pedersen et al., 2005; Henseleit et al., 2005). Like Nkx2.2, Nkx6.1 appears to be a marker of multipotent pancreatic progenitor cells (Chen et al., 2007). Pdx1 expression precedes Nkx6.1 and 6.2, and pdx1 null mutant mice have only a few Nkx6.1-positive cells, but with no change in the number of Nkx6.2-positive cells. These results suggest that Nkx6.1 is likely downstream of pdx1, whereas Nkx6.2 is not (Pedersen et al., 2005). At around E15, Nkx6.1 expression becomes restricted to insulin-positive cells (Sander et al., 2000), whereas Nkx6.2 becomes restricted to glucagon-positive and amylase-positive cells. Nkx6.1 continues to be expressed in mature β-cells, suggesting an ongoing role in mature β-cell function (Sander et al., 2000). Nkx6.2 is extinguished in the developing pancreas after E15.5.

Single and double null mutant studies for Nkx6.1 and Nkx6.2 have been performed and suggest that both molecules have important roles and suggest that both molecules have important roles in pancreas development (Henseleit et al., 2005). Nkx6.1 null mutant mice have an 85% reduction in β-cells, whereas Nkx6.1/Nkx6.2 double null mutant mice have a 92% reduction, suggesting an important role for Nkx 6.1 in the generation of β-cells, with some compensatory ability of Nkx6.2 to generate β-cells (Sander et al., 2000; Henseleit et al., 2005). The β-cell defect was specifically at the secondary transition, since the insulin-positive cell population was unable to expand after E13 (Sander et al., 2000). Interestingly, β-cell loss in Nkx6.1 null mutant mice could be rescued by transgenic expression of either Nkx6.1 or Nkx6.2 under the pdx1 promoter, but not under the ngn3 promoter. These results suggest that Nkx6.2 can replace Nkx6.1 function if expressed in the proper cell, and also that early Nkx6.1 expression, prior to ngn3 expression, is necessary for β-cell formation (Nelson et al., 2007), though other later roles for Nkx6.1 in β-cell function seem likely. Nkx6.1 normally is able to repress Nkx6.2 expression, and in the absence of Nkx6.1, Nkx6.2 is upregulated specifically in the early pdx1-positive progenitor cells, perhaps being involved in the early commitment of β-cells. Early Nkx6.2 expression appears to be key to endocrine cell generation since Nkx6.2 is expressed in ngn3-positive cells just prior to ngn3 expression, and then Nkx6.2 quickly turns off in those cells, perhaps due to ngn3-induced suppression (Henseleit et al., 2005). Although Nkx6.2 null mutant mice had normal pancreatic development, Nkx6.1/Nkx6.2 double null mutant mice had a 65% reduction in α-cells, suggesting an important role for Nkx6.2 in α-cell formation, and also suggesting that Nkx6.1 is fully capable of compensating for the absence of Nkx6.2 in α-cell development.

MafA and mafB

MafA and mafB are members of a large family of basic leucine-zipper transcription factors that are active in many developmental processes. MafA has been identified as specific to pancreatic β-cells (Olbrot et al., 2002; Matsuoka et al., 2003); and is a critical regulator of the insulin gene. The β-cell specificity of mafA is attributed to a specific region ("region 3") of its promoter that binds pdx1, Nkx2.2, and HNF3β/FoxA2 (Raum et al., 2006). MafA is first expressed in insulin-positive cells that form during the secondary transition (Matsuoka et al., 2004; Nishimura et al., 2006). MafA is not necessary for β-cell formation and mafA null mutant mice have overall normal pancreatic development. MafA may, however, play a role in β-cell function in a mature animal (Zhang et al., 2005). It appears that the mafA-positive insulin-positive cells in the secondary transition of development may derive from mafB/insulin double-positive progenitor cells (Artner et al., 2006, 2007; Nishimura et al., 2006). MafB is expressed early in pancreatic endocrine development in some ngn3-positive cells, and then in insulin and glucagon-positive cells (Gu et al., 2004; Artner et al., 2006). As the pancreas develops, mafB is turned off in developing insulin-positive cells as they transition from immature to mature β-cells (Nishimura et al., 2006). This transition from mafB to mafA appears to depend on mafB function, since mafB binds and activates the *mafA* gene (Artner et al., 2007). In mafB null mutants, there is delayed development of early insulin-positive and glucagon-positive cells, and a 50% reduction in insulin-positive and glucagon-positive cells overall, with an abundance of hormone-negative cells that appear otherwise to be of the endocrine lineage. Thus, mafB appears to be a key regulator of α- and β-cell maturation (Artner et al., 2007).

