Review

Transcriptional networks controlling pancreatic development and beta cell function

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Abstract

Transcription factors provide the genetic instructions that drive pancreatic development and enable mature beta cells to function properly. To understand fully how this is accomplished, it is necessary to unravel the regulatory networks formed by transcription factors acting on their genomic targets. This article discusses recent advances in our understanding of how transcriptional networks control early pancreas organogenesis, embryonic endocrine cell formation and the differentiated function of adult beta cells. We discuss how mutations in several transcription factor

genes involved in such networks cause Maturity onset diabetes of the young (MODY). Finally, we propose that pancreatic gene programs might be manipulated to generate beta cells or to enhance the function of existing beta cells, thereby providing a possible treatment of different forms of diabetes.

Keywords Chromatin immunoprecipitation · Gene networks · Haplo-insufficiency · Hnf1 α · Hnf1 β · Hnf4 α · Islets of Langerhans · MODY1 · MODY3 · Ngn3 · Nkx2.2 · Pdx1 · Stem cells · Type 1 diabetes mellitus · Type 2 diabetes mellitus

Introduction

Transcription factors control the ability of stem cells to maintain their undifferentiated state [1, 2]. They also control the developmental process that leads to the formation of beta cells [3], and provide the genetic program for adult beta cells to function properly [4, 5,

6, 7]. It was originally thought that single dedicated transcriptional activators might be responsible for directing the specific phenotype of each cell type. The accumulated evidence points instead to a combinatorial mode of action, whereby unique sets of transcription factors determine the identity and function of a cell. For a set of transcription factors to be expressed in a specific cell, mechanisms need to be in place to initiate and maintain the transcription of their genes. This is achieved through networks formed by transcription factors that directly regulate the promoters of genes encoding other transcription factors [8, 9]. Thus, each cell type possesses not only a unique combination of transcription factors, but also a specific gene regulatory network structure.

With the advent of the sequences of entire genomes of key model organisms and the technologies to analyse them, we can realistically expect to obtain most of the relevant experimental information pertaining to pancreatic transcription networks. Deciphering the logic of such networks is highly relevant to the pathophysiology and treatment of diabetes. Knowledge of

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Abbreviations: ChIP, chromatin immunoprecipitation · E, embryonic day · Hnf, hepatocyte nuclear factor · Lrh1, liver receptor homologue 1 · MODY, Maturity onset diabetes of the young · Ngn3, neurogenin 3 · Pklr, L-pyruvate kinase

Fig. 1. Analysis of transcription factor promoter interactions using the chromatin immunoprecipitation (ChIP) assay. The ChIP assay can show that a native transcription factor binds to an endogenous gene in a specific cell type [15]. Living cells are exposed to formaldehyde to crosslink proteins bound to DNA. The cells are lysed, chromatin is sheared by sonication, and an antibody against a DNA-binding protein is used to immunoprecipitate the chromatin fragments to which the protein is bound. After DNA purification, specific DNA segments are assayed for enrichment in the immunoprecipitate. This is achieved by PCR amplification of the candidate target gene segment and a negative control DNA, which is not expected to be enriched (A and B respectively). The amplification pattern is compared with that obtained in unenriched (“input”) genomic DNA [167]. Alternatively, ChIP-enriched DNA can be hybridised to microarrays containing a large number of promoter or intergenic fragments, a technique known as ChIP-on-chip [168, 169]. A^{for}, A forward; A^{rev}, A reverse oligonucleotide primer

pancreatic gene programs could enable *in vitro* recapitulation of embryonic development to produce functioning insulin-producing cells. It could also enable *in vivo* stimulation of beta cell regeneration for the treatment of Type 1 diabetes [10, 11, 12]. In addition it could help us to understand the pathophysiology of monogenic forms of diabetes caused by mutations in genes encoding transcription factors [7, 13, 14]. An in-depth understanding of beta cell transcription programs could also help in the development of drugs to treat insulin secretory dysfunction in Type 2 diabetes.

In this article we discuss the process of pancreatic development and beta cell differentiation from the perspective of its transcriptional control. Particular focus is placed on emerging evidence revealing the *in vivo* structure of transcription networks within distinct cells of the developing and adult pancreas, and on providing insights into how such knowledge can affect our understanding of pancreatic gene regulation.

Interpreting experimental data on transcription networks

The information currently available on pancreatic gene networks is still very incomplete. As this information increases, so will its apparent complexity. To interpret the functional logic of such large networks, it will ultimately be crucial not only to obtain sufficient experimental data, but also to define the significance and level of certainty for each of the network components.

A major source of information on transcription networks is studies using mice with targeted inactivation of a transcription factor gene. Using either microarrays or more focused analysis of candidates, such knock-out models can identify which genes are activated or repressed by a transcription factor of interest in specific tissues and developmental stages. Yet these studies do not distinguish between direct and indirect effects. The inactivation of a transcription factor in an animal can modify the function of a cell and of the entire organism, and this can exert profound changes in the expression of genes that are not directly bound by the transcription regulator. Thus, to understand the structure of a gene network, we need to complement differential expression data with biochemical evidence indicating which effects reflect direct regulation.

