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Review

Specifying pancreatic endocrine cell fates

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Abstract

Cell replacement therapy could represent an attractive alternative to insulin injections for the treatment of diabetes. However, this approach requires a thorough understanding of the molecular switches controlling the specification of the different pancreatic cell-types *in vivo*. These are derived from an apparently identical pool of cells originating from the early gut endoderm, which are successively specified towards the pancreatic, endocrine, and hormone-expressing cell lineages. Numerous studies have outlined the crucial roles exerted by transcription factors in promoting the cell destiny, defining the cell identity and maintaining a particular cell fate. This review focuses on the mechanisms regulating the morphogenesis of the pancreas with particular emphasis on recent findings concerning the transcription factor hierarchy orchestrating endocrine cell fate allocation.
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1. Introduction

The pancreas, an abdominal gland lying dorsal to the stomach in humans, plays a crucial function in maintaining nutritional homeostasis, through the synthesis/secretion of enzymes and hormones. The mammalian pancreas includes three tissue-types: acinar, ductal and endocrine. The exocrine pancreas consists of acinar cells secreting digestive enzymes, such as lipases, proteases and nucleases, which are emptied into the pancreatic duct forming an elaborately branched network of tubules composed of epithelial duct cells. The latter produce bicarbonate ions and electrolytes, which together with the exocrine enzymes, are transported through the main duct into the duodenum, where they contribute to food processing (Githens, 1994). The endocrine tissue is organized into small spherical clusters of cells called islets of Langerhans, representing only a small proportion of the entire pancreas. Each islet is composed of four cell-types, α-, β-, δ- and pancreatic polypeptide (PP) cells, which produce and secrete the hormones glucagon, insulin, somatostatin and pancreatic polypeptide, respectively. A fifth endocrine peptide hormone was recently discovered: ghrelin, a growth hormone-releasing and orexigenic peptide originally characterized in enteroendocrine cells of the gastro-intestinal tract, is also produced in the endocrine tissue by most α-cells, but also by a new independent islet cell type called the ε-cell (Heller et al., 2005; Prado et al., 2004; Wierup et al., 2002). The function of ghrelin in the pancreas remains unclear.

During mouse embryogenesis, the pancreas develops at the foregut/midgut junction from a pre-patterned region of the primitive gut endoderm (bracket in Fig. 1A). The first morphological indications of pancreas genesis are distinguishable around Embryonic day 9.5 (E9.5) with the emergence of two evaginations called pancreatic buds (Fig. 1B), first in the dorsal, and subsequently in the ventral position of the gut tube (Pictet et al., 1972). Not only is the development of these two pancreatic outgrowths temporally distinct, but they seem to result from independent regulatory signalling pathways (Slack, 1995). Next to the formation of the dorsal and ventral pancreatic primordia, the pancreatic epithelium further proliferates invading the

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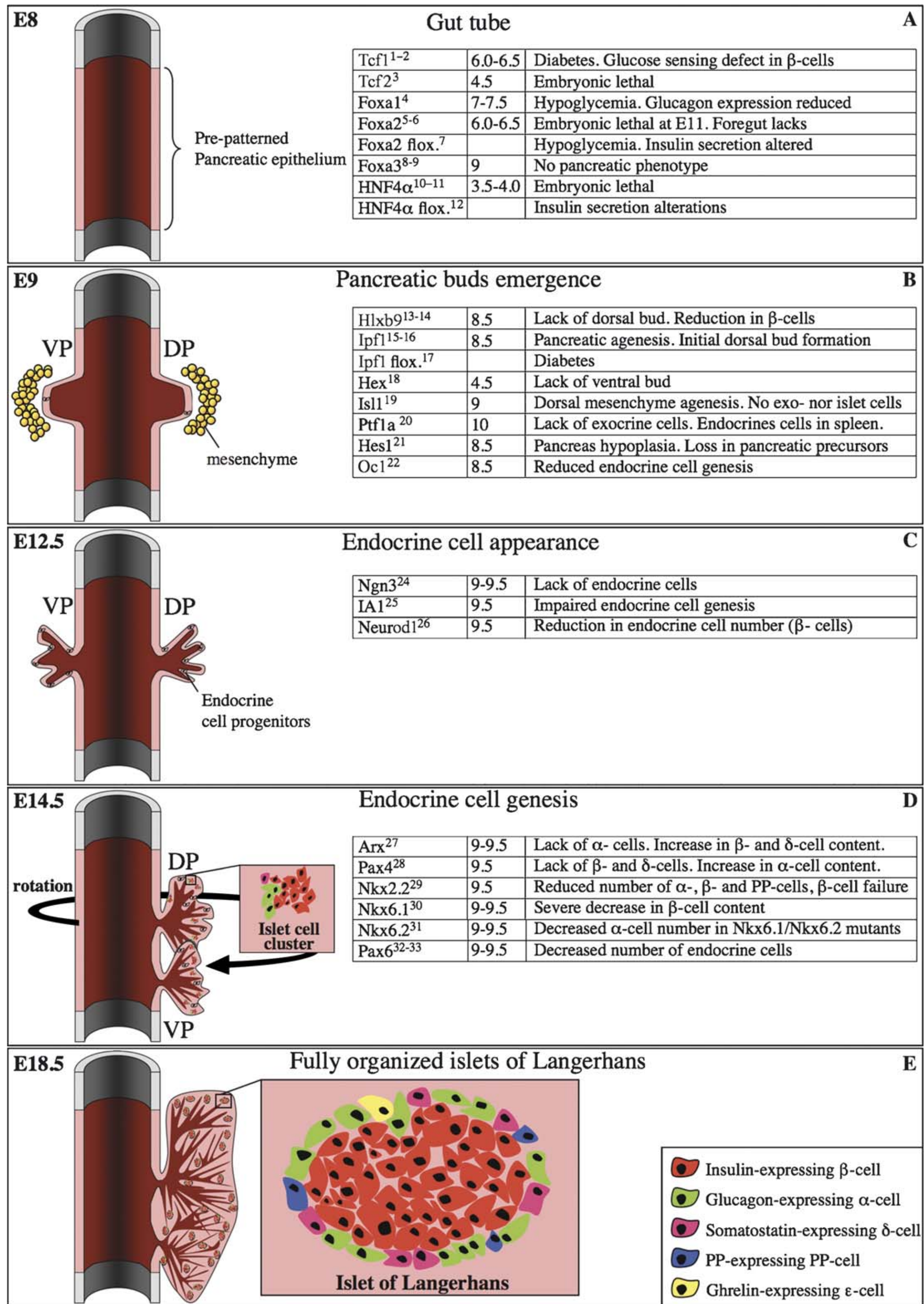