HNF cascade

Three HNF factors: HNF6/onecut1, vHNF1 (also called TCF2 and HNF1β), and HNF3β (now FoxA2) together appear to have an interwoven, complex set of interactions that all impact on pancreatic development, starting from the earliest pancreatic specification, all the way through to mature pancreatic cell differentiation and function. HNF6 or onecut1 is a cut homeodomain protein that is expressed in the endoderm as early as E8 at the foregut-midgut junction (Lemaigre et al., 1996; Poll et al., 2006; Pierreux et al., 2004)

under the control of vHNF1/HNF1β, which is present in the early endoderm and bound to HNF6 regulatory elements (Poll et al., 2006; Haumaitre et al., 2005, and Barbacci et al., 1999). Aggregated chimeras were used to generate vHNF1 null mutant embryos. These embryos were found to secondarily lack HNF6 expression, and to have ventral pancreatic agenesis and an atrophic dorsal pancreas (Haumaitre et al., 2005). A similar pancreatic hypoplasia also occurs in both humans and zebrafish that have vHNF1/HNF1β mutations (Barbacci et al., 2004; Sun and Hopkins, 2001). HNF6, in turn, can regulate expression of HFN3β/FoxA2, which is necessary for gut formation (Weinstein et al., 1994) and binds and activates the *pdx1* gene (Wu et al., 1997). HNF3β/FoxA2 is not, however, necessary for pancreas formation since endoderm-specific deletion of HNF3β/FoxA2 had no effect on pancreas development (Lee et al., 2005).

After early prepancreatic endodermal expression, HNF6, vHNF/HNF1β, and HNF3β/FoxA2 are then all expressed in the early pancreatic epithelium at E9 to E10 (Rausa et al., 1997; Jacquemin et al., 2003; Maestro et al., 2003). HNF6 appears to be a key determinant of pancreas specification, and it was found that HNF6 null mutant mice had a severely reduced field of pancreas-specified endoderm (Jacquemin et al., 2003). These HNF6 null mutant mice are thus born with a hypoplastic pancreas (Jacquemin et al., 2000, 2003). The reduced pancreatic field in these HNF6 null mutant mice may be due to inadequate pdx1 expression since HNF6 can bind the *pdx1* gene.

HNF6 in the early pancreatic epithelium may also activate vHNF1 to initiate formation of endocrine progenitors in early pancreatic epithelium (Maestro et al., 2003). Although the *ngn3* gene can be bound and activated by HNF6, and ngn3-positive cells are reduced in HNF6 null mutant mice (Jacquemin et al., 2000), the control of endocrine cell generation by HNF6 appears to be through vHNF1/HNF1β expression in pancreatic epithelial cells. As a parallel, HNF6 has been shown to act upstream of vHNF1/HNF1β in bile duct formation (Clotman et al., 2002). Since ngn3-positive cells are thought to have a low proliferative potential, and vHNF1/HNF1β-positive pancreatic epithelial cells appear to be strongly proliferative, it suggests that HNF6-induced vHNF1/HNF1β-positive epithelial cells are a key highly expandable endocrine progenitor pool that gives rise to the less proliferative ngn3-positive cells. The CPA1/PTF1a-double positive cells that recently were demonstrated to be possible ngn3-negative endocrine progenitors (see Fig. 15 and "Overview of endocrine pancreas lineage selection" section) (Zhou et al., 2007) may in fact be regulated by vHNF1/HNF1β, since PTF1a has a vHNF1/HNF1β-binding site (Haumaitre et al., 2005). In addition, vHNF1/HNF1β regulates multiple endocrine-inducing signals (BMP, FGF, retinoic acid) in zebrafish (Song et al., 2007). Late in gestation, HNF6 turns off, which is necessary for what seems to be a normal β-cell inhibition of non-β-cell endocrine growth (Gannon et al., 2000).

The major function of HNF3β/FoxA2 in pancreatic development appears to be in the maturation of endocrine cells (Lee et al., 2002, 2005). HNF3β/FoxA2 is necessary for pdx1 expression in β-cells (Gerrish et al., 2000; Sund et al., 2001). Endoderm-specific HNF3β/FoxA2 ablation led to development of very few glucagon-positive cells,

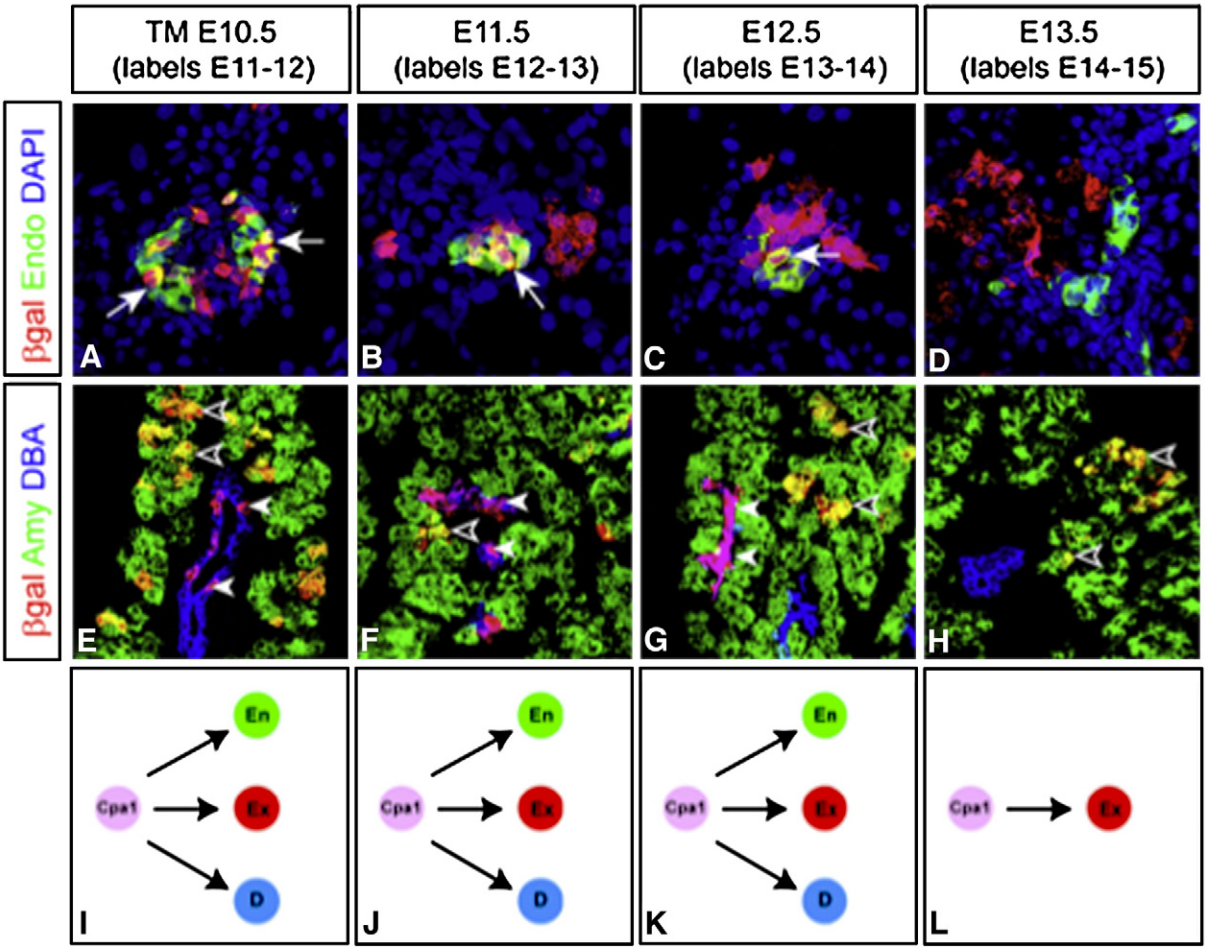

Fig. 15. Carboxypeptidase 1 (cpa1) expressing cells can serve as multi-potent progenitor cells early in embryonic development. Here, the cpa1-positive cells and their progeny are lineage-tagged to express β-galactosidase. Injection of tamoxifen at E10.5, E11.5, and E12.5 induces conditional lineage-tagging at E11–12, E12–13, and E13–14, respectively, showing that cpa1-positive cells will give rise to endocrine cells (yellow cells in A, B, and C), duct cells (red cell in ducts of E, F, and G), as well as acinar cells (yellow cells in E, F, and G). After E14 of gestation, the cpa1-positive cells are only able to give rise to acinar cells (yellow cells in H) (with permission, Zhou et al., 2007).

despite the presence of Arx, brain4, and pax6, suggesting that HNF3β is also necessary for glucagon hormone expression in developing and mature α cells (Lee et al., 2005).

Sox9

Sox9 may serve as a key mediator of the commitment of ngn3-positive endocrine progenitors. Sox9 is expressed during pancreatic development (Piper et al., 2002; Lioubinski et al., 2003; Lee and Saint-Jeannet, 2003; Seymour et al., 2007) and it seems that Sox9 marks a population of pancreatic progenitor cells that can give rise to all pancreatic cell types (Akiyama et al., 2005), and importantly Sox9 is necessary for maintaining the progenitor state as loss of function Sox9 mutants showed pancreatic hypoplasia due to the depletion of a progenitor cell pool (Seymour et al., 2007). Sox9 is expressed in hormone-negative epithelial cells in a similar expression pattern as vHNF1/HNF1β and HNF6, and there are direct interactions between Sox9 protein and the regulatory elements of these two *HNF* genes, suggesting a role for Sox9 in mediating HNF6 and vHNF1/HNF1β control over the formation of ngn3-positive cell populations (Lynn et al., 2007b; Seymour et al., 2007). Consistent with cooperative functions of Sox9 and HNF6, conditional deletion of Sox9 in pancreatic epithelial cells led to the formation of cystic duct structures similar to HNF6 null mutants, with absence of pancreatic endocrine cells (Seymour et al., 2007). In addition, Sox9 can bind and upregulate the *ngn3* gene, and Sox9 appears to be transiently co-expressed with ngn3 (Lynn et al., 2007b; Seymour et al., 2007). Given that precursors to ngn3-positive cells are likely hes1-positive, with ngn3 then turning on when hes1 turns off (Jensen et al., 2000c; Zhou et al., 2007), Sox9 was found to frequently co-localize with hes1 (Seymour et al., 2007), perhaps mediating the transition of endocrine progenitors from a non-endocrine hes1-positive/ngn3-negative state to a hes1-negative/ngn3-positive endocrine-committed state. Persistent expression of Sox9, likely through signal integration with notch signaling, appears to maintain the progenitor population of epithelial cells.

Myt1, GATA factors, HB9, Sox4, Isl1, Hex, Prox1, and Brain4

Myt1 was identified in an embryonic pancreas screen, and the gene encodes two different zinc-finger transcription factors (Myt1a and 1b) that can potentially interact with ngn3 (Gu et al., 2004). These two factors are expressed in the developing pancreas in a pattern similar to ngn3 (Gu et al., 2004; Wang et al., 2007). A dominant-negative form of Myt1a expressed in the ngn3 domain can block α- and β-cell development by 30% and 40%, respectively. Myt1 null mutant mice developed abnormal multi-hormone-expressing pancreatic endocrine cells. Further roles for Myt1 may have been masked by compensatory expression of paralogs Myt1l and Myt3. Thus, overall Myt1 appears to be an important regulator of pancreatic endocrine cell differentiation.

The homeodomain protein Hlxb9, or HB9, is expressed in the pancreatic domain of the foregut, preceding pdx1 expression (Li and Edlund, 2001). Subsequently it is present in the early pancreatic epithelium, and then after E13 is localized to islet cells, and in the adult islet specifically only in β-cells (Harrison et al., 1999; Li et al., 1999). The normal downregulation of HB9 after E12 in the epithelium appears to be critical for further pancreatic development since transgenic overexpression of HB9 using a pdx1-HB9 construct led to global pancreatic hypoplasia and pancreatic intestinalization (Li and Edlund, 2001). HB9 is important for dorsal pancreatic development and formation of all β-cells since an HB9 null mutant mice developed specifically without a dorsal pancreas, and the remaining pancreas showed a 65% reduction in β-cells.

Sox4 is expressed in early pancreatic buds, and then focuses to the islets. Null mutant embryos died by E14.5, but null mutant cultured pancreatic explants had reduced endocrine cell differentiation and a lack of islet formation (Wilson et al., 2005). In zebrafish, Sox4 has two paralogs, 4a and 4b. Sox4b is localized to endocrine cells, and 4b morphants specifically lacked α-cells due to down-regulation of Arx (Mavropoulos et al., 2005).

Isl1 is a lim homeodomain protein discovered based on its ability to bind the insulin gene, and is expressed both in early pancreatic epithelium, as well as in the dorsal mesenchyme in a pattern similar to pbx-1 (Ahlgren et al., 1997; Kim et al., 2002). Isl1-positive cells are post-mitotic and likely downstream of neuroD and upstream of pax6 (Jensen et al., 2000a). Isl1 is necessary for survival of the dorsal mesenchyme as null mutant embryos have no dorsal mesenchyme over the dorsal bud. These embryos lack a dorsal pancreas, but die at E9.5, making ventral pancreas development difficult to evaluate. Embryonic pancreas explant cultures, however, showed that even with replenishment of the absent dorsal mesenchyme by adding back wild-type mesenchyme, only exocrine/acinar pancreas developed, still with no pancreatic endocrine cells. Thus, like pbx-1, isl1 in the epithelium is necessary, cell autonomously, for development of all endocrine cells, whereas isl1 in the mesenchyme appears to be necessary for production of an exocrine/acinar-inductive factor (Ahlgren et al., 1997).

GATA4 and GATA6 are zinc-finger transcription factors that have been shown to be expressed in the developing pancreas (Ketola et al., 2004; Ritz-Laser et al., 2005; Decker et al., 2006). Null mutant mice for these GATA factors die early in development, precluding pancreatic analysis. To circumvent this early lethality, two approaches were used. First, GATA4 or GATA6 were fused to an engrailed repressor domain, and expressed under the pdx1 promoter to mimic a pancreas-specific null mutation (Decker et al., 2006). GATA4-engrailed mice were normal, but expression of GATA6-engrailed under a pdx1 promoter led to severe pancreas disruption or agenesis, suggesting a key role for GATA6 in pancreas specification. A second approach was to use the tetraploid embryonic stem cell aggregation technique to generate GATA4 and GATA6 null mutant mice. In both cases, significant disruption or absence of the ventral pancreas was seen (Watt et al., 2007).

Hex, a hox-related homeodomain protein is expressed in the early pre-pancreatic foregut, and then in the pancreatic epithelium from E13 to E16 (Bort et al., 2004). Prior to pancreas formation, hex is expressed in the anterior leading edge of the ventral foregut endoderm. There hex induces proliferation of the endoderm leading to the hex-expressing endoderm growing further away from the cardiogenic mesenchyme, which in turn allows development of the ventral pancreas (Jung et al., 1999; Deutsch et al., 2001b).

Prox1 is a homeodomain protein that marks pancreatic and liver progenitor cells in the endoderm (Burke and Oliver, 2002), and continues to be expressed in the developing pancreas, becoming focused to ngn3-positive cells and endocrine cells and ducts by E15. Prox1 appears to play an important role in pancreatic development since null mutant mice die at E15 with a small pancreas, and with loss of secondary transition endocrine cells due to premature cell cycle exit (Wang et al., 2005).

Brain4 is a pou-domain protein that was identified based on its ability to bind and activate the glucagon gene (Hussain et al., 1997). It is restricted to α-cell progenitors (Jensen et al., 2000a; Heller et al., 2004) and persists in mature α-cells and in a few PP-cells in the late-gestation embryonic pancreas. Brain4 is not necessary for α-cell formation, as evidenced by the presence of α-cells in the brain4 null mutant mouse (Heller et al., 2004). Brain4 can, however, drive glucagon expression in β-cells in a pdx1-brain4 transgenic mouse.

MicroRNA

MicroRNA's have recently been implicated in pancreatic development. MicroRNA's are small 20-to-22 base RNA molecules that are derived from larger primary RNA transcripts through intranuclear (Drosh enzyme) and cytosolic (Dicer enzyme) processing. These small RNA molecules are able to regulate gene expression at the post-transcriptional level through either silencing or mRNA degradation. MicroRNA's

were found to play a role in normal β-cell function (Poy et al., 2004), and more recently have been studied in developing pancreas. In zebrafish, inhibition of a specific microRNA (mi-375) was found to disrupt normal islet formation, resulting in scattered islet cells (Kloosterman et al., 2007). In mouse embryos, pancreatic mIR-375 was localized to the ducts and epithelium (Lynn et al., 2007a). Pancreas-specific deletion of Dicer, which results in failure to produce mature microRNA, led to a global disruption of pancreatic architecture, with ductal ectasia and a particular loss of β-cells, reminiscent of the HNF6 null mutant phenotype (Lynn et al., 2007a). These effects were thought to be mediated through enhanced notch signaling with elevated hes1 levels.

Along similar lines, a role for microRNA's in post transcriptional regulation of ngn3, the hes1 target gene, has been identified in pancreatic regeneration. Ngn3 was surprisingly absent in regenerating islets after pancreatectomy (Lee et al., 2006). This absence in the pancreatectomy model was recently explained as possibly due to microRNA suppression specifically of the 3′UTR of neurogenin3 transcripts, since a 200-fold increase in the level of neurogenin3 mRNA was seen in these post-pancreatectomy specimens, but without detectable ngn3 protein (Joglekar et al., 2007).

Other RNA binding and inhibiting molecules include Vg1RBP, which was identified as an RNA binding protein that binds to an untranslated region of a newly identified *shirin* gene. *Xenopus* morphants for this binding protein had no insulin or pdx1 expression, and ectopic expression of this RNA binding protein led to ectopic pancreas (Spagnoli and Brivanlou, 2006).

Summary and overview of endocrine pancreas lineage selection

The greatest stimulus to pancreatic developmental biological research comes from a desire to first engineer progenitor or stem cells into mature β-cells for the treatment of diabetes, and second to better understand the mechanisms of exocrine lineage selection that may play a role in the pathogenesis of pancreatic ductal adenocarcinoma. Towards these two goals, a better understanding of specific mechanisms and pathways by which progenitor cells evolve into specific pancreatic cell lineages would seem critical.

In the early foregut, HNF family. by the 10 somite (or E8 stage of mouse development), the regions of the foregut that will become dorsal and ventral pancreas. At the transcription factor level, this specification comes in the form of pdx1/PTF1a co-expression, which diverts cells away from duodenal, hepatic, and bile duct fates. In the absence of pdx1 or PTF1a, an evagination with a few endocrine cells still forms, though it is likely that these cells are only vestigial in nature.

After this early evagination of dorsal and ventral pancreas, with the need for pancreatic determination no longer an issue, multiple factors then seem to determine the selection between acinar, endocrine, and ductal lineages. The overlying mesenchyme, with FGF's, canonical Wnts, and notch signaling, together with the presence of laminin and certain proteoglycans, all play a key role in favoring acinar and ductal differentiation instead of endocrine differentiation. Notch signaling is a critical mediator of this ductal/acinar-versus-endocrine decision. Absence of notch-induced hes1 activity allows ngn3 expression and subsequent commitment to the endocrine lineage.

Notch signaling seems to actively drive duct formation in certain progenitor cells, and such ductal progenitor cells specifically express pdx1 only within the E19 to E12 gestational window (Gu et al., 2002). Beyond the initial notch suppression of endocrine lineages, later persistence of notch signaling will actually prevent acinar differentiation, due to the notch-induced RBP-Jκ binding of PTF1a. As progenitor epithelial cells commit to the acinar lineage, notch-regulated RBP-Jκ is displaced by notch-independent RBP-Jl to start to form the mature PTF1 transcriptional complex, which allows acinar maturation to occur. The decision between acinar and endocrine commitment during the E11 to E14 gestational period appears to occur at the growing tips of the epithelial branches (Zhou et al., 2007) (see Fig. 15). There, PTF1a/CPA1-positive cells will make the commitment (likely under control of notch signaling) to either leave the growing tip and become ngn3-positive, or instead to stay at the tips as a progenitor cell. Then, after E14, those remaining PTF1a/CPA1-positive tip cells are committed to the acinar lineage. The control of the flow of ngn3-negative epithelial cells to become ngn3-positive, endocrine-committed cells seems to involve important interactions between HNF6, vHNF1/HNF1β, and sox9, possibly in that order hierarchically, to determine ngn3-positive-commitment.

Within the endocrine cell compartment, ngn3-positive cells reside at the top of the hierarchy, and all pancreatic endocrine cells go through an ngn-3 positive stage. Beyond ngn-3-positive-commitment, several studies have investigated the mechanism by which the five separate endocrine lineages (α-, β-, γ-, PP-, and ε-cells) are determined among the ngn3-positive cells. Early in pancreatic differentiation, cells are known to be double-positive for insulin and glucagon, and possibly other peptides (reviewed in Teitelman (2004)). It appears, however, through lineage-tagging studies, that these insulin-glucagon double positive cells do not give rise to mature α- or β-cells (Herrera, 2000), and likely undergo apoptosis. Early studies have shown the presence of endocrine cells that express PYY (Upchurch et al., 1994). These PYY-expressing cells were originally identified as possibly PP-expressing cells (Herrera et al., 1991) or NPY-expressing cells (Teitelman et al., 1993), based on what may have been cross-reacting antibodies. PYY may be a marker of early endocrine committed cells, since it is co-expressed with all endocrine cells in the early pancreas, and then is suppressed in many maturing cells, especially maturing β-cells, but is still expressed at low levels in many mature endocrine cells (Liu et al., 2006; Myrsen-Axcrona et al., 1997). Various experimental lineage-tracing approaches suggest that the precursors of α- and β-cells go through a PP-positive phase (Herrera et al., 2002).

At the transcription factor level, there is an early decision between the β/δ-cell-lineage versus the α/PP-cell-lineage. This early decision is mediated by Arx and pax4, which are both expressed in ngn3-positive cells. Arx and pax4 inhibit the effects of one another, but perhaps unknown additional factors are needed to determine the overall balance between these two factors. Arx dominance in a cell favors the α/PP-cell lineage, whereas initial pax4 dominance within a cell favors β/δ cells (see Fig. 14). Interestingly, once the β/δ-cell decision is made, pax4 becomes a δ-cell suppressor specifically in β/δ-committed cells, since Arx/pax4 double-knockout animals have an overabundance of δ-cells. The identification of a transcription factor (or factors) required for the specification of δ-cells, PP-cells or ε-cells remains to be determined.

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