Traditionally the analysis of direct interactions between transcription factors and promoters has been carried out through *in vitro* binding assays and transient transfection studies in cultured cell lines. Such studies can be invaluable for detailed mechanistic analysis of promoters. However, they do not prove that an interaction occurs at physiological concentrations of the factor in the native chromatin environment of the target gene. Moreover, they cannot address in which cells the interaction occurs *in vivo*. In this respect, chromatin immunoprecipitation (ChIP) has emerged as a method for demonstrating that a transcription factor binds to a gene segment in a specific cell type (Fig. 1) [15]. Other useful *in vivo* tools are

Fig. 2. Schematic representation of three stages of pancreatic development in the mouse embryo. At E9-E10 (*left*) dorsal and then ventral pancreatic buds arise from foregut endoderm. At this stage most epithelial cells are multipotent precursors expressing markers such as Pdx1 and Nkx2.2 (see also Fig. 3). The representative immunofluorescence image (E10.5) shows dorsal (*dp*) and ventral (*vp*) buds marked by anti-Nkx2.2 (*green*), while intense reactivity for anti-Hnf1 α (*red*) identifies primitive liver (*li*). At E14-E17 (*middle*), the pre-differentiated epithelium has formed a well-defined ductal network distinguished from acinar and endocrine cells by the expression of Hnf1 β . Numerous Ngn3 $^{+}$ cells arise from the Hnf1 β^{+} ductal epithelium at this time, later giving rise to endocrine cells. The immunostaining image (E14.5) depicts ducts formed by Hnf1 β^{+} cells (*red*), Ngn3 $^{+}$ cells (*blue*) and endocrine cells expressing exclusively Nkx2.2 (*green*) (some cells co-express these markers, see main text). On the right is a schematic representation of the mature pancreas. Immunofluorescent stainings were provided by M. A. Maestro

Transcriptional networks in very early pancreatic bud multipotent cells

In the mouse embryo, the first visible signs of pancreatic development appear at approximately embryonic day (E) 9 (Fig. 2) [3, 22]. Two pancreatic buds emerge independently from the ventral and dorsal regions of the foregut endoderm. The ventral bud is formed from cells that escape the inhibitory effect of fibroblast growth factor and bone morphogenetic protein signalling from the cardiac and septum transversum mesoderm [23, 24]. In contrast, signals from the notochord promote dorsal pancreas development through a process involving the repression of Sonic hedgehog expression in the dorsal endoderm [25, 26]. It is likely therefore that important aspects of the transcriptional programs orchestrating the specification and development of ventral and dorsal buds are distinct.

Fate-mapping and immunolocalisation studies have shown that most epithelial cells of the early buds are pre-differentiated precursors of exocrine and endocrine cells [27, 28, 29]. These cells express a combination of transcription factors, including Pdx1, Ptf1a (p48), Nkx6.1, Nkx2.2, Hb9, Hex, hepatocyte nuclear factor (Hnf) 6 and Foxa2 (Hnf3 β) [29, 30, 31, 32, 33, 34, 35].

Knock-out studies show that many of these factors play essential roles in early buds (Fig. 3). Thus Pdx1 and Ptf1a are indispensable for the formation or progression of both buds [29, 36, 37, 38]. On the other hand, Hb9 and Isl1 are only required for the specification or differentiation of the dorsal pancreatic bud, while Hex is selectively needed for specification of the ventral counterpart, which is consistent with the different mechanisms underlying the formation of the two buds [33, 35, 39, 40]. Interestingly, at this stage

transgenics using wild-type and mutated regulatory DNA segments linked to a reporter gene, or targeted mutations of *cis* elements to determine their role in the transcription of a gene at distinct developmental stages [16, 17, 18].

A key issue in ChIP and expression profiling experiments is that relationships between transcription factors and target genes are highly cell-specific, and thus need to be demonstrated in specified cell types or developmental stages [19, 20, 21]. Throughout this review, therefore, the emphasis is on direct regulatory interactions that have been shown to occur during defined cellular stages of the developing pancreas.

Fig. 3. Essential roles and expression patterns of transcription factors during different embryonic stages of the beta cell lineage. At the top, arrows point to the developmental steps at which the indicated transcription factors have been shown to be indispensable in genetic knock-out studies of mice, irrespective of when these genes are expressed. At the bottom the relative expression levels of different transcription factors throughout pancreatic development are indicated schematically

Isl1 is predominantly present in mesenchymal cells, and its mutant phenotype is due to lack of dorsal pancreas mesenchyme [39]. *Hex* contributes to the growth of ventral foregut endoderm so that part of it is at a site that is permissive for pancreatic organogenesis [35]. The findings for *Isl1* and *Hex* show that the critical roles of transcription factors in pancreatic development can be mediated by completely indirect mechanisms that do not involve cell-autonomous activation of pro-differentiation genes in precursor epithelial cells.

There is thus considerable information on which transcription factors are expressed in pancreatic precursors and which ones are essential for early organogenesis. However, very little information is available at present on how transcriptional networks are organised within these cells. For example, nothing is known about the transcription factors that directly activate *Ptf1a*, *Nkx6.1*, *Nkx2.2* and *Hlxb9* (*Hb9*) genes in the pancreatic primordium. There are also no data about their direct targets at this stage.

Information on the regulators and targets of *Pdx1* in early bud networks is at a more advanced stage. The importance of understanding the network linked to *Pdx1* in pancreas organogenesis cannot be overstated. *Pdx1* is not only essential for early pancreas for-

mation [36], but can also activate pancreatic developmental programs if misexpressed in the right cellular context. Two laboratories have shown that *Pdx1* can induce beta cell properties when expressed in adult or fetal liver cells [41, 42]. Another study demonstrated that a fusion protein containing the *Xenopus* orthologue of *Pdx1* and the VP16 transcriptional activation domain can induce the formation of pancreatic tissue in the liver when misexpressed using the transthyretin promoter [43]. In this experiment VP16 provided a very strong activation domain, which probably acts as a surrogate for other activators that need to act in concert with *Pdx1* at common genomic targets.

So which transcription factors control the activation of *Pdx1* in early bud cells? It was recently shown that the activation of retinoic acid receptors during gastrulation specifies the location of *Pdx1* expression along the anterior–posterior boundary of the endoderm in zebrafish [44]. Moreover, *Hb9* (in keeping with its role in the specification of dorsal pancreas) activates *Pdx1* in the dorsal region of the foregut by an unknown mechanism, while *Hex1* is indirectly required for *Pdx1* expression in the ventral foregut [33, 35, 40]. Another recent study reported that the onecut homeodomain protein *Hnf6* regulates *Pdx1* in the primordium [45]. *Hnf6* is expressed in the foregut endoderm prior to *Pdx1*. Embryos lacking *Hnf6* exhibit reduced expression of *Pdx1* at E10 and delayed development of both ventral and dorsal pancreas, resulting in pancreatic hypoplasia (as well as other abnormalities discussed later) [45]. In vitro binding and co-transfection studies indicate that *Hnf6* binds and transactivates mouse *Pdx1* 5' flanking DNA [45], suggesting that *Hnf6* is the first known direct regulator of *Pdx1* expression during early bud formation (Fig. 4).

Fig. 4. Genetic networks during defined cellular stages of pancreatic development. The four compartments represent consecutive embryonic cellular stages analogous to those shown in Figure 3. Gene promoters are represented by a rectangle and a thick arrow. Binding of a transcription factor to a promoter is represented by a thin line. A perpendicular line replaces an arrow when an interaction is inhibitory. Only interactions shown to occur in vivo and for which there is biochemical evidence of a direct effect are depicted

The role of two other candidate regulators of *Pdx1* remains less well established. High-affinity binding of *Pdx1* to the *Pdx1* gene has been reported, consistent with an autoregulatory mechanism that operates once the gene is turned on [46, 47]. However *Pdx1*^{-/-} mice exhibit apparently normal expression of a *LacZ* reporter knocked into the *Pdx1* locus in early bud cells [38]. Similarly, although *Foxa2* (*Hnf3β*) is expressed in the foregut and early pancreatic cells, and binds the *Pdx1* gene [47, 48], *LacZ* transgenics containing a mutation of a *Pdx1* gene–*Foxa* binding site affect expression in beta cells rather than in pancreatic rudimentary epithelial cells [49]. Cultured *Foxa2*^{-/-} embryoid bodies exhibit reduced expression of *Pdx1* mRNA [50], but evidence of a similar role in the early embryonic pancreas is still lacking.

A different but equally crucial question is that of how *Pdx1* controls pancreas organogenesis. The identification of the genomic targets of *Pdx1* in early pancreatic buds should provide important clues in this regard. So far, the only direct target of *Pdx1* reported at this stage is the nuclear receptor named liver receptor homologue 1 (*Lrh1*, NR5A2) [51]. This receptor is expressed in early bud epithelium, differentiated ducts and acinar cells [52]. *Pdx1* was shown to bind to the *Lrh1* gene in vitro and in vivo in embryonic pancreas,

and to transactivate episomal and endogenous *Lrh1* in pancreatic carcinoma cells [51]. Furthermore, *Lrh1* mRNA is reduced in pancreatic buds of *Pdx1*^{-/-} embryos at E9.5 (although, surprisingly, this study reported reduced expression in liver) [51]. Unfortunately *Lrh1*^{-/-} embryos die before the pancreas is formed [51], so further experiments will be needed to define the possible role of *Lrh1* as a downstream *Pdx1* target. Other *Pdx1*-dependent genes are *Hlxb9* (*Hb9*), which is induced by ectopic expression of *Pdx1* in chick embryo endoderm, and Sonic hedgehog, a negative regulator of pancreatic specification that is suppressed by *Pdx1*. However, it is not known if these effects are direct [53].

The regulatory network controlling the formation of neurogenin 3⁺ cells from embryonic duct precursor cells

The embryonic mouse pancreas undergoes major changes by E13.5 to E17.5. The two buds fuse, and signs of acinar and endocrine differentiation become evident, while the remaining pre-differentiated cellular epithelium is organised into prominent branching ductal structures (Fig. 2) [3]. In addition to these morphological changes, the pre-differentiated duct epithelium differs from earlier *Pdx1*⁺ bud epithelial cells at the level of gene expression. *Pdx1*, *Nkx2.2* and *Nkx6.1* are visibly down-regulated (though still expressed), while *Hnf6* and *Hnf1β* expression is maintained [34] (Fig. 2, Fig. 3, bottom panel). In fact, *Hnf1β* is slightly induced and serves as a specific marker for this ductal epithelial domain because it is not visibly expressed in differentiated acinar or endocrine cells [34].

The predominant wave of pancreatic endocrine cell generation takes place precisely at this embryon-

ic stage [54, 55]. Endocrine cells originate from lineage-committed progenitors that are marked by the helix-loop-helix transcription factor neurogenin 3 (Ngn3) [56]. Ngn3⁺ cells are present intercalated with Hnf1 β ⁺ cells in the epithelium that lines the lumen of embryonic ducts, and in their immediate vicinity (Fig. 2) [34]. Ngn3⁺ progenitors are short-lived cells lacking a significant self-renewal capability [27]. This raises the question of which cells provide the precursor pool for Ngn3⁺ cells. Several observations, including the existence of frequent Hnf1 β ⁺/Ngn3⁺ transition cells and the paucity of Hnf1 β ⁺/Ngn3⁻ cells in embryonic duct epithelium, support the idea that Hnf1 β ⁺ cells are the direct precursors of Ngn3⁺ cells [34]. The embryonic Hnf1 β ⁺ duct cell domain probably also provides the precursors of differentiated pancreatic duct cells, as pancreatic ducts are essentially composed of Hnf1 β ⁺ epithelial cells from E13.5 up to birth [34]. Thus, Hnf1 β ⁺ ductal cells at E13.5 to E17.5 can be regarded as a distinct stage of embryonic precursors that immediately precedes the initiation of lineage-specific differentiation (Fig. 2, Fig. 3).

What are the transcriptional mechanisms controlling the generation of Ngn3⁺ cells within the Hnf1 β ⁺ epithelial domain? The current evidence points to the presence of positive and negative mechanisms regulating mutually exclusive pathways either to differentiation or to self-renewal of the Hnf1 β ⁺ epithelium. *Hes1*, a basic helix loop helix transcriptional repressor that is an effector of the Notch receptor signalling pathway, is a major negative regulator of endocrine cell commitment [57]. Thus, *Hes1* homozygous null mutant embryos exhibit accelerated differentiation of Ngn3⁺ and endocrine cells, resulting in pancreatic hypoplasia due to early depletion of precursors [57]. This effect may be mediated through direct repression of the *Ngn3* gene by *Hes1* [58]. *Hes1* is activated by Notch receptor signalling, and accordingly the knock-outs for the Notch pathway genes delta-like ligand 1 (*dll1*) or RBP-J κ /CBF-1 exhibit phenotypes closely related to that of the *Hes1* knock-out [59]. In contrast, overexpression of activated Notch in embryonic precursors using *Pdx1* promoter-driven transgenics suppresses both endocrine and exocrine differentiation [60, 61].

The inactivation of Notch/*Hes1* in pancreatic duct precursors is therefore a decisive event for endocrine (and exocrine) differentiation. Accordingly, *Hes1* is absent in endocrine cells [62]. However, the mechanisms whereby Notch/*Hes1* is turned off in some precursor cells are unknown. One speculative scenario is that Ngn3 participates in this process, forming a negative cross-regulatory feedback loop. The activation of Ngn3 beyond a threshold in a given cell could thus result in the inhibition of its own repressor, hence consolidating commitment to the endocrine lineage. On the other hand, because Ngn3 turns on Notch ligand genes such as delta-like 1, activation of Ngn3 in a precursor cell is

thought to suppress differentiation in the surrounding cells as a mechanism to modulate the number of endocrine cells being generated [59, 63, 64].

The most prominent known positive regulator of Ngn3⁺ cell formation is Hnf6 [65]. Hnf6 is normally expressed in Hnf1 β ⁺ duct epithelium and acinar cells, but not in endocrine cells [30, 34, 65]. The *Hnf6* knock-out develops a pancreas but almost completely fails to generate Ngn3⁺ progenitors and endocrine cells during embryogenesis [65]. Moreover, Hnf6 interacts in vitro with a 5' flanking *cis* element of the *Ngn3* promoter and transactivates it in transient transfections [65]. It thus forms part of the program that induces the formation of Ngn3⁺ cells in concert with the inactivation of Notch signalling.

Another candidate key component of the Hnf1 β ⁺ precursor cell gene program is Hnf1 β . Embryos deficient in Hnf6 have markedly reduced Hnf1 β in primitive pancreatic ducts, demonstrating that Hnf6 is an important regulator of Hnf1 β in these cells [34]. Interestingly, Hnf6 also controls Hnf1 β in the bile duct epithelium, and both Hnf6-deficient mice and liver-specific *Hnf1 β* null mutants exhibit arrested bile duct differentiation, indicating that the Hnf6–Hnf1 β hierarchy regulates bile duct differentiation [66, 67]. However, the exact role of Hnf1 β in pancreatic ducts is still unclear. Whereas homozygous germ-line Hnf1 β mutations cause embryonic death before the pancreas is formed [68, 69], heterozygous mice have not been reported to exhibit a phenotype. Yet humans with diabetes owing to heterozygous *Hnf1 β* mutations do exhibit pancreatic hypoplasia, which would be the expected phenotype, if Hnf1 β acts as a regulator of pluripotent pancreatic precursor cells [70]. The Hnf6–Hnf1 β hierarchy thus links two transcription factors with important pancreatic developmental phenotypes, suggesting that it is an integral component of an as yet incompletely understood genetic program that regulates the function of ductal precursor cells.

Intriguingly, the embryonic bile duct epithelium shares many of the transcriptional components with its pancreatic equivalent, including the Hnf6–Hnf1 β hierarchy, expression of *Hes1*, and even *Pdx1* [34, 38, 66, 71]. A striking recent report showed that *Hes1*-deficient embryos exhibit conversion of bile duct epithelium into a pancreas-like structure containing both acinar and islet endocrine cells, in a process that involves activation of *Ngn3* [71]. Thus, embryonic pancreas and bile duct epithelium share a common intrinsic program capable of driving pancreatic differentiation. Unlike pancreatic duct cells, bile ductal cells normally suppress this program due to their inability to switch off *Hes1* expression. An understanding of the reasons for this difference could provide important clues on how to sort out the mechanisms underlying pancreatic differentiation. This finding also offers an extremely attractive potential route to induce the formation of beta cells in Type 1 diabetes.

Neurogenin 3 orchestrates a network that drives endocrine differentiation

Mice lacking Ngn3 have no pancreatic endocrine cells [56], indicating that Ngn3 is not just a marker of progenitors, but also an essential effector. In fact, Ngn3 is sufficient to induce endocrine differentiation in appropriate cellular contexts [53, 59, 63, 72]. Thus, transgenic overexpression of Ngn3 in early Pdx1⁺ cells results in massive endocrine differentiation, mostly glucagon cells [59, 72]. Similarly, electroporation of chick embryo endoderm with Ngn3 and Pdx1 gives rise to endocrine cells [53]. Again, glucagon but not insulin cells are generated, even if beta-cell-promoting factors such as Pax4 are added, indicating that the refinement of the endocrine subtype fate requires additional unidentified ingredients. In a different model, adenovirus was used to express Ngn3 in purified adult human ductal cells, resulting in expression of endocrine differentiation markers such as PC1, and even low levels of insulin [63].

The pro-endocrine role of Ngn3 results from the activation of several genes encoding endocrine differentiation regulators. Thus, in the human duct study described above, forced expression of Ngn3 induced expression of endocrine transcription factors NeuroD1, Pax4, Nkx2.2 and Pax6 [63], while in the *Ngn3* knock-out [56] a similar set of genes became inactive.

Many of the Ngn3-dependent, pro-endocrine regulators are direct genomic targets. In vitro Ngn3 binds and transactivates the 5' flanking region of *NeuroD1* (Beta2), another cell-restricted basic helix–loop–helix transcription factor gene that regulates the survival and terminal differentiation of beta cells [73]. NeuroD1 is likely to be directly responsible for part of the pro-endocrine effects of Ngn3, given that its misexpression in precursor cells of transgenic embryos or in human ducts partially mimics the effects of Ngn3 [63, 72].

Neurogenin 3 also binds and transactivates a *cis* element in the *Pax4* promoter [74], while *Ngn3*^{−/−} mice fail to express Pax4 mRNA [56], suggesting that Pax4 is also a direct target of Ngn3. During embryogenesis, Pax4 is only transiently expressed in progenitors (Fig. 3). Its function is to sub-specify the beta cell type within the endocrine progenitor pool [75]. Interestingly, one role of Pax4 is to directly or indirectly suppress the expression of another transcription factor, Arx, whose function is to promote the alpha rather than beta cell fate of Ngn3⁺ progenitors [76].

Another Ngn3 target, *Nkx2.2*, also drives endocrine differentiation. The regulation of *Nkx2.2* is somewhat complex. *Nkx2.2* is expressed at low levels in pre-differentiated epithelial cells, then exhibits a sharp induction in Ngn3⁺ cells and remains highly expressed in endocrine cells [32, 34, 64, 77] (Fig. 3). *Nkx2.2* is controlled by alternative promoters at different cellular stages [78]. Immediately after the Ngn3⁺ stage,

promoter 1a drives *Nkx2.2* expression in endocrine cells [78]. It has been suggested that Ngn3 and NeuroD1 consecutively activate *Nkx2.2* through this promoter in progenitor and endocrine cell stages, a suggestion based on the presence of E boxes that are bound and transactivated by these factors [78]. In contrast, promoter 1b is activated in Ngn3⁺ progenitor cells and probably requires a separate activation mechanism parallel to the induction of Ngn3, while the *cis* regulatory elements involved in the earlier bud cells are less well characterised [78].

Even though *Nkx2.2* is present in pancreatic epithelium prior to the activation of Ngn3, its first essential role is not until the immediate post-Ngn3 stage (Fig. 3). The inactivation of *Nkx2.2* gives rise to endocrine-like cells lacking insulin or Glut2 but expressing other endocrine markers such as amylin and Isl1, which suggests that the endocrine differentiation process is blocked or diverted [32]. In contrast, inactivation of another homeodomain transcription factor, *Nkx6.1*, results in a severe reduction of insulin-producing cells [31]. Remarkably, *Nkx2.2/Nkx6.1* double knock-outs entirely mimic the *Nkx2.2* mutant pancreatic phenotype [31], suggesting that the essential role of *Nkx6.1* takes place after the step controlled by *Nkx2.2*.

Interestingly, Ngn3 has also been shown to negatively regulate its own promoter in transient transfection studies, providing a potential mechanism for self-inactivation that is consistent with its transient expression during pancreatic development [79].

Pdx1, a critical regulator of the differentiated beta cell phenotype

Once beta cells are formed during pancreatic development, genetic programs are needed to carry out their specialised functions. One obvious function of such programs is to support high levels of insulin gene transcription. Another equally important task is to activate a vast number of genes involved in (i) the sensing of extracellular signals such as nutrients, hormones and neurotransmitters; (ii) the coupling of these signals to the process of secretion; and (iii) the regulation of beta cell mass or the ability to respond to immune-mediated damage [80, 81, 82].

In addition to its role in early organogenesis, Pdx1 is also a key regulator of differentiated beta cells. Shortly after the Ngn3⁺ cell stage, Pdx1 is sharply induced in insulin-producing cells and remains highly expressed throughout adult life (Fig. 3). Targeted disruption of the *Pdx1* gene in mature beta cells leads to a diabetic phenotype due to impaired beta cell function [6, 83, 84]. This is associated with reduced activity of islet-specific genes regulated by Pdx1 such as insulin, IAPP, *Nkx6.1* and Glut2 [6, 84]. A similar expression profile results from dominant negative

suppression of *Pdx1* in INS1 beta cells [85]. In vitro binding and transient transfection studies suggest that *Pdx1* interacts directly with the 5' flanking regions of these four genes [86, 87, 88, 89], while ChIP experiments have confirmed that *Pdx1* binds in vivo to the insulin and IAPP genes in cultured beta cell lines [90, 91].

The key role of *Pdx1* in beta cells is further emphasised by mouse and human genetic studies showing that beta cells are sensitive to *Pdx1* gene dosage. Thus heterozygous mutant mice exhibit beta cell dysfunction and diabetes associated with defective expression of *Glut2*, *IAPP* and *Nkx6.1* [92, 93], as well as increased apoptosis [94], whereas in humans heterozygous IPF1 (*Pdx1*) mutations lead to Maturity onset diabetes of the young (MODY) 4 [95].

How is cell-specific, high-level *Pdx1* expression achieved in beta cells?

Given the pre-eminent role of *Pdx1* in differentiated beta cell function, it is not surprising that the regulation of the *Pdx1* gene in these cells has attracted considerable interest. Numerous transgenic and cell culture studies have by now established that the cell-specific transcription of the *Pdx1* gene is mediated by several *cis* regulatory areas located at more than 1.6 kb from the transcription site, most of which are evolutionarily conserved [48, 96, 97].

Several regulators of *Pdx1* in beta cells have been identified, one of which is *Foxa2*. As mentioned earlier, in vitro binding studies and transient transfection studies have identified multiple *Foxa2*-binding sites in the distant 5' flanking regions of human, mouse and rat *Pdx1* genes [47, 48, 50, 97, 98], while ChIP analysis has shown that *Foxa2* binds to the mouse *Pdx1* distant enhancer region in beta cell lines [49]. Transgenic experiments have shown that the so-called Area II of the *Pdx1* distant 5' flanking region, which contains one of the high-affinity *Foxa2*-binding sites, can drive beta-cell-specific expression, whereas mutation of the *Foxa2*-binding site reduced beta cell expression penetrance of another transgene [49]. Furthermore, beta-cell-specific ablation of *Foxa2* with the Cre/loxP system results in down-regulation of islet *Pdx1* mRNA and protein levels [99].

Recently, the beta-cell-enriched MafA basic-leucine zipper protein was found to bind to a conserved *Pdx1* Area II sequence in vitro and in vivo in a beta cell line [100]. Transient transfection studies with substitution mutants of the *Pdx1* gene Maf-binding site suggest that MafA exerts positive regulation of this gene in cultured beta cell lines [100]. The same region is bound by *Pax6* in beta cell lines, both in vitro and in vivo, and mutational analysis of the *Pax6*-binding site suggests that it too is a positive regulator of the *Pdx1* gene [49].

As mentioned earlier, some data point to autoregulation of *Pdx1*, a finding based on the in vitro [47] and in vivo binding of *Pdx1* to a conserved distant enhancer region of *Pdx1* in cultured beta cells [46, 90]. The physiological consequences of this loop in beta cells are still unclear, as *Pdx1*^{+/-} islets exhibit, as expected, roughly 50% expression of *Pdx1* mRNA, whereas some existing studies have proposed either positive or negative regulatory effects [46, 47, 83, 93].

Hnf1α, a POU-homeodomain protein involved in *MODY3*, binds in vitro to the mouse and human *Pdx1* genes [46, 98]. The genetic data on the functional consequences of this interaction are somewhat controversial. Whereas one study showed a 2.9-fold reduction of *Pdx1* mRNA in islets of *Hnf1α*^{-/-} mice [21], two others failed to show this in young *Hnf1α*^{-/-} mice [19, 101] (the latter [101] did find a reduction after 6 weeks of age). Moreover, dominant negative suppression of *Hnf1α* in INS1 cells fails to inhibit *Pdx1* mRNA [102]. Potential explanations of these discrepancies include (i) the effects of diverse genetic backgrounds, and (ii) the suggestion that *Hnf1α* is required after a certain postnatal stage and/or that the observed effects are secondary to hyperglycaemia.

So far, in short, no single factor has been found to be absolutely indispensable for *Pdx1* expression in beta cells. On the other hand, recent studies have revealed a combination of cell-specific transcriptional activators that bind to the *Pdx1* gene, many of which are involved in ensuring high levels of expression in beta cells.

A signalling cascade acting through *Pdx1*

Recent studies have provided interesting insights into how signalling cascades can mediate their effects through the modulation of beta cell transcription networks. A revealing example is the regulation of *Pdx1* by *Irs2* and *Foxo1*, two transducers of key extracellular signals such as insulin and IGF1 [103].

Several genetic experiments support the proposal that *Irs2* is required for Akt to phosphorylate the forkhead transcription factor *Foxo1*, which is then excluded from the nucleus and prevented from inhibiting the *Pdx1* gene. *Irs2*^{-/-} mice thus exhibit reduced phosphorylation of *Foxo1* and diminished expression of *Pdx1* [104, 105]. Haplo-insufficiency for *Foxo1* partially restores the expression of *Pdx1* and reverses beta cell failure caused by *Irs2* deficiency. Moreover, a constitutively active *Foxo1* mutant inhibits *Pdx1* expression in beta cells [106].

The mechanism whereby *Foxo1* exerts this negative effect on the *Pdx1* gene is not known, although one suggestion is that it inhibits *Foxa2*-dependent regulation of *Pdx1* [104]. On the other hand, the extent to which the *IRS2* and *Foxo1* findings relate to

Fig. 5. Basic regulatory motifs in genetic networks. Certain patterns of regulation between transcription factor genes are over-represented in gene networks. Circles represent transcription factor genes, arrows show that a gene product exerts direct control of another gene or its own gene. This figure is largely based on data presented in references [128, 129]

insulin and IGF1-dependent signalling has yet to be established. Beta-cell-specific knock-outs for insulin- and IGF1-receptor genes have reduced beta cell function, but this has not been reported to result in reduced *Pdx1* expression [107, 108, 109, 110]. Future studies will thus need to further define the precise signals and regulatory contexts that drive *Irs2/Foxo1* regulation of the *Pdx1* gene.

Multi-input motifs control the transcription of beta-cell-specific genes

Much of what we know about the transcriptional control of beta cells stems from a wealth of studies that analysed the *cis* and *trans* elements involved in insulin gene transcription [111, 112]. Three major pancreatic transcription factors were discovered precisely because of their ability to bind to several evolutionarily conserved sites in the insulin gene promoter, namely *Pdx1* [113], *MafA* [114, 115] and *NeuroD1/Beta2* [116], although these same factors were also independently cloned through other routes [117, 118, 119, 120, 121]. Subsequent *in vivo* studies have verified that all three factors occupy insulin gene chromatin in cultured beta cell lines [90, 91, 115]. As discussed earlier, *Pdx1* is essential for normal insulin gene expression in mouse beta cells [6, 83, 84]. The *MafA* knock-out has not been reported on, although cell culture studies indicate that one major role may be related to glucose induction of insulin gene transcription [120], while transgenic studies analysing a DNA rat insulin promoter 3 element, to which *MafA* binds, suggest that it contributes to cell-specific expression [122]. Surprisingly, homozygous *NeuroD1* mutant mice showed that this gene is not indispensable for insulin gene expression, despite numerous functional studies in cultured cell lines indicating that

it plays a regulatory role in rodent and human insulin genes [116, 123, 124, 125]. To date, no detailed analysis of these mice has determined whether insulin mRNA content is partially reduced or whether the physiological regulation of insulin gene expression is impaired.

Two other factors that bind the insulin gene *in vitro* and *in vivo* in cultured beta cell lines are *Nkx2.2* and *Pax6* [91]. As discussed earlier, *Nkx2.2* is essential for insulin gene activity in mice [32], and in one of the two *Pax6* mutants marked reductions of insulin gene expression were observed [126]. Thus, analogously to the situation described for the *Pdx1* gene, beta-cell-specific activation of the insulin gene is dependent on a unique combination of nuclear factors occupying its 5' flanking region in beta cells.

Remarkably, a nearly identical collection of transcription factors (i.e. *Pdx1*, *Pax6*, *NeuroD1*, *Nkx2.2*) has been reported to occupy a set of beta cell promoters that includes glucokinase, *IAPP* and *Pax4* [91, 127] (Fig. 4). This pattern, whereby multiple factors control a common set of genes, is a characteristic motif encountered in transcription networks and referred to as a multi-input motif [128, 129] (Fig. 5). It has been suggested that such multi-input motifs confer the ability to provide coordinated gene responses to diverse regulatory signals [128], but they can also more simply underlie the combinatorial code required to activate a particular set of cell-specific genes. On the other hand, *MafA* and *Pax6* both regulate the insulin gene as well as *Pdx1* [49, 91, 100, 115], which itself is a regulator of the insulin gene. Feed-forward motifs of this sort are thought to serve as a switch, capable of responding to sustained rather than transient inputs (the distal target gene is activated only if a previous input has enabled expression of the intermediate gene) [128, 129, 130, 131]. It could therefore be speculated that such motifs modulate insulin transcription as a function of long-term metabolic demands.

Collectively these results show that the transcription of the insulin gene results from the concerted action of a complex non-linear beta-cell-specific network. While several major components of this network have been identified, the regulatory logic of its structure is not yet understood.

Several MODY genes play key roles in a common differentiated beta cell network

The genetic analysis of MODY has provided a mine of previously unsuspected information on beta cell transcription networks. We now know that MODY is caused by heterozygous mutations in at least five genes encoding transcription factors: *HNF4A/HNF4A* (MODY1), *HNF1A/TCF1* (MODY3), *PDX1/IPF1* (MODY4), *HNF1B/TCF2* (MODY5) and *NEUROD1/BETA2* (MODY6) [95, 124, 132, 133, 134] (reviewed in references [14] and [13]). MODY2, which is so far the only subtype not related to a transcription factor [135], is caused by mutations in the glucokinase gene.

MODY is essentially a haplo-insufficient phenotype, based on functional studies showing that most mutations are loss-of-function defects and that disease-causing mutations exist in promoter regions [7, 136, 137, 138, 139, 140]. Mutation carriers develop a severe impairment of insulin secretion and diabetes after at least 10 years of age [141, 142, 143]. Interestingly, beta cell dysfunction does not result from the sheer lack of insulin, as elegantly shown in a recent human therapeutic trial. This trial [144] revealed that MODY3 patients retain a considerable ability to secrete insulin in response to sulphonylureas. This suggests that Hnf1 α controls specific differentiated functions of beta cells, rather than solely the beta cell mass or insulin gene transcription.

Hnf1 α ^{-/-} mice are viable, and have thus turned out to be a very fruitful model to understand the function of MODY transcription factors in beta cells [19, 20, 21, 145, 146, 147]. These mice have abnormal glucose-induced insulin release and develop diabetes shortly after birth, in addition to other metabolic abnormalities in the liver and kidney [145, 146]. Reduced nutrient-induced insulin release in Hnf1 α -deficiency has been linked to impaired islet glucose metabolism [102, 147, 148]. A number of beta cell targets of Hnf1 α have been identified, e.g. *Glut2* and L-pyruvate kinase (*Pklr*), but the role of specific gene defects in the stimulus–secretion coupling phenotype of Hnf1 α -deficient islets is unclear [19, 20, 102, 147, 148]. Interestingly, mRNA levels of several beta cell targets such as *Glut2* and *Pklr* are not reduced in hepatocytes or pre-differentiated pancreatic cells from *Hnf1 α ^{-/-}* mice, suggesting that the role of *Hnf1 α* is highly dependent on the developmental and cell-type-specific context [19].

Islets from *Hnf1 α ^{-/-}* mice exhibit decreased expression of several transcription factor mRNAs, including *Hnf4 α* , *Hnf4 γ* , *Shp* and *Foxa3*, pointing to the existence of a broad Hnf1 α -dependent transcriptional network [20, 21]. Being also a MODY gene, *Hnf4 α* is a particularly interesting transcription factor target. Importantly, *Hnf4 α* mRNA is dependent on Hnf1 α specifically in differentiated pancreatic cells, but not in the liver [20]. This is because in the pancreas the

Hnf4 α gene is almost exclusively driven by the P2 promoter, which is bound and controlled by Hnf1 α , whereas in the liver and most other tissues *Hnf4 α* is predominantly driven by the P1 promoter, which does not require Hnf1 α [20, 149, 150].

The tissue specificity of Hnf1 α -dependent expression of *Hnf4 α* is concordant with the common beta cell phenotype in MODY due to mutations in these two genes. This suggests that Hnf1 α control of *Hnf4 α* P2 promoter could be a critical regulatory link in beta cells. This is strongly reinforced by the identification of a natural human mutation in the *HNF4A* P2 promoter that selectively impairs binding by Hnf1 α and causes diabetes [138]. What is remarkable about this mutation is that although Hnf1 α regulates multiple genes in beta cells, the selective disruption of its regulation of *Hnf4 α* has similar consequences to mutations that completely disrupt Hnf1 α function [138]. On the other hand, this Hnf1 site mutation is one of three reported genetic defects that disrupt P2 promoter function and cause MODY [149, 151], and together they provide compelling confirmation that this is the biologically significant *Hnf4 α* promoter in the pancreas, despite the fact that P1-driven transcripts can be detected by PCR in human islet RNA [152] (S.F. Boj and J. Ferrer, unpublished).

Regulation in the opposite direction, i.e. the regulation of *Hnf1 α* by *Hnf4 α* , also occurs. However, in this case there is no beta cell selectivity. The germ-line *Hnf4 α* knock-out is lethal at a very early embryonic stage [153], but Hnf1 α expression is reduced in *Hnf4 α ^{-/-}* embryoid bodies [136], in *Hnf4 α* -deficient hepatocytes from chimeric mutant embryos [154], and in a Cre/loxP-based *Hnf4 α* liver-specific knock-out (Iannis Taliandidis, unpublished observations). In vitro and in vivo transgenic studies have shown that regulation of the *Hnf1 α* gene by *Hnf4 α* is dependent on an evolutionarily conserved *cis* sequence element in the *Hnf1 α* promoter [17, 155, 156]. Support for the notion that *Hnf4 α* controls *Hnf1 α* expression both in liver and in pancreatic cells comes from a human mutation in this same conserved *cis* element that co-segregates with MODY in a large pedigree [140]. Furthermore, dominant negative suppression of *Hnf4 α* in INS1 cells decreases expression of Hnf1 α , resulting in inhibition of the same mRNAs as dominant negative suppression of Hnf1 [4]. Thus, Hnf1 α is dependent on *Hnf4 α* in numerous cell types, but specifically in pancreatic cells the two genes are interdependent.

The Hnf1 α –Hnf4 α positive cross-regulatory loop could act as a cellular memory mechanism that maintains the expression of the two genes and their targets in differentiated pancreatic cells. Other self-sustaining cross-regulatory circuits connecting MODY genes are also likely to operate in beta cells. For example, *Hnf1 α* mRNA is reduced in *Pdx1^{+/-}* mice [93], while in some studies discussed earlier *Pdx1* and *NeuroD1* mRNA was reduced in *Hnf1 α ^{-/-}* mice [21]. A regula-

tory interaction of some sort between *Pdx1* and *Hnf1 α* is further supported by the finding that *Pdx1*^{+/-}*Hnf1 α* ^{+/-} double heterozygous mice exhibit a more severe beta cell defect than expected from the sum of the single heterozygous defects [93]. The regulation of *Pdx1* by *Hnf4 α* has not yet been analysed, but *Pdx1* interacts in vitro with a TAAT element in the *Hnf4 α* P2 promoter and transactivates it in transient transfections [149]. A mutation in this *cis* element causes MODY in a large pedigree, indicating that the sequence is essential for *Hnf4 α* promoter activity [149].

MODY as a paradigm of a haplo-insufficient defect resulting from the breakdown of cell-specific networks

One of the outstanding questions in the genetics of MODY is why heterozygous mutations in genes like *HNF1 α* or *HNF4 α* cause a phenotype essentially restricted to beta cells, whereas homozygous mutations give rise to phenotypes affecting numerous other cell types [7, 153, 157, 158]. The resolution of this paradox may reside in the fact that these transcription factors form part of different networks in distinct cell types. The network operating in beta cells may be sensitive to haplo-insufficiency, whereas other networks need not be affected.

It is thus worth reflecting on the consequences of reduced gene dosage in the context of the pancreas-selective *Hnf1 α* –*Hnf4 α* circuit configