Pancreas and beta-cell development: from the actual to the possible

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The development of insulin-producing pancreatic beta (β)-cells represents the culmination of a complex developmental program. Cells of the posterior foregut assume a pancreatic identity, cells within the expanding pancreatic primordia adopt an endocrine fate, and a subset of these precursors becomes competent to generate β-cells. Postnatally, β-cells are primarily maintained by self-duplication rather than new differentiation. Although major gaps in our knowledge still persist, experiments across several organisms have shed increasing light on the steps of β-cell specification and differentiation. Increasing our understanding of the extrinsic, as well as intrinsic, mechanisms that control these processes should facilitate efforts to regenerate this important cell type in humans.

Introduction
The pancreas serves two major functions: (i) the production of digestive enzymes, which are secreted by exocrine acinar cells and routed to the intestine by a branched ductal network; and (ii) the regulation of blood sugar, which is achieved by endocrine cells of the islets of Langerhans. Type 1 diabetes results from the autoimmune destruction of one islet cell type – the insulin-producing β-cells. Although insulin treatment has saved countless diabetics from early death, it represents an ameliorative treatment rather than a cure. A true cure for diabetes – and a triumph for the concept of ‘regenerative medicine’ – might be achieved by replacing lost β-cells. This was demonstrated in an animal model several decades ago: rats rendered diabetic by the β-cell toxin streptozotocin could be cured by injection of isogenic islets (Ballinger and Lacy, 1972).

Clinical diabetes researchers have since made considerable progress in translating this approach to human patients, although numerous hurdles remain (Naftanel and Harlan, 2004). Not least of these is the scarcity of transplantable islets, which explains the interest in generating β-cells artificially and the corollary interest in understanding how β-cells normally develop. Here, I review pancreas development from the perspective of the β-cell; several additional reviews should be consulted for a more comprehensive view (Edlund, 2002; Jensen, 2004; Slack, 1995). In addition, as the mouse provides the pre-eminent model for pancreatic development, I will focus on this species, although not exclusively. In utero development of the human pancreas has obviously received less attention, but studies suggest that it resembles that of the mouse (Piper et al., 2004).

Because of its obvious medical importance, the pancreas has been subject to decades of close study. The differentiation of exocrine, and to a lesser extent endocrine, cells can be observed in simple histological sections, which allowed these processes to be studied and manipulated before the availability of immunohistochemical methods. As discussed below, the pancreas was among the first organs in which the importance of epithelial-mesenchymal interactions was recognized (Golosow and Grobstein, 1962), and the development of enzymatic and radio-immunoassays for pancreatic gene products conferred remarkable quantitative precision to studies of the differentiation in this organ (Pictet and Rutter, 1972). The pancreas is also among the relatively few organ systems in which systematic promoter-mapping has informed embryology: two of the transcription factors most crucial for pancreas development, Pdx1 (pancreatic and duodenal homeobox 1; also known as Ipfl1) and Ptf1a (pancreas specific transcription factor 1a; also known as PTF1-p48), were identified as DNA-binding proteins on pancreatic promoters (Krapp et al., 1996; Ohlsson et al., 1993). As we shall see, few of the unsolved problems in pancreas and β-cell development are conceptually unique, and further work on this fascinating (and frustrating) organ should shed a more general light on the processes of specification, differentiation and regeneration.

The anatomy of pancreas development
The pancreas is often described as two organs in one, due to the distinct function and organization of its endocrine and exocrine components. In higher vertebrates, it might more properly be thought of as four organs, as it comprises anatomically distinct dorsal and ventral lobes. Referred to in humans as the tail and head, respectively, these two pancreatic lobes arise as thickenings along the dorsal and ventral surfaces of the posterior foregut, near the prospective hepatic endoderm (Fig. 1A). These thickenings are histologically recognizable by approximately 9 days of development (E9.0-E9.5) in the mouse (Wessells and Cohen, 1967). Retaining luminal continuity with the gut tube, these structures evaginate into the surrounding mesenchyme as dense epithelial buds, which subsequently expand, branch and differentiate to yield a fully functional organ system prior to birth (Fig. 1A, Fig. 2). Gut rotation brings the two lobes into close apposition; in humans, their ducal systems undergo partial fusion, although this process is less obvious in rodents.

The bulk of the mature pancreas is comprised of acinar cells, connected to the intestine via a highly branched ductal tree, while islets are primarily scattered through the central regions of the organ. Several separate endocrine cell types comprise the islet: β-cells are the most prominent (50-80% of the total, depending on species) (Brissova et al., 2005; Cabrera et al., 2006), and tend to segregate to the islet core, with other cell types arranged closer to the mantle (see Fig. 3R). Glucagon-producing α-cells are the next most-common cell type; the remaining islet cells, each comprising a small minority of the total, include δ-cells, which produce somatostatin, PP cells, which produce pancreatic polypeptide, and the recently-described ε-cells, which produce ghrelin (Prado et al., 2004; Wierup et al., 2002).

The visible anatomy of the developing pancreas is prefigured by the molecular anatomy of differential gene expression, and few genes are better studied in this regard than the homeodomain

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transcription factor Pdx1. The initial expression of Pdx1 (E8.5-E9.0) marks the pre-pancreatic endoderm before it has visibly thickened (Ahlgren et al., 1996; Guz et al., 1995; Offield et al., 1996) (Fig. 2), and corresponds to the classically defined period of pancreatic specification (Wessells and Cohen, 1967). Early Pdx1 expression is therefore a useful marker of pancreatic identity, although it expands over the next several days of development to encompass the posterior stomach, duodenum and bile duct. Another transcription factor, Ptf1a, is also expressed in the early pancreas, and its endodermal expression remains pancreas-specific throughout development (Kawaguchi et al., 2002).

Genetic-lineage tracing (Box 1) shows that Pdx1+ cells represent progenitors of all the mature pancreatic cell types, including duct, islet and acinar cells (Gu et al., 2002) (Fig. 1B). Pdx1 is expressed broadly in the pancreas during the first several days of pancreas development, as the organ grows and branches (Figs 2, 3). Ptf1a expression is similarly broad at the early stages, and Kawaguchi et al. (Kawaguchi et al., 2002) have found, through lineage tracing with a Ptf1aCre knock-in allele, that Ptf1a+ cells contribute to all three mature lineages. These authors also observed that a minor population of duct and islet cells was not labeled by Ptf1aCre, possibly due to the fact that Ptf1a expression becomes restricted to acinar precursor cells by approximately E13.5 (Fig. 2).

Ptf1a and Pdx1 each play crucial roles in pancreas specification, as discussed below, yet were identified for their roles in adult cell type-specific gene expression. Consistent with its later phase of expression, Ptf1a was identified as an acinar gene activator (Krapp et al., 1996), and Ptf1a-deficient pancreata entirely lack acinar cells (Krapp et al., 1998). Pdx1, meanwhile, was identified as a regulator of the rat insulin 1 (Ins1) gene (Ohlsson et al., 1993), and from E15.5 onwards its expression becomes mainly restricted to β-cells. (Note that rodents have two insulin genes, whereas primates have only one; as the regulation and pancreatic expression of these genes are nearly identical, I refer to them collectively as insulin.) The transitions of Pdx1 and Ptf1a expression coincide with the overall conversion of progenitors to mature endocrine and exocrine cells (Figs 2, 3). This conversion is also reflected in the dynamic expression of the bHLH transcription factor neurogenin 3 (Neurog3, also known as Ngn3), which specifically marks precursors of islet cells (Gu et al., 2002) (Fig. 1). Ngn3+ cells appear in small numbers in the early organ, dramatically increase during mid-embryogenesis, and finally decline

**Box 1. Lineage tracing and pancreas development.** Genetic-lineage-tracing techniques have produced considerable insight into the development of the pancreas and other tissues. The most common lineage-tracing approach uses Cre recombinase, which can delete DNA segments that are flanked by loxP (so-called floxed) sites (Branda and Dynecki, 2004). Mouse strains have been developed in which the ubiquitous expression of a reporter gene, such as LacZ, is prevented by a floxed sequence being placed between the promoter and the reporter (Soriano, 1999); Cre-mediated deletion of this sequence results in heritable marking of the Cre-expressing cell. When Cre expression is driven by a promoter that is active in progenitor cells, the descent of those cells in various classes of differentiated offspring can be traced (Gu et al., 2002; Kawaguchi et al., 2002) (see Fig. 1). Conversely, if Cre is co-expressed with a differentiation marker, such as insulin, one can determine whether differentiated cells change their phenotype in the course of embryogenesis or adulthood. Such an approach was used to refute the hypothesis that mature α- and β-cells are derived from progenitors that co-express glucagon and insulin (Herrera, 2000). Lineage tracing can therefore offer valuable insights that are not obvious from historical studies. A major limitation to the technique, however, is its relatively low resolution: if a particular Cre driver labels multiple differentiated cell types, it is impossible to distinguish whether that driver was active in multipotent progenitors or in separate classes of tissue-restricted precursor cells. A further limitation is that recombination converts an analog input (i.e. the level of Cre expression per cell), into a digital output (either the reporter gene is activated or not). A given Cre transgene can therefore fail to label a tissue of interest either because its promoter is completely silent in the progenitors of that tissue, or because the level of its expression falls below a crucial threshold for recombination. Cre transgenes often give incomplete labeling of specific tissues or cell types, possibly due to this sensitivity threshold.

**Fig. 1. Pancreatic anatomy, lineages and genes.** (A) The dorsal and ventral pancreata (dp and vp, respectively) arise at approximately E8.5 in the mouse (top), from two strips of gut endoderm (marked dp, vp) that are located adjacent to the forming liver (li) within the developing gut endoderm. At E10.5 (middle), the pancreatic primordia bud out into the surrounding mesenchyme and occupy a position between the stomach (st) and intestine (in). Subsequent gut rotation, from E12.5 onward (bottom), brings the two lobes into closer apposition, although each maintains its original ductal connection to the intestine and/or common bile duct (cbd). (B) Lineage tracing indicates that all mature pancreatic cell types derive from progenitors that express Pdx1 and/or Ptf1a (purple), and that a subset of these progenitors go on to express Ngn3 and differentiate into islet cells. Genes listed in red are required for various aspects of the indicated steps, as described in the text.
towards birth (Figs 2, 3) (Gradwohl et al., 2000; Schwitzgebel et al., 2000). Similar to neurons, therefore, islet cells are ordinarily generated during a restricted developmental window; this process is termed neogenesis, in order to distinguish it from the proliferation of pre-existing islet cells.

Ngn3 is expressed in duct-like epithelial cells that are centrally located within the developing pancreas; as these cells differentiate, they downregulate Ngn3, exit the epithelium and aggregate into proto-islet structures (Pictet and Rutter, 1972; Schwitzgebel et al., 2000). Importantly, the spectrum of endocrine differentiation changes through embryogenesis, with α-cells being born quite early (see Box 2) and other cell types, including β-cells, not being generated in significant numbers until E13.5 or later (Herrera et al., 1991; Pictet and Rutter, 1972) (Figs 2, 3). Interestingly, β-cell differentiation occurs simultaneously with that of acinar cells, albeit in a different region of the organ (Fig. 2), during a period termed the secondary transition, which is marked by a massive differentiation of β-cell and acinar cells, as well as by the progressive restriction of Pdx1 and Ptf1a expression to these respective cell types. (E) The organ has assumed its mature form by birth, with distinct islets of Langerhans scattered among exocrine acini and ducts.

Fig. 2. Stages of pancreas development. Schematic cross-sections of developing embryos and organs, representing the progression of pancreas development. (A) Concomittant with specification of the organ, Pdx1 and Ptf1a initiate expression in two restricted domains of the gut endoderm (en). Nearby tissues, including notochord (nt) and aorta (ao), may promote this specification process (Kim et al., 1997; Lammert et al., 2001; Yoshitomi and Zaret, 2004). Mesenchyme (mes) surrounds the thickening buds as the first Ngn3+ pro-endocrine cells appear. (C) Subsequent outgrowth produces a dense epithelial bud, in which early α-cells begin to differentiate. (D) Further growth and branching precedes the secondary transition, which is marked by a massive differentiation of β-cell and acinar cells, as well as by the progressive restriction of Pdx1 and Ptf1a expression to these respective cell types. (E) The organ has assumed its mature form by birth, with distinct islets of Langerhans scattered among exocrine acini and ducts.

Fig. 3. Dynamics of endocrine specification and differentiation. (A-F) Brightfield photomicrographs of Pdx1 immunostaining at various stages of mouse dorsal pancreas (dp) development. From E11.5-E15.5, Pdx1 is expressed throughout the pancreatic epithelium (as well as in the posterior stomach, st), and is subsequently downregulated in acini (ac) and ducts (du) while being maintained in islet β-cells (is). (G-L) Confocal immunofluorescence photomicrographs at equivalent stages, for the pan-epithelial marker E-cadherin (green) and the islet precursor marker Ngn3 (red). Ngn3 expression is rare at E11.5, dramatically peaks during the secondary transition (E13.5-E15.5) and declines again at E17.5, becoming undetectable in neonatal and adult pancreas. Arrowheads indicate proto-acinar clusters at the periphery of the branched epithelium, from which Ngn3 expression is consistently excluded. (M-R) Confocal detection of glucagon (green) and insulin (red). Glucagon+ α-cells are relatively common at E11.5 and E13.5, whereas large numbers of insulin+ β-cells are not detected until after E13.5. From E17.5 onwards, endocrine cells aggregate into recognizable islets, with β-cells at their cores and α-cells distributed peripherally. Scale bar in all images, 50 μm.
‘secondary transition’ (Pictet and Rutter, 1972). Newly differentiated islet cells are non-dividing, although they resume low levels of proliferation towards birth (Sander et al., 2000).

The observation that islets arise from duct-like progenitors has led to the longstanding hope that the duct of the mature pancreas could be coaxed into renewed β-cell neogenesis. As we shall see, it remains unclear whether this occurs in the normal pancreas, or whether it can be induced in vitro. The same uncertainty applies to essentially all potential sources of transplantable β-cells. The only tissue that unambiguously exhibits β-cell neogenesis is the embryonic pancreas, and reproducing this feat elsewhere will probably require that we understand how it happens in situ.

### Specification of the early pancreas

#### Local versus global signals

As early Pdx1 expression marks the newly-specified pancreas, one approach to the problem of pancreas specification is to ask what lies upstream of Pdx1. Promoter-mapping has identified putative activators of Pdx1, including the winged-helix transcription factor forkhead box A2 (Foxa2) (Wu et al., 1997) and the one cut domain, family member 1 (Onecut 1, also referred to as Hnf6) (Jacquemin et al., 2003). Deletion of Foxa2 throughout the early mouse endoderm does not impair Pdx1 activation, however, although subsequent α-cell differentiation is severely impaired (Lee et al., 2005), whereas Hnf6-knockout mice still activate Pdx1 expression, albeit with a slight delay (Jacquemin et al., 2003). Mouse embryos lacking the transcription factors SRY-box containing gene 17 (Sox17) (Kanai-Azuma et al., 2002) or the homeodomain factor Tcf2 (transcription factor 2, also known as Hnf1β) (Haumaire et al., 2005; Sun and Hopkins, 2001) also exhibit absent or severely reduced Pdx1 expression, respectively, although it is unknown whether either factor directly regulates Pdx1. As none of these transcription factors are expressed in a domain as restricted as that of Pdx1, it is most likely that they act cooperatively, potentially in concert with additional intrinsic and extrinsic regulators that are, as yet, undiscovered.

The first extrinsic signals to be implicated in pancreas specification were transforming growth factor-β (TGFβ) proteins of the activin or nodal families, and retinoic acid (RA). Experiments in the frog *Xenopus laevis* (Gamer and Wright, 1995; Henry et al., 1996), later confirmed in the mouse (Tremblay et al., 2000), show that TGFβ signaling is required for endoderm formation. Experiments in frog embryos have also shown that transient exposure to activin and RA can induce pancreas development from isolated animal cap ectoderm (Moriya et al., 2000). Recent studies suggest that TGFβ signaling induces definitive endoderm in mouse and human embryonic stem (ES) cells (D’Amour et al., 2003; Kubo et al., 2004; Yasunaga et al., 2005), and that RA treatment promotes Pdx1 expression and pancreas specification in ES cell-derived endoderm (D’Amour et al., 2006; Micallef et al., 2005). These results strongly support the idea that studying early pancreas development can inform efforts to generate new β-cells.

RA is widely considered to be a mediator of global anteroposterior (AP) patterning, and RA-deficient zebrafish exhibit endoderm anteriorization, which eliminates the liver as well as the pancreas (Stafford and Prince, 2002). In *Xenopus* and mouse, however, RA is required only for dorsal pancreas development, and is dispensable for the development of both the ventral pancreas and liver (Chen et al., 2004; Martin et al., 2005; Molotkov et al., 2005). Although embryologists like to think globally, the best-characterized interactions in pancreas specification seem to act locally. A possible exception, recently described, is fibroblast growth factor 4 (Fgf4), which is expressed in the posterior mesoderm, following gastrulation, and promotes posteriorization of the endoderm (Wells and Melton, 2000). The pancreas appears to arise from cells that receive intermediate levels of Fgf4, and manipulations of Fgf4 levels in the early embryo can expand or contract the pre-pancreatic domain (Dessimoz et al., 2006).

### Distinctive dorsal and ventral specification programs?

Both lobes of the pancreas are sensitive to local signals, positive and negative. RA, synthesized by the aldehyde dehydrogenase family 1, subfamily A2 (Aldh1a2, also referred to as Raldh2) gene, represents one of these positive signals, although its target may actually be outside the endoderm. Tissue-recombination studies have shown that expression of the homeodomain factorIsl1 transcription factor, LIM/homeodomain (Isl1) in the peripancreatic mesenchyme is essential for dorsal pancreas development (Ahlgren et al., 1997). This mesenchymal expression is lost in Raldh2 mutants, possibly accounting for their lack of dorsal pancreas (Martin et al., 2005). The precise spatial relationships between endoderm and mesoderm are also crucial, as revealed by studies of mice lacking the homeodomain transcription factors bagpipe homeobox 1 (Bapx1) (Asayesh et al., 2006) and hematopoietically-expressed homeobox (Hhex) (Bort et al., 2004). These genes act outside the pancreas, but mediate morphogenetic movements that separate the dorsal (Bapx1) and ventral (Hhex) pancreata from inhibitory cues. Other genes that are differentially required for dorsal bud and ventral bud development, including N-cadherin and the receptor tyrosine kinase Kdr (previously known as Flk1), also act outside the endoderm (Esni et al., 2001; Yoshitomi and Zaret, 2004). Hlx/b9 (homeobox gene B9)-knockout mice lack a dorsal pancreas, and, because this gene is co-expressed with Pdx1 in the early pancreas (albeit ventrally as well as dorsally), its knockout is often cited as evidence for intrinsic differences in the specification of the dorsal and ventral lobes (Harrison et al., 1999; Li et al., 1999). Hlx/b9 is also expressed in the notochord, however, which itself is required for dorsal pancreas development (Kim et al., 1997), and the knockout phenotype may actually be due to defects in this tissue, rather than in the pancreas itself.

The idea of distinctive dorsal and ventral programs is also supported by Pdx1 and Tcf2 knockouts – in which the dorsal bud forms and expands somewhat into the mesenchyme, whereas the ventral bud is undetectable (Haumaire et al., 2005; Offield et al.,

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**Box 2. Distinct regulation of early and late islet cell development.** Glucagon* cells appear as early as E9.5 in the developing mouse pancreas, and a subset of these cells appears to co-express insulin (Teitelman et al., 1993). As noted in Box 1, lineage tracing indicates that these double-positive cells are not precursors of mature α- and β-cells (Herrera, 2000), and their functional significance remains unclear. These early islet cells do not express the complement of markers typical of their mature counterparts (Pang et al., 1994; Wilson et al., 2002), and they still form in several knockout mice in which later islet development is impaired (Ahlgren et al., 1996; Collombat et al., 2003; Offield et al., 1996; Sander et al., 2000). A distinct population of early islet cells, expressing insulin exclusively, also forms in zebrafish and *Xenopus* (Afelek et al., 2006; Field et al., 2003), whereas early islet cells are not found in the human embryonic pancreas (Piper et al., 2004). Together, these observations suggest that the secondary transition represents the major phase of islet development, and that regulatory programs governing early and late islet populations are only partially overlapping.
1996) – as well as by Ptf1α knockouts, in which at least a fragment of the dorsal bud forms and begins to expand while the prospective ventral bud ends up contributing to intestine (Kawaguchi et al., 2002). It should be emphasized, however, that much of the Ptf1α−

dorsal bud assumes an intestinal fate as well, suggesting that the difference between the buds is of degree rather than kind. Indeed, the dorsal pancreatic bud starts out physically larger than the ventral, and may therefore have a ‘head start’ in the tissue movements that separate prospective pancreatic tissue from nearby inhibitory cues. In Pdx1, Tcf-2 and Ptf1α mutants, this slight edge may suffice for some of the dorsal pancreas to expand into the permissive mesenchyme, while the ventral pancreas cannot escape signals that divert it towards an intestinal or biliary fate. Future work will probably identify additional signals that act directly on the endoderm, and these findings might be used to achieve the directed differentiation of pancreas and β-cells.

Master regulators of pancreas specification

Perhaps the need for signals that promote pancreas and β-cell development could be bypassed altogether, if we could transduce cells from another organ with a ‘master regulator’ of pancreas development. A number of investigators have taken this approach with the adult liver, using Pdx1 as a candidate master regulator. Initial work indicated that adenoviral misexpression of Pdx1 can induce a small number of insulin+ cells in the liver (Ber et al., 2000); subsequent studies have extended these findings, in some cases demonstrating limited activation of ectopic gene expression as well (Ber et al., 2003; Kojima et al., 2003; Miyatsuka et al., 2003; Sapir et al., 2005). The Pdx1 protein directly binds and activates the insulin promoter (Ohlsson et al., 1993), as well as that of at least one acinar enzyme gene (Swift et al., 1998), and it remains unclear whether the observed induction of pancreatic genes by Pdx1 represents true transdifferentiation or an artificial ‘forcing’ of pancreas-specific gene expression.

From a therapeutic standpoint, this may be irrelevant, and reprogramming of liver cells by the expression of Pdx1 and/or other β-cell regulatory factors (described below) remains an attractive possibility. From our perspective as developmental biologists, however, these studies cannot settle the question of whether Pdx1 acts as a true specification factor for the pancreas. Indeed, efforts to induce ectopic pancreas via Pdx1 misexpression in the embryo have proven unsuccessful (Afelik et al., 2006; Grapin-Botton et al., 2001; Heller et al., 1998; Horb et al., 2003). By contrast, expression of the hybrid protein Pdx1-VP16, in which Pdx1 is fused to the strong transcriptional activator VP16, can fully convert embryonic Xenopus liver to pancreas, including both exocrine and endocrine components (Horb et al., 2003). Similar results were obtained upon transducing human hepatoma cells with Pdx1-VP16, and this fusion construct is now being successfully applied by liver-to-β-cell researchers (Imai et al., 2005; Kaneto et al., 2005; Li et al., 2005).

Endogenously, of course, Pdx1 does not have the benefit of a VP16 activation domain, but it may exert similar effects via collaboration with Ptf1α. This is suggested by recent studies of mice that lack hairy and enhancer of split 1 (Hes1), a transcriptional repressor that mediates Notch signaling in the pancreas and elsewhere (Fukuda et al., 2006; Sumazaki et al., 2004). In addition to their intra-pancreatic phenotype, as discussed below, Hes1 mutants develop patches of ectopic pancreas tissue in posterior stomach, duodenum and bile duct. These tissues normally express Pdx1, and, in the absence of Hes1, they also express Ptf1α, precisely where pancreatic tissue later forms. Moreover, Ptf1α is required for ectopic pancreas development in Hes1 mutants, suggesting that its co-expression with Pdx1 may be necessary and sufficient for pancreatic specification; this hypothesis is supported by a recent misexpression study in Xenopus (Afelik et al., 2006). The fact that Hes1 normally represses Ptf1α suggests that manipulation of the Notch pathway could provide a tool for the directed development of pancreas in vitro, although it remains to be shown that the requirement for Hes1 in the early gut reflects the action of Notch signaling per se.

Establishing endocrine and exocrine compartments: everything in its right place

Are cells of the early pancreas multipotent, capable of contributing to both the endocrine and exocrine compartments, or do they arise already committed to one or the other lineage? The only direct evidence bearing on this question is a single report, based on retroviral ‘tagging’ in vitro, which showed that single cells in the E11.5 dorsal bud can give rise to both acinar and islet descendents (Fishman and Melton, 2002). This conclusion is supported by indirect evidence from embryological and genetic manipulations that alter the balance of endocrine and exocrine development. These include studies of epithelial-mesenchymal interactions, which for decades have been known to promote growth and acinar differentiation of the bud-stage pancreas (Golosow and Grobstein, 1962). Cultured without mesenchyme, pancreatic epithelium shows little proliferation and fails to produce acinar cells (Horb and Slack, 2000); more-recent studies have shown that endocrine differentiation is actually enhanced in the absence of mesenchyme, as though multipotent progenitors choose islet fates by default (Duvillie et al., 2006; Gittes et al., 1996; Miralles et al., 1998). Alternatively, the removal of mesenchyme may enhance the proliferation of an endocrine-restricted progenitor population; improved lineage-tracing techniques, permitting robust labeling of single cells and their progeny, will be required to distinguish between these possibilities.

A similar enhancement of endocrine development is observed in pancreata lacking one of several Notch signaling components [see Lai (Lai, 2004), for a recent review of the Notch pathway]. The overall role of Notch signaling in the pancreas appears to be to delay the differentiation of progenitor cells until the secondary transition, when an unknown mechanism renders them competent to assume β-cell, acinar and other later-born fates. Knockouts of the Delta-family ligand delta-like-1 (Dll1), the Notch DNA-binding partner recombining binding protein suppressor of hairless (Rbpsuh, also referred to as RBPJκ and CBF1) or the target gene Hes1 all exhibit pancreatic hypoplasia due to premature differentiation of progenitor cells into endocrine α-cells (Apelqvist et al., 1999; Jensen et al., 2000). Sustained Notch signaling, by contrast, inhibits acinar, as well as islet, differentiation (Esnin et al., 2004; Hald et al., 2003; Murtaugh et al., 2003). The mesenchyme appears to enhance Notch activity within the epithelium through the secretion of growth factors such as Fgf10 (Bhushan et al., 2001; Duvillie et al., 2006; Hart et al., 2003; Miralles et al., 2006; Norgaard et al., 2003). By extending the temporal window of epithelial Notch signaling, the mesenchyme thus indirectly promotes acinar and β-cell development, although it may also produce additional signals that more directly influence the exocrine-endocrine decision (Li et al., 2004).

Whereas Notch signals negatively regulate endocrine development, certain members of the TGFβ superfamily appear to promote endocrine specification, possibly at the expense of exocrine. Thus, early pancreatic-bud explants treated with the ligand TGFβ1 in vitro develop increased numbers of islet cells, and fewer acini (Sanvito et al., 1994). TGFβ1 activates the MAD homolog 2
(Smad2) and Smad3 signal transduction pathway (reviewed by Massague et al., 2005), which is also activated by activin family ligands, and treatment of early buds with the activin antagonist follistatin enhances acinar development, while inhibiting that of islets (Miralles et al., 1998). This result suggests that endogenous activin–family ligands normally promote endocrine development; consistent with this hypothesis, the developing pancreata of mice that lack the type IIB activin receptor have severely reduced islet mass, but apparently normal acinar tissue (Kim et al., 2000).

Paradoxically, however, mouse embryos heterozygous for the Smad2 gene, which should be impaired for activin and TGFβ signaling, exhibit increased numbers of Ngn3+ islet precursor cells (Harmon et al., 2004). Precisely how and when activin/TGFβ-family members act in the developing pancreas therefore remain important open questions.

The central intrinsic regulator of endocrine specification is Ngn3, the expression of which is necessary and sufficient for endocrine development in the pancreatic endoderm (Apelqvist et al., 1999; Gradwohl et al., 2000; Grapin-Botton et al., 2001; Schwitzgebel et al., 2000) (although see Box 3). The scattered distribution of Ngn3+ cells within the epithelium (see Fig. 3G-L), reflects, in part, repression by Notch activity, because Ngn3+ cell numbers increase in the absence of Dll1 or Rbpsuh, prefiguring precocious endocrine differentiation (Apelqvist et al., 1999; Fujikura et al., 2006). Furthermore, Ngn3 is a target for direct repression by Hes1 (Lee et al., 2001). In addition to Dll1, the developing pancreatic epithelium expresses Notch ligands of the Serrate/Jagged family, and two recent studies confirm that this ligand network is involved in endocrine development. First, morpholino knockdown studies in zebrafish embryos show that loss of specific Jagged-family ligands causes ectopic islet cell differentiation (Zecchin et al., 2006). Notch activation by Serrate/Jagged ligands is inhibited by glycosyltransferases of the Fringe family (Haltiwanger and Stanley, 2002), and a second recent study demonstrates that the expression of one family member, manic fringe homolog (Mfng), partially overlaps with that of Ngn3 in the developing pancreas (Xu et al., 2006). Moreover, misexpression of Mfng was found to induce endocrine cells (primarily α-cells), apparently via the inhibition of Notch and the upregulation of Ngn3. Together, these studies emphasize the crucial role of the Notch-Ngn3 regulatory axis in islet development, and highlight the need to better understand Notch ligand distribution and function in the developing pancreas.

Transcription of Ngn3 presumably reflects the input of positive, as well as negative, regulators, the best-characterized of which is Hnf6 (Jacquemin et al., 2000). Hnf6 directly binds and activates the Ngn3 promoter in vitro and is genetically required for its expression. Hnf6 is expressed throughout the developing pancreas, however, as well as in domains of the foregut that lack Ngn3 expression (Landry et al., 1997; Rausa et al., 1997), and Hnf6−/− mutants exhibit numerous additional defects in pancreatic organogenesis, to which their loss of Ngn3 may be secondary (Jacquemin et al., 2003; Pierreux et al., 2006).

Neither Notch activity nor Hnf6 expression can obviously account for another aspect of Ngn3 expression, rarely discussed but easy to see: its segregation into a central, ‘pro-endocrine’ domain, and exclusion from a peripheral ‘pro-exocrine’ region (Fig. 3). Although there are many candidates, no signals have yet been identified that can account for such a restricted expression pattern, or indeed for the overall morphological organization of the developing pancreas. Ngn3+ cells rapidly activate a battery of transcription factors that constitute a ‘core program’ of endocrine development, in that they appear to be expressed in all endocrine precursors, and are required, quantitatively or qualitatively, for the development of many or all islet cells (Fig. 4A, Table 1). These include Isil, neurogenic differentiation 1 (Neurod1) and insulinoma-associated 1 (Insma1/IA1) (Ahlgren et al., 1997; Gierl et al., 2006; Naya et al., 1997). Ngn3 is

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**Box 3. Islet development without Ngn3?** A recent human genetics study raises the startling possibility that human β-cells can develop without Ngn3 function (Wang et al., 2006). Three unrelated children were identified with congenital malabsorptive diarrhea, and biopsies revealed an almost complete absence of endocrine cells in their intestines, a phenotype similar to that of Ngn3-null mice (Jenny et al., 2002). Sequencing revealed that these patients carry homozygous missense mutations in the bHLH gene NGN3, which encode NGN3 completely non-functional in overexpression experiments. Importantly, the affected children were not hyperglycemic at birth, although two of them later (aged 8 years) developed type 1 diabetes of unknown etiology. (The third child developed severe liver disease and died at age 3.) The pancreata of these children were not examined in infancy to confirm the presence of β-cells, and genetic experiments (e.g. targeted replacement of wild-type mouse Ngn3 with one of these NGN3 mutants) will be required to confirm that these mutations represent nulls. Nonetheless, the most straightforward interpretation is that another pathway can compensate for the lack of NGN3 in human pancreas development (Wang et al., 2006). Ngn3-null mice are completely devoid of islet cells at birth, and die shortly thereafter (Gradwohl et al., 2000). If this early death, possibly caused by malabsorptive diarrhea, as in humans, could be prevented, it is formally possible that a novel pathway of Ngn3-independent islet development could be uncovered postnatally (Wang et al., 2006). Such experiments would be especially important if the NGN3 mutants identified in this study prove to be true nulls.

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**Fig. 4. Pathways of islet-subtype specification.** A hypothetical lineage diagram for Ngn3+ endocrine precursors, which give rise to all islet cell types (here, a single Ngn3+ cell is depicted giving rise to each subtype, whereas, in reality, the potential of a given Ngn3+ cell may be more restricted). In red are genes required for various steps of this process; some of which (top) appear to function similarly in all subtypes, constituting a core program of endocrine development, whereas others (bottom) are differentially required for specific subtypes (also see Table 1). Although more genes have been implicated in β-cell development than in that of other subtypes, this is probably due to the greater effort focused on this cell type, rather than an inherently greater complexity in its developmental program.
required for the expression of all known islet-specific transcription factors (Collombat et al., 2003; Gradwohl et al., 2000; Mellitzer et al., 2006), and presumably their collective absence accounts for the lack of islet cells in Ngn3−/− pancreata. Precisely how these factors individually promote endocrine development is not well understood. For instance, NeuroD1 is thought to activate the insulin promoter, yet NeuroD1−/− pancreata still generate insulin-expressing β-cells, which are later eliminated by apoptosis (Naya et al., 1997; Naya et al., 1995).

We began this section by considering whether early Pdx1+ progenitors are truly multipotent, and a similar question applies to Ngn3+ cells. Although conventional lineage diagrams depict single Ngn3+ cells giving rise to multiple islet cell types (Fig. 4), it is equally plausible that five (or more) independent classes of Ngn3+ cells exist, one for each mature cell type in the islet. Distinguishing these models, however, will require lineage-tracing techniques of considerable higher resolution than are currently available.

**β-cell precursors: what do they know and when?**

Table 1 lists genes that exhibit expression patterns and knockout -cell precursors: what do they know and when? Depending on the expression, regulation and function of the transcription factor paired box gene 4 (Pax4) in β-cell development. First, Pax4 is expressed exclusively during embryogenesis, and tracing the initiation and perdurance of its expression (by staining for β-galactosidase protein in Pax4−/− mice) indicates that it is expressed strongly in β-cell precursors but only transiently, if at all, in the α-cell lineage (Wang et al., 2004). Second, Pax4 is not expressed in the absence of Ngn3 (Gradwohl et al., 2000), and Ngn3 appears to be a direct upstream activator of the Pax4 promoter (Smith et al., 2003). Immediately upon activation, Pax4 appears to be co-expressed with another transcription factor, aristaless related homeobox (Arx), also an Ngn3 target gene, but thereafter Pax4 and Arx each begin to repress the expression of the other. Precursor cells subsequently partition into separate populations that express either Pax4 or Arx, and these populations develop into β- or δ-cells or α-cells, respectively (Collombat et al., 2003). Finally, Pax4 mutants develop an excess of α-cells at the expense of β- and δ-cells (Sosa-Pineda et al., 1997). Together, these results support the conventional model of β-cell specification, depicted in Fig. 4, in which Ngn3+ cells adopt a β-cell fate, as opposed to an α-cell fate, partly due to maintained Pax4 expression [the role of Pax4 in the somatostatin lineage is apparently more complex; Collombat et al. (Collombat et al., 2005)].

The major unknown in this scheme is how the antagonism between Pax4 and Arx is resolved in such a way as to generate a reproducible balance between α- and β-cell numbers. One possibility is that, once Ngn3 activates expression of Pax4 and Arx, extrinsic signals regulate the repressive activity of these transcription factors, tipping the balance of their mutual repression and favoring one lineage over another. Identifying such signals would certainly enhance the prospects for controlled β-cell development in vitro. Another possibility is that the expression levels of these two factors are subtly biased from the outset, such that β-cells develop from precursors that initiate higher levels of Pax4, and vice-versa for α-cells and Arx. According to this hypothesis, Pax4-Arx cross-repression would essentially refine a pre-existing pattern established before Ngn3 expression. There are no strong candidate molecules that would support either hypothesis (e.g. transcription factors that would cooperate with Ngn3 in activating higher levels of Pax4 in β-cell precursors), but further investigation of Pax4 and Arx should continue to illuminate islet subtype specification.

The question of when β-cell specification begins is relevant to other factors involved in the process. Several transcription factors thought to act relatively late in β-cell specification, including Nkx2.2, Nkx6.1 and Hlxb9, are expressed in not only β-cell precursors but also widely throughout the early progenitor population (Harrison et al., 1999; Li et al., 1999; Sander et al., 2000; Sussel et al., 1998). This early expression may be an epiphenomenon, or it may represent the first stage of a progressive process by which Ngn3+ cells acquire β-cell differentiation competence. In liver development, by analogy, transcriptional activators of hepatocyte genes actually begin to modify the chromatin of their targets within multipotent progenitor cells, well

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**Table 1. Genes required for endocrine development**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Islet cell types affected in knockout</th>
<th>References</th>
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| Islet core program
| Isl1       | All (absent)                         | Ahlgren et al., 1997      |
| NeuroD     | All (reduced)                        | Naya et al., 1997         |
| Insulin1A1 | α, β- and δ-cells (reduced)          | Gierl et al., 2006        |
| Islet subtype specification
| Pax6       | α, β- and δ-cells (reduced); e-cells (increased) | Heller et al., 2005; Sander et al., 1997 |
| Pax4       | β-cells (strongly reduced); δ-cells (absent); α-cells (increased) | Collombat et al., 2005; Sosa-Pineda et al., 1997 |
| Arx        | α-cells (absent); β- and δ-cells (increased) | Collombat et al., 2003 |
| Nkx2.2     | β-cells (absent); α- and PP-cells (reduced); e-cells (increased) | Sussel et al., 1998; Wang et al., 2004 |
| Nkx6.1     | β-cells (absent)                     | Sander et al., 2000       |
| Hlxb9      | β-cells (reduced)                    | Harrison et al., 1999; Li et al., 1999 |
| Foxa2      | α-cells (strongly reduced)            | Lee et al., 2005          |

Genetic analyses implicate these genes in various aspects of islet development, either acting in a general program that generates most or all endocrine cells, or to promote the development of specific endocrine cell subtypes. As indicated, the latter class includes genes whose deletion phenotype causes decreases in one or more cell types balanced by increases in others, indicating that they act to bias the cell fate decision of a multipotent progenitor. Not listed are genes whose deletion compromises islet cell function, without preventing specification or differentiation.
before those cells have committed to a hepatocyte fate (Zaret, 2002). In the case of Nkx2.2, separate regulatory elements appear to drive its expression in early progenitor cells, in Ngn3⁺ precursors and in differentiated β-cells (Watada et al., 2003). If Nkx2.2 is required prior to Ngn3 expression, then deleting the progenitor-specific element—which has not yet been precisely mapped—should reproduce the β-cell deficiency observed in Nkx2.2⁻/⁻ mice. If similar elements can be defined for Nkx6.1 and Hnf4α, the same prediction holds for these factors as well.

The pancreas is not unique in generating different cell types over time from a common progenitor population: a similar phenomenon occurs in retinal development, where an intrinsic ‘clock’ appears to control the changing developmental competence of progenitor cells (Livesey and Cepko, 2001). Studies of retinal development have benefited from the use of very fine-scale lineage-tracing techniques (Turner and Cepko, 1987), and analogous approaches might be used to discern whether a similar timing mechanism exists in the pancreas, as well as to illuminate other mysteries of endocrine specification and differentiation.

**Adult β-cells: keep on keeping on**

A flurry of papers in the past several years has shown that the maintenance of β-cell function and numbers in postnatal life relies on mechanisms that appear to be distinct from those used to generate these cells in utero. With respect to function, β-cell differentiation is accompanied by activation of a novel transcriptional network that is involved in the maintenance of β-cell-specific gene expression (Boj et al., 2001). Haplo-insufficient mutations in components of this network cause the human syndrome maturity-onset diabetes of the young (MODY), which is characterized by the progressive impairment of insulin secretion (reviewed by Servitja and Ferrer, 2004). Among the most crucial members of the MODY gene network is Pdx1 (Stoffers et al., 1997), which appears to activate a number of β-cell-specific genes, in addition to insulin (Carty et al., 1997; Chakrabarti et al., 2002; Waeber et al., 1996; Watada et al., 1996). Consistent with these observations, β-cell function is also rapidly lost upon β-cell-specific inactivation of Pdx1 in mice (Ahlgren et al., 1998; Holland et al., 2005), highlighting the crucial role that this developmental factor plays in adulthood. (Indeed, while the targets of Pdx1 in mature β-cells are relatively well-characterized, those mediating its functions in the developing organ are unknown.)

Additional regulators of β-cell function include Neurod1, of which heterozygous mutations also produce MODY in humans (Malecki et al., 1999), as well as the basic leucine-zipper (bZIP) transcription factor Mafa (v-maf musculoaponeurotic fibrosarcoma oncogene family, protein A) (Zhang et al., 2005). It should be emphasized that, although both of these genes were originally implicated in pancreas function through their binding to essential regulatory elements of the insulin promoter (Kataoka et al., 2002; Matsuoka et al., 2003; Naya et al., 1995; Olibert et al., 2002), neither gene is absolutely required for insulin expression in vivo (in contrast to Pdx1). Issues of redundancy remain to be resolved regarding the function of these proteins at the insulin promoter; Mafa, for example, is co-expressed with the related CMAF (v-maf oncogene homolog) gene, which also activates the insulin promoter in vitro (Nishimura et al., 2006). Finally, global and tissue-specific knockouts in mice reveals that the Foxa1 and Foxa2 transcription factors are required for glucose-stimulated insulin secretion by β-cells, although not for insulin mRNA expression (Lantz et al., 2004; Vatamaniuk et al., 2006). Importantly, prenatal β-cell development occurs normally in the absence of Mafa, Foxa1 or Foxa2, emphasizing the distinction between genes that regulate specification and differentiation and those that regulate mature cellular function. It will be of considerable interest to determine whether Maf-family or Foxa-family mutations underlie any diabetes-related conditions in humans.

With respect to β-cell numbers, mice that lack the cyclin-dependent kinase Cdk4 have a normal complement of β-cells at birth, but the failure of these cells to subsequently proliferate produces progressive hyperglycemia (Rane et al., 1999). A similar phenomenon occurs in mice deficient for cyclin D1 and/or cyclin D2 (Georgia and Bhushan, 2004; Kushner et al., 2005), and in mice with a pancreas-specific deletion of the Foxm1 (forkhead box M1) transcription factor (Zhang et al., 1999). Essentially opposite results are obtained in mice heterozygous for the tumor-suppressor gene Men1 (multiple endocrine neoplasia 1), or in those carrying a gain-of-function cdk4 allele: normal pancreas development occurs until birth, followed by progressive β-cell hyperplasia (Crabtree et al., 2001; Rane et al., 1999). These results imply that, whereas β-cell production in utero depends on progenitor cell differentiation, their postnatal maintenance and expansion occurs primarily through proliferation. This has been confirmed by a genetic-lineage-tracing study, in which differentiated β-cells were labeled in adult mice and followed over several months. Despite an increase in total β-cell numbers during this time, the proportion of labeling did not decrease, indicating that the new cells arose from pre-existing β-cells (Dor et al., 2004).

The signals controlling β-cell proliferation are becoming increasingly well-understood. Mice lacking the insulin receptor in β-cells exhibit a severe reduction in adult β-cell mass (Otani et al., 2004), whereas β-cells deleted for Pten, a negative regulator of insulin-receptor signaling, undergo hyperplasia (Stiles et al., 2006). If this and other pathways could be manipulated in vitro, clinicians might be able to grow unlimited numbers of β-cells from scarce donor islets, providing transplantable material without even having to worry about developmental biology. Indeed, recent studies of diabetic mice suggest that blocking autoimmunity at early stages of the human disease could restore normoglycemia through the expansion of residual β-cells (Chong et al., 2006; Nishio et al., 2006; Suri et al., 2006).

Does β-cell neogenesis ever occur in the adult (e.g. through a pancreatic stem cell)? In uninjured mice, as summarized above, the weight of evidence is negative. Islet cell mass can regenerate following partial pancreatectomy, however, and strong correlative evidence from rodent studies suggests that at least part of this regenerative response reflects neogenesis from pre-existing duct cells or acini (reviewed by Bonner-Weir and Weir, 2005; Lardon and Bouwens, 2005). It should be noticed that the lineage-tracing study described above failed to find evidence for β-cell neogenesis following partial pancreatectomy (Dor et al., 2004), and an independent study found that Ngn3 expression, which marks islet precursors in utero, could not be detected in pancreata recovering from partial pancreatectomy (Lee et al., 2006). Nonetheless, neither of these studies focused on the precise regions of the pancreas damaged during surgery, in which neogenesis might be most pronounced (Sharma et al., 1999). Past and present studies may also have differed in the severity or type of injury models that were applied. It now seems imperative to replicate past correlative studies with genetic-lineage-tracing techniques to answer this controversial and fundamental question.

Even if β-cell neogenesis does not occur endogenously, it may be experimentally inducible ex vivo. For example, transducing cultured human duct cells with Ngn3 activates many islet-specific
genes, including insulin, albeit at low levels (Gasa et al., 2004; Heremans et al., 2002). A recent report indicates that non-endocrine epithelial cells of the adult human pancreas (presumably ductal or acinar) can be induced to adopt a β-cell fate by signals from fetal pancreas (Hao et al., 2006). Similar findings in the rat (Dudek et al., 1991) suggest that an embryonic differentiation program can be reactivated in adult pancreatic ducts, consistent with the overlap in gene expression that is seen between mature ducts and embryonic progenitors (Pierreux et al., 2006; Piper et al., 2004). Moreover, a recent study that combined in vitro culture with genetic-lineage tracing suggests that adult acinar cells can be coaxed to a β-cell fate (Minami et al., 2005), a result consistent with independent work documenting the plasticity of differentiation in mature acini (Means et al., 2005). As ducts and acini are currently discarded during human islet isolation, the prospect of spinning this dross into gold is highly attractive.

Conclusion
The past decade has seen remarkable progress in understanding the molecular program of islet specification and differentiation. Nevertheless, we still cannot answer a number of questions raised in the premolecular era. Why, for instance, do α-cells begin to differentiate days earlier than β-cells? Why, in turn, does β-cell differentiation occur simultaneously with that of acinar cells, yet in a spatially distinct region of the developing organ? What is the anatomical relationship between the duct-like progenitors of the embryo and the mature ducts of the adult, and can the functional program of the former be re-established in the latter? The application of molecular genetics to the study of β-cell development has itself raised almost as many questions as it has answered. When, for example, do Ngn3+ cells become terminally specified, and to how many different cells and cell types can a single Ngn3+ cell contribute? Although novel technical approaches are clearly required, for example a method of easily tracing the progeny of single progenitor cells, the history of the field encourages optimism that those approaches will be found and exploited.

Another set of open questions in pancreas development concerns the role of extracellular signaling pathways in cell fate specification. To date, the only strong genetic evidence for such a role comes from studies of the Notch, and to a lesser extent TGFβ, pathways. Testing the in vivo role of other signals will require that we carefully consider the different stages of β-cell specification, across which a single molecule might have multiple roles. The field will also benefit from the development of better in vitro culture techniques. It is easy enough to buy a growth factor and add it to a cultured pancreatic bud, yet relatively few consistent results have been reported from such studies, probably owing to minor technical differences.

One of the strengths of pancreas development research is its diversity of approaches, and maintaining that diversity is essential for the field to progress towards the controlled generation of human β-cells. Although the mouse offers numerous experimental advantages, important contributions have been made by studies in the embryos of the chick, fish and frog (e.g. Afefik et al., 2006; Dessimoz et al., 2006; Zecchin et al., 2006), and work on human tissue remains essential as well. Each stage of β-cell development, as summarized in Figs 1, 2, offers a unique starting point for potential therapeutic β-cell neogenesis: re-specification of hepatocytes, for example, via Pdx1-VP16 expression, or β-cell induction from pancreatic duct cells by Ngn3. It may also be possible to fully recapitulate the process of pancreas and β-cell development in ES cells, and the recent efforts of D’Amour et al. (D’Amour et al., 2006) along these lines suggest that such an approach will hinge on a robust understanding of normal developmental mechanisms. I must emphasize that, even with an unlimited supply of perfect β-cells, we will have to clear numerous hurdles before curing diabetes, not least of which is the autoimmune reaction that causes the disease in the first place. Nonetheless, the future study of pancreas development will not only enrich our basic understanding of organogenesis, but should also advance translational efforts towards that ultimate end.

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