Fig. 1. Chronological progression of the embryonic mouse pancreas morphogenesis. A multi-part table presenting selected key genes involved in the development of the pancreas, their earliest expression and a short description of the phenotypes of the corresponding loss-of-function mutant mice, as well as the corresponding references is provided. 1 – (Shih et al., 2001), 2 – (Pontoglio et al., 1998), 3 – (Coffinier et al., 1999), 4 – (Kaestner et al., 1999), 5 – (Ang and Rossant, 1994), 6 – (Weinstein et al., 1994), 7 – (Sund et al., 2001b), 8 – (Kaestner et al., 1998), 9 – (Shen et al., 2001), 10 – (Chen et al., 1994), 11 – (Duncan et al., 1997), 12 – (Gupta et al., 2005), 13 – (Harrison et al., 1999), 14 – (Li et al., 1999), 15 – (Jonsson et al., 1994), 16 – (Ahlgren et al., 1996), 17 – (Ahlgren et al., 1998), 18 – (Bort et al., 2004), 19 – (Ahlgren et al., 1997), 20 – (Krapp et al., 1998), 21 – (Apelqvist et al., 1999), 22 – (Jacquemin et al., 2000), 23 – (Pin et al., 2001), 24 – (Gradwohl et al., 2000), 25 – (Mellitzer et al., 2006), 26 – (Naya et al., 1997), 27 – (Collombat et al., 2003), 28 – (Sosa-Pineda et al., 1997), 29 – (Sussel et al., 1998), 30 – (Sander et al., 2000), 31 – (Henseleit et al., 2005), 32 – (St-Onge et al., 1997), 33 – (Sander et al., 1997).

surrounding mesenchyme, the ventral bud subsequently rotates and eventually merges with its dorsal counterpart to form the pancreas around E18 (Fig. 1C–E).

Concurrently with pancreas morphogenesis, the endocrine tissue develops. Endocrine cells are derived from progenitors whose generation begins approximately at E9.5 in the early gut endoderm. A few scattered cells rapidly initiate glucagon expression and within one day, a subset of these cells co-produce insulin and sometimes PYY (Herrera et al., 1991; Teitelman et al., 1993; Upchurch et al., 1994). Importantly, these early glucagon- and insulin co-expressing cells most likely do not contribute to the definitive endocrine pancreas (Herrera, 2000). It rather seems that these will die during embryonic development, their detailed fate and potential function remaining unclear. At ∼E14.5, during the secondary transition as defined by Pictet et al. (1972), a peak of endocrine and acinar cell genesis, marked by the appearance of exocrine digestive enzymes, will lead to the generation of numerous mature insulin- or glucagon-expressing cells. Within the next 24 h, the first somatostatin-producing δ-cells emerge (Pictet et al., 1972). Finally, at E18, shortly before birth, PP-cells appear whilst endocrine cells begin to form well-organized islets of Langerhans. Following birth, for about 2–3 weeks, the islets will be rearranged and further matured.

Over recent years, the characterization of mechanisms regulating endocrine pancreas development has been subjected to intensive experimental work aiming to gain insight into the genetic determinants of pancreas morphogenesis and cytodifferentiation. The analysis of mouse loss-of-function phenotypes has represented a powerful tool for the attribution of a particular function to a specific gene, and has demonstrated the importance of certain transcription factors in pancreatic cell fate determination. Thus, the deficiency in a number of these factors has been linked to improper formation and/or function of pancreatic cell-types (Fig. 1, Table).

A major goal of diabetes-related research is to further understand pancreas development at the molecular level in order to provide cues and potential tools to improve the design of rational protocols for the *in vitro* generation of functional β-cells from stem- and/or progenitor cells. In the present review, we will summarize the current knowledge of the endocrine pancreas specification program with particular emphasis on the crucial role of homeodomain-containing transcription factors.

2. Specification of the pancreas anlage

As discussed above, the first indications of pancreas development are discernable at approximately E9.5. However, the specification of the foregut endoderm towards a pancreatic fate occurs as early as E8.5-E9 (Deutsch et al., 2001; Li et al., 1999; Slack, 1995) (Fig. 1A and B). At this stage, interactions between endoderm and surrounding mesodermal tissues induce endodermal cells localized in the vicinity of the midgut/foregut junction, to adopt a pancreatic destiny (Deutsch et al., 2001; Hebrok et al., 1998; Kim et al., 1997; Lammert et al., 2001; Wells and Melton, 2000). The mechanisms governing early pancreas genesis involve the concerted activities of members of the Retinoic Acid, Fibroblast Growth Factor, Sonic HedgeHog and Bone Morphogenetic Protein signalling pathways, and have recently been excellently reviewed (Hebrok, 2003; Jensen, 2004; Kumar and Melton, 2003; Yamaoka and Itakura, 1999; Zaret, 2000, 2001). Dependent on the various permissive and instructing signalling pathways responsible for the regionalization of the endoderm, a specific program of gene expression defines the fate of pancreatic progenitors. This program controls the expression of transcription factors acting (positively or negatively) on the synthesis of selected target proteins, thus defining cell identity and function during pancreas morphogenesis and cytodifferentiation.

3. The pancreatic fate specification program

Prior to the emergence of pancreatic buds, the endodermal cells in the foregut/midgut junction are committed to a pancreatic fate, exhibiting the potential to generate all of the different pancreatic cell-types (Wessells and Cohen, 1967), although recent findings suggest that this process may be reversible (Kumar et al., 2003). Several transcription factors, expressed in this early pre-pancreatic endoderm, control the first steps of pancreas initiation. These include the homeodomain-containing proteins Hlxb9, Ipf1, the bHLH factor Ptf1a (Ahlgren et al., 1996, 1998; Harrison et al., 1999; Jonsson et al., 1994; Krapp et al., 1998; Li et al., 1999; Offield et al., 1996), as well as Isl1, albeit not cell-autonomously in early stages (Ahlgren et al., 1997).

Hlxb9 expression is initiated around E8 in the gut endoderm, the notochord and subsequently in β-cells (Harrison et al., 1994, 1999). In mice, the loss of functional *Hlxb9* alleles provokes a dorsal pancreatic bud agenesis, whereas the ventral bud develops normally, despite the β-cell content appearing significantly diminished (Harrison et al., 1999; Li et al., 1999). This observation further outlines the differential genesis of the two pancreatic buds and unravels a crucial role of *Hlxb9* in the development of the dorsal evagination.

Similarly to *Hlxb9*, the homeodomain-containing protein Isl1 is also selectively necessary for this process, as *Isl1*-deficient mice exhibit an impaired dorsal bud genesis (Ahlgren et al., 1997). This requirement for Isl1 does not appear cell-autonomous, since mutant pancreatic epithelium grows when recombined with wild-type (*Isl1*-expressing) mesenchyme. Strikingly, the expression of *Isl1* in endocrine cells and the lack of these in Isl1-depleted pancreata, suggest a second cell-autonomous function for this gene (Ahlgren et al., 1997). However, due to the early lethality of mutant embryos, further analysis will await the generation of conditional *Isl1* mutant mice.

Ipf1 also exhibits different functions depending on the developmental stage of the pancreas. It is first detected, at E8.5, in the endodermal region giving rise to the two pancreatic evaginations (Guz et al., 1995). Ipf1 plays an important cell-autonomous role in the expansion of the pancreatic epithelium. The growth of the two pancreatic buds is impaired in *Ipf1*-deficient mice, although the dorsal bud undergoes limited proliferation (Ahlgren et al., 1996; Jonsson et al., 1994; Offield et al., 1996). Tissue recombination studies suggest that the failure of epithelium growth resides in its inability to respond to mesenchymal signalling (Ahlgren et al., 1996). Remarkably, *Ipf1* expression is lacking in the remnant β-cells of *Hlxb9*-deficient mice, suggesting that Hlxb9 lies upstream of Ipf1 (Harrison et al., 1999; Li et al., 1999). However, ventral *Ipf1* expression is maintained in *Hlxb9* mutants and *Ipf1* can induce *Hlxb9* expression when ectopically expressed in chicken endoderm (Grapin-Botton et al., 2001 #557), implying that the epistatic relationship between Hlxb9 and Ipf1 depends on context. Importantly, the ectopic expression of *Ipf1* in non-pancreatic chick gut endoderm was also shown to induce *Nkx6.1* expression as well as formation of pancreatic bud-like structures, further demonstrating the crucial role exerted by this factor in the initiation of pancreas development, although additional mechanisms clearly act in this process (Grapin-Botton et al., 2001; Pedersen et al., 2005). Recent studies *in vivo* have elegantly demonstrated that a tight control of *Ipf1* gene activity is required for the proper genesis of various organs of the posterior foregut, including the pancreas (Fujitani et al., 2006). At later stages, Ipf1 displays a second role (Ahlgren et al., 1998) in regulating insulin expression in β-cells (see thereafter).

Several factors were shown to tightly regulate Ipf1 activity, including TCF1, Oc1 and Foxa family members (Gerrish et al., 2001; Marshak et al., 2000; Sharma et al., 1997; Wu et al., 1997). In addition, the targeted disruption of these genes underlines their involvement in early pancreatic development and endocrine specification. Hence, mice deficient for the *Tcf1* gene have smaller islets and reduced insulin secretion (Lee et al., 1998; Pontoglio et al., 1998). Haumaitre et al. (2005) recently demonstrated that the loss of *Tcf2* results in pancreas agenesis, whereby the dorsal pancreatic bud is transiently formed, but lacking *Ipf1* expression. In the absence of *Foxa2*, an impaired formation of the foregut and development of β- and α-cells is noted, while the expression of *Ipf1* is lost (Ang and Rossant, 1994; Lantz et al., 2004; Lee et al., 2002; Sharma et al., 1997; Sund et al., 2001a; Weinstein, 2002; Wu et al., 1997). In contrast, Foxa1 and Foxa3 are involved in glucose homeostasis and liver function, respectively (Kaestner et al., 1998, 1999). Finally, mouse embryos lacking Oc1 activity (Jacquemin et al., 2000, 2003) display a delayed onset of *Ipf1* expression and a reduced endocrine cell differentiation, together with a reduced level of *Ngn3* expression (see thereafter). Hence, these findings underline the importance of transcription factors in orchestrating the initial steps of pancreas genesis and specification, as well as the central role exerted by Ipf1 in these processes. These factors in turn exhibit specifying, regulative and maintaining activities. However, the subsequent events leading to the development of differentiated islet cells requires the function of additional transcription factors.

4. The endocrine fate determination

The pancreatic buds contain undifferentiated precursor cells that are specified towards the endocrine or exocrine lineages. A factor capable of driving pancreatic precursors towards the endocrine cell fate is the bHLH transcription factor Neurogenin-3 (Ngn3) (Gradwohl et al., 2000; Grapin-Botton et al., 2001; Gu et al., 2002). The expression pattern of *Ngn3*, loss-/gain-of-function experiments and lineage tracing, demonstrate that, within the pool of undifferentiated cells of the pancreatic buds, *Ngn3* specifically labels endocrine progenitors. Notably, the *Ngn3*-deficient pancreas is devoid of any endocrine tissue or putative endocrine precursors, while the exocrine and ductal counterparts appear to develop normally (Gradwohl et al., 2000). Accordingly, the ectopic expression of *Ngn3* in pancreatic progenitors leads to the differentiation of the entire pancreas into endocrine cells, albeit these are mostly glucagon-producing cells (Apelqvist et al., 1999; Schwitzgebel et al., 2000).

During embryogenesis, a number of bHLH-containing factors act to initiate a variety of ontogenetic processes. This is also true for pancreas development, where Ngn3 activates the promoter of *Neurod1* (BETA2), another bHLH-encoding gene. *Neurod1*-deficient mice display reduced numbers of hormone-producing cells, particularly in the β-cell population (Naya et al., 1997). It is important to note that, unlike the situation in *Ngn3*-deficient mice, endocrine cells are generated in the absence of *Neurod1*, however, they undergo massive apoptosis. The observation that mice lacking *Ngn3* fail to express BETA2, whereas *Ngn3* expression is unaffected in BETA2-depleted mice, further suggests that BETA2 acts downstream of Ngn3 in the endocrine differentiation program (Gradwohl et al., 2000). Similar to *Ngn3* (Grapin-Botton et al., 2001), the over-expression of *Neurod1* leads to the differentiation of progenitor cells into endocrine cells (Schwitzgebel et al., 2000; Serup et al., unpublished observations). However, BETA2 is not able to induce Ngn3 in chick endoderm (Serup et al., unpublished observations), indicating that these two bHLH factors are directly and unidirectionally linked within the pro-endocrine gene expression cascade. In that context, the recent identification of a zinc-finger-containing transcription factor, IA1, shed further light on the initial steps underlying the endocrine cell differentiation. Using a combination of ectopic expression and knock-down experiments, Mellitzer et al. (2006) elegantly established that this factor represents a direct target of Ngn3, subsequently allowing endocrine cell genesis through the activation of *Neurod1* and several genes known for their involvement in pancreas morphogenesis.

Following the initiation of the endocrine program, a set of transcription factors are necessary to convert Ngn3-labelled cells into α-, β-, δ- or PP-cells. In order to describe the mechanisms implicated, several models have been proposed. For the purpose of simplification, most display the differentiation processes as a linear cascade of transcription factor activation, eventually leading endocrine progenitors to adopt a specific islet cell identity. However, given the fact that a particular transcription factor might operate at various developmental stages, in different processes and occasionally using distinct mechanisms of action, the resulting network of gene product interactions will likely become extremely complex.

5. Differential specification of endocrine progenitors

Using a loss-of-function approach, the pathways controlling the differential selection of the endocrine fates in mice were linked to the function of homeodomain-containing factors, including Nkx2.2, Nkx6.1, Pax4 and Arx, all of which are co-expressed with Ngn3 and act early during endocrine development.

Nkx2.2 belongs to the NK class of homeodomain-encoding genes and its expression is initiated at E9.5 in the dorsal epithelium (Sussel et al., 1998). Concurrently with endocrine cell differentiation, *Nkx2.2* expression becomes progressively restricted to α-, β- and PP-cell subtypes, but is excluded from δ-cells. Accordingly, mice lacking *Nkx2.2* display a loss of β-cells and a decrease in α- and PP-cell populations, whereas the number of δ-cells remains unaffected. Interestingly, *Nkx2.2* mutant islets also contain a population of immature β-cells lacking the late β-cell specific markers *glut2* and *glucokinase*. Thus, Nkx2.2 appears to control both the late differentiation of β-cells, as well as PP- and α-cell genesis.

Nkx6.1, an additional member of the NK class of homeodomain-containing proteins, is detectable at E9.5 in both pancreatic buds (but is absent in *Ipf1* mutants), where it persists until approximately E13. Following this stage, *Nkx6.1* expression becomes specifically restricted to β-cells (Oster et al., 1998; Pedersen et al., 2005; Sander et al., 2000). The lack of *Nkx6.1* results in a diminished β-cell genesis, reminiscent of the phenotype observed in *Nkx2.2* mutants (Sander et al., 2000). Recently, the analysis of animals depleted in both Nkx6.1 and its paralog Nkx6.2, demonstrate a compensatory effect of Nkx6.2 over Nkx6.1 function, as β-cell genesis is almost completely abolished in double mutants (Henseleit et al., 2005). Surprisingly, the content of α-cells was also found markedly reduced in pancreata lacking both genes. In *Nkx2.2*-deficient mice, *Nkx6.1* expression is lost in endocrine cells (*Nkx6.2* expression has not been yet assessed in these mutants), indicating that Nkx2.2 lies upstream of Nkx6.1 in endocrine differentiation processes (Sander et al., 2000). Additional findings support this assumption. Indeed, the alterations observed in β-cells are similar, both in *Nkx6.1* and *Nkx2.2* mutant mice, and a deficiency in *Nkx6.1* does not affect *Nkx2.2* expression. The phenotypic modifications detected in the pancreas of mice lacking *Nkx2.2* and *Nkx6.1* are similar to those exhibited by *Nkx2.2* mutant mice (Sander et al., 2000). Finally, biochemical evidence demonstrates that Nkx2.2 directly controls *Nkx6.1* expression by interacting with its promoter (Watada et al., 2000). In conclusion, these findings sustain the requirement of Nkx2.2 for proper islet cell development and unravel the function of Nkx6 factors for β- and α-cell differentiation.

Pax4, a paired-box-encoding gene, plays a central role in the differential specification of endocrine precursor cells. Its expression is initiated around E9.5 in both pancreatic buds and becomes progressively restricted to β-cells until ~E15 (Dohrmann et al., 2000; Smith et al., 1999; Sosa-Pineda et al., 1997). Mice depleted in Pax4 are normally born, but rapidly develop severe diabetes as a consequence of hypoinsulinemia, and eventually die 2 days *postpartum*. Closer examination indicates that β- and δ-cells, as well as their associated developmental marker genes, lack in these animals (Sosa-Pineda et al., 1997). Most interesting is the observation that the loss of β- and δ-cells is compensated by a proportional increase in α-cell content. Thus, it was previously concluded that the lack of Pax4 might either unravel a default α-cell fate for endocrine precursors, or alternatively a β-/δ-cell destiny-inducing function for Pax4. If the former were true, Pax4 should be capable of forcing *Ngn3*-expressing endocrine progenitors towards a β- or δ-cell fate. However, several studies failed to validate such a model (Grapin-Botton et al., 2001; Wilson et al., 2003). *Pax4* appears to be a direct downstream target of Ngn3, which can interact with and activate the *Pax4* promoter, *in vivo* (Smith et al., 2000) and *in vitro* (Wilson et al., 2003). A recent study of mice lacking Pax4 and Nkx2.2 factors indicated that, in the absence of either or both factors, the expression of *Ipf1*, *Hlxb9*, *Nkx6.1* and *insulin* genes is lost (Wang et al., 2004). Albeit the study of α-cell development in this context has not been performed, these findings sustain the notion that Pax4 and Nkx2.2 act upstream of these factors in early β-cell differentiation and insulin production.

Interestingly, the recent identification of a gene involved in the specification of endocrine progenitors brought interesting insight into the function of Pax4 and, to a more general extent, into the processes governing the choice between the alternate islet subtype destinies (Collombat et al., 2003). This gene, *Arx*, a member of the paired-like-encoding gene family and the *Aristaless* subfamily, is located on the X chromosome. Its Ngn3-dependent expression is initiated at E9.5, becomes rapidly restricted to α-, β-precursor- and δ-cells and persists following birth. *Arx*-deficient animals are born normally, but develop early-onset hypoglycemia, dehydration, weakness and die approximately 2 days after birth (Collombat et al., 2003). This phenotype is associated with a drastic hypoglucagonemia, secondary to a loss of mature α-cells, concomitantly with a proportional increase of β- and δ-cell populations, leaving islet

morphology intact. Further characterization demonstrated an early requirement of Arx for the α-cell fate acquisition and a repressive action upon β- and δ-cell destinies. These phenotypic changes are reminiscent of those found in *Pax4* mutant animals, except that *Pax4*- and *Arx*-depleted pancreata display inverted alterations in α-, β- and δ-cell ratios. In fact, the abnormalities described in *Arx*- and *Pax4*-deficient pancreata were linked to mutually antagonistic roles for these two transcription factors: the amount of *Pax4* mRNA is up-regulated in *Arx* mutant mice, whereas the *Arx* transcript and protein contents are increased in *Pax4*-deficient pancreata (Collombat et al., 2003). Importantly, Arx and Pax4 are co-localized in the early endocrine pancreas, but not at later developmental stages, suggesting that both factors are co-expressed during early endocrine differentiation. One of them will ultimately predominate; if it is Pax4, β- and δ-cell fates will be specified, whereas Arx will favor the α-cell commitment. Upon the inactivation of both *Arx* and *Pax4* genes, the islets of Langerhans lack α- and β-cells, which are replaced by cells exhibiting characteristics normally associated with δ-cells (Collombat et al., 2005). These animals develop a severe hyperglycemia and eventually die around post-natal day two. As surprising is the observation that the excess δ-cells initiate an ectopic PP expression following birth, the PP-cell genesis appearing normal. Further studies have revealed that this activation of PP expression is triggered by the onset of feeding. Hence, it was proposed that Pax4 initially instructs endocrine precursors towards a β-/δ-cell fate, and later acts in promoting the β-cell destiny at the expense of the δ-cell lineage. Finally, Arx and Pax4 were recently found to mutually inhibit each other's transcription through direct physical interaction with the pertinent promoter (Collombat et al., 2005). Taken together, the antagonistic activities of Arx and Pax4 provide new insights into the molecular mechanisms governing the selection of a particular endocrine cell fate. Early during pancreas morphogenesis, endocrine precursor cells express Arx and Pax4. Since both directly inhibit each other's transcription, such a co-expression implies that they might initially be produced in an inactive form. Hereafter, an unknown factor supposedly selectively activates Arx or Pax4, thereby leading endocrine progenitors towards either an α-cell or a β-/δ-cell fate, respectively, the activated protein directly inhibiting the transcription of the other. Hence, Arx appears to specify the α-cell fate, whereas unlike previously assumed (Collombat et al., 2003; Sosa-Pineda et al., 1997), Pax4 first allows the commitment towards a β-/δ-cell fate by repressing *Arx* and subsequently inducing β-/δ-precursor cells towards a β-cell fate, through the inhibition of the δ-cell destiny. Therefore, it seems likely that a third unknown factor instructs β-/δ-cell precursor cells to become δ-cells by antagonizing Pax4 activity. It is important to notice that the number of ghrelin^{+}/glucagon^{-} ε-cells remain unchanged following the depletion in Arx and/or Pax4 (Heller et al., 2005), indicating that the development of this newly identified cell subtype does not seem to depend on any of these factors. Thus, the increase in ghrelin-labelled cell content reported previously in *Pax4* mutants (Prado et al., 2004), can be attributed to an augmentation in the number of ghrelin^{+}/glucagon^{+} α-cells.

6. Maintenance of the islet cell subtype

In addition to Nkx2.2, Nkx6.1, Pax4 and Arx factors, a second set of transcription factors, including Pax6, Isl1, Ipf1 and Pou3f4 act later in differentiating endocrine cells.

Accordingly, the loss of the paired-domain-encoding gene, *Pax6*, normally present in all endocrine cells, promotes a dramatic reduction in the entire islet cell population (Sander et al., 1997; St-Onge et al., 1997). The remaining cells form disorganized islets and produce significantly less hormone compared to their wild-type counterparts, an effect that can doubtlessly be attributed to the presence of regulatory sequences specifically recognized by the Pax6 factor within the *insulin*, *glucagon* and *somatostatin* promoters (Hill et al., 1999; Sander et al., 1997; St-Onge et al., 1997). Interestingly, mice homozygous for a targeted deletion of both *Pax4* and *Pax6* genes, fail to develop mature endocrine cells (St-Onge et al., 1997). Thus, Pax6 may play a role in sustaining the hormone-expressing cell phenotype, rather than determining the endocrine destiny. In agreement with this conclusion, the total endocrine cell content is not modified in animals where *Pax6* is conditionally inactivated in the early pancreas, but hormone production and late endocrine marker expression is altered (Ashery-Padan et al., 2004). However, in this latter work, the δ- and PP-cell contents were found to be seemingly unaltered, albeit no quantification was presented. A recent study documenting the interplay between Nkx2.2, Pax4 and Pax6 factors during β-cell differentiation, demonstrates the synergistic roles of Pax4 and Nkx2.2 in the initiation of β-cell differentiation. This underlines the role of Nkx2.2 in maintaining *Pax6* expression during the advanced steps of this process (Wang et al., 2004). Such a function can most likely be extended to all of the endocrine cell-types, as suggested by the work from Sander et al. (1997).

The assessment of the late role of Isl1 in endocrine cell differentiation is complicated by the fact that this factor operates at different developmental stages. However, in *Isl1*-deficient mice, the expression of *Pax6* is lost, whereas *Nkx2.2* and *Neurod1* are normally expressed (Ahlgren et al., 1997; Dong et al., 1991; Andersen et al., 1999a,b), indicating that Isl1 may act upstream of Pax6, but downstream of Neurod1.

Similarly to Isl1, Ipf1 is firstly involved in pancreatic bud morphogenesis, but also exhibits a second function in the final stages of mature endocrine β-cell differentiation. Indeed, Ipf1 is reactivated in β-cells and a few δ-cells, where it is instrumental in maintaining the differentiated phenotype (Schwitzgebel et al., 2000). Accordingly, the expression of numerous β- and δ-cell-specific factors appears to depend on Ipf1 activity (Chakrabarti et al., 2002; Leonard et al., 1993; Macfarlane et al., 2000; Miller

et al., 1994; Ohlsson et al., 1993; Waeber et al., 1996; Watada et al., 1996a,b). Using a conditional loss-of-function approach (Ahlgren et al., 1998), Ipf1 was suggested to maintain the β-cell phenotype, through the activation of the β-cell-specific genes *insulin*, *IAPP*, *Glut2*, and additionally, through the inhibition of *glucagon* gene expression. However, the α-cell origin of β-cells using lineage tracing experiments remains to be assessed. Nevertheless, the expression of a dominant-negative form of Ipf1 in the ins-1 islet tumor cell line induces a down-regulation in the levels of *insulin*, *IAPP*, and *Glut2* gene expression. Finally, a tight control of *Ipf1* levels in adult β-cells appears essential for normal glucose homeostasis (Ahlgren et al., 1998; Dutta et al., 1998; Lantz et al., 2004; Thomas et al., 2001).

In a similar fashion to Ipf1, Pou3f4, a member of the class III family of the POU homeodomain-containing transcription factor, was thought to maintain the α-cell fate (Jensen et al., 2000). Indeed, this factor is expressed only in α-cells, where it is able to stimulate the glucagon gene expression by interacting with regulatory regions lying within the *glucagon* gene promoter (Hussain et al., 1997, 2002). When ectopically expressed in *Ipf1*-producing cells, Pou3f4 promotes ectopic expression of the *glucagon* gene (Hussain et al., 2002). However, mice deficient for *Pou3f4* do not exhibit any obvious defect in α-cell development and function (Heller et al., 2004). Therefore, it was concluded that Pou3f4 may play a role during the late differentiation of the α-cell phenotype, notably by promoting *glucagon* gene expression, but it is likely that additional factors possess similar and redundant activities. For instance, MafB, belonging to the v-maf musculoaponeurotic fibrosarcoma oncogene family, was shown to transactivate the glucagon promoter, whereas its paralog MafA has a similar function on insulin production (Artner et al., 2006; Kataoka et al., 2004; Matsuoka et al., 2004).

7. Endocrine cell lineage analysis

As outlined previously, numerous transcription factors act in a concerted fashion to induce endodermal cells at the foregut–midgut junction to adopt a pancreatic cell fate. Coincidently, the expression of *Ipf1* is initiated. Using Cre-LoxP recombination-based lineage tracing or ablation strategies, *Ipf1*-expressing cells were further identified as progenitors of pancreatic cells (Gannon et al., 2000; Gu et al., 2002; Herrera, 2000). Interestingly, an unsuspected role of Ptf1a was highlighted by the work of Kawaguchi et al. (2002), demonstrating the co-expression of *Ipf1* and *Ptf1a* as a prerequisite for proper pancreatic cell fate acquisition and subsequent proliferation and differentiation.

Additional studies, using a similar approach, have demonstrated that the subsequent activation of Ngn3 will specifically label cells giving rise to the endocrine tissue (Gu et al., 2002; Herrera, 2002). It was previously believed that islet cells arise from the precursors observed during early development, which often co-secrete glucagon, insulin, and occasionally PYY. Specifically, the development of a particular endocrine cell subtype was assumed to arise from the selective activation and inhibition of hormone-expressing genes, resulting in the generation of insulin-, glucagon-, somatostatin- and PP-expressing cells (Guz et al., 1995). However, Herrera (2000) clearly demonstrated that mature glucagon-expressing α-cells and insulin-producing β-cells originate from precursors that have not previously expressed glucagon or insulin. These findings provide evidence that α- and β-cells on one side, and the early insulin- and glucagon-secreting cells on the other side, correspond to two distinct lineages, the latter most likely eliminated by apoptosis, since their irreversible labelling was lacking at late pancreatic developmental stages (Herrera, 2000). Furthermore, it is also clear that the endocrine subtype is determined prior to any expression of hormone genes. In line with this conclusion, through a detailed immunohistochemical study, Jensen et al. (2000) proposed that α- and β-cells develop from Ngn3-expressing precursors, but not from the early insulin-/glucagon-secreting cells. Lastly, the phenotypes observed following the deficiency in *Nkx6.1*, *Arx*, *Pax4* and both *Arx* and *Pax4* genes (Collombat et al., 2003; Heller et al., 2005; Sander et al., 2000; Sosa-Pineda et al., 1997), are all associated with alterations in mature endocrine cell numbers, but leave these early hormone-co-expressing cells unaffected. Numerous studies indicate that the steps following *Ngn3* expression in the islet differentiation program include the induction of *IA1* and the subsequent expression of *Neurod1* (Gradwohl et al., 2000; Huang et al., 2000; Lee et al., 1995; Mellitzer et al., 2006; Naya et al., 1997). Thereafter, the commitment to any of the four endocrine cell subtypes depends on the differential expression of additional transcription factors. Until recently, a consensual model involved Ipf1, Nkx6.1 and Pax4 for a commitment towards a β-cell fate, whereas Pou3f4 was seen as responsible for the α-cell destiny. This model needs some adjustment, as illustrated by recent studies described in this review. It seems that, next to the activation of Ngn3, IA1 and Neurod1, the first fate-choice presented to endocrine precursors corresponds to the selection between an α- or a β-/δ-cell fate, Arx promoting the former, whereas Pax4 induces the latter (Fig. 2). However, little is known concerning the PP-cell lineage. Using PP-cell ablation, a loss of β- and δ-cells was observed (Herrera et al., 1994) and cell lineage tracing revealed that a PPCre transgene also marked β-cells (Herrera, 2000). These data may indicate that PP- and β-cells share a common progenitor, but further analysis would be required. Indeed, these results could also reflect a low-expression of PP in early β-/δ-precursor cells and/or a paracrine role of PP-cells on δ-cell maintenance. Additional Cre-LoxP recombination-based lineage tracing studies using ArxCre, Pax4Cre, $PPCre^{ERT}$ and $GlucagonCre^{ERT}$ lines will doubtlessly provide more insight into the mechanisms involved.

Following the commitment to either an α- or a β-/δ-cell fate, Pax4 promotes β-/δ-cell precursor cells towards the

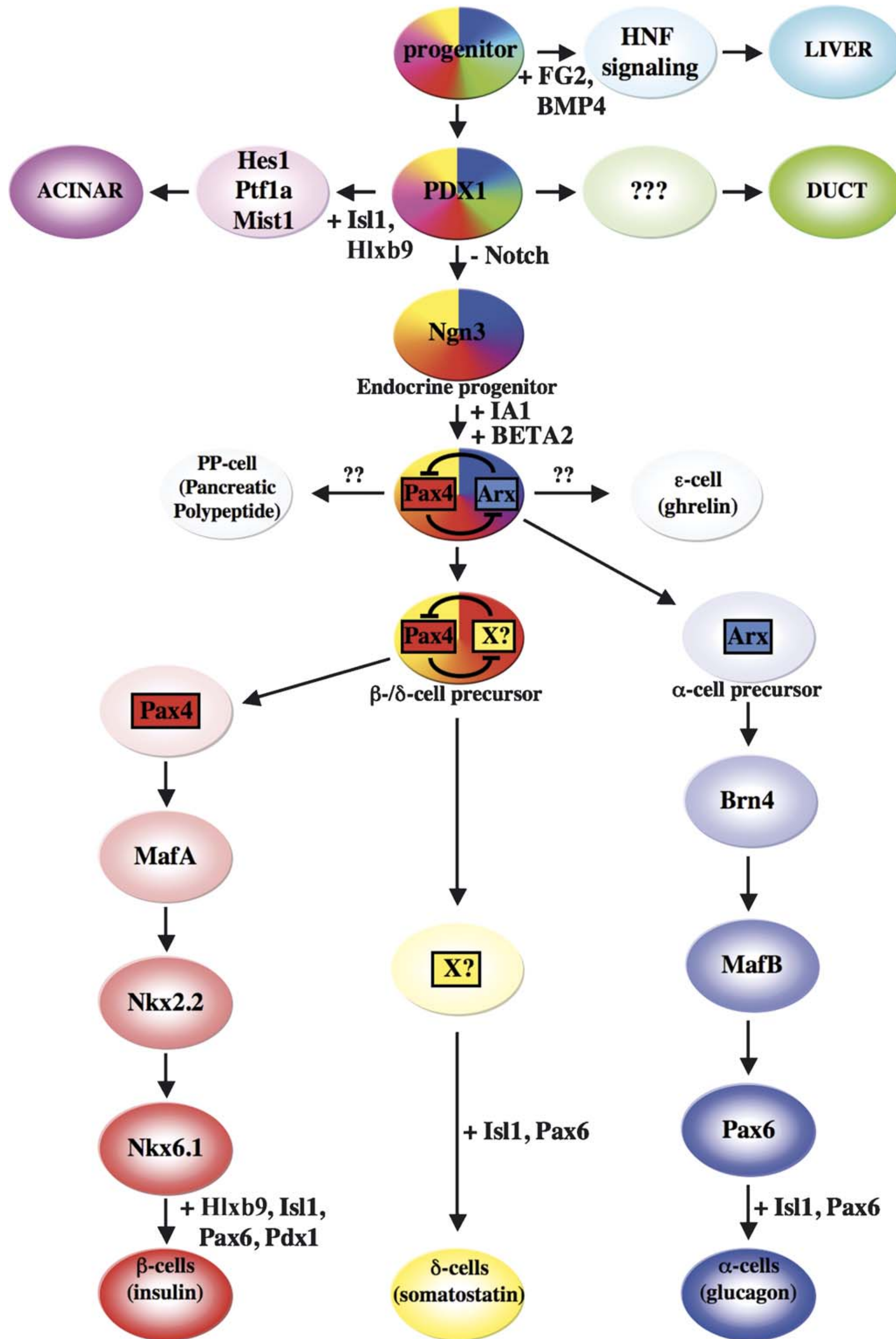

Fig. 2. Schematic model representing the transcription factors implicated in the specification of the endocrine pancreas, based on temporal expression and the phenotypic consequences of specific gene deletions. Circles represent endocrine cells at particular developmental stages. The different transcription factors expressed in a particular cell type are indicated within the circles. Arrows correspond to different (hypothetical) endocrine differentiation steps. The colours represent the different cell lineages or the potential for a given cell to give rise to these lineages.

β-cell destiny while repressing the δ-cell lineage. A third unknown factor is envisioned to promote the δ-cell commitment, by inhibiting the β-cell fate through a mutual repressive mechanism between Pax4 and this factor, as described for Arx and Pax4. Following these key decisions, numerous additional factors will further promote the differentiation and maintain the phenotype of the different endocrine subtypes (Fig. 2).

8. Conclusion

One of the ultimate goals of diabetes research is to provide a proper substitute to insulin injections, and thereby regulate blood glucose levels more accurately. A putative hope is seen in the isolation of pancreatic adult stem cells. However, the work of Dor et al. (2004) elegantly established that, during adult life, β-cells seemingly do not arise from pancreatic-duct or stem cells, but rather from pre-existing β-cells. This study opened new perspectives, where isolated β-cells could be multiplied. The *in vitro* generation of pancreatic β-cells from embryonic stem or progenitor cells represents another promising alternative. The recent findings discussed in this review brought new insights into our understanding of endocrine cell development, pancreas dysfunction, and may eventually provide key tools for stem cell-based therapies. However, despite such major advances, numerous important issues remain unanswered, a few highlighted here. The first concerns the origin of the signalling pathways instructing Ngn3-expressing progenitor cells towards a particular endocrine cell fate. Although Arx and Pax4 function allow the allocation to specific endocrine cell lineages, the genetic determinants acting upstream of these remain hitherto unknown. Equally important is the question of whether any adult stem and/or duct cells could participate in β-cell regeneration. Dor et al. (2004) clearly established the absence of β-cell regeneration during normal life or in pancreatectomized mice. However, this needs to be demonstrated in human, especially in diabetic subjects. Finally, the possibility of cell transdifferentiation is a matter of interest. Evidences of cell identity reprogramming were reported both *in vitro* and *in vivo* (Ahlgren et al., 1998; Deutsch et al., 2001; Shen et al., 2000), albeit under specific conditions. The characterization of the molecular and epigenetic mechanisms controlling these different cell identity switches belongs to the challenging questions to be addressed to allow the development of rational protocols for stem cell-based therapies.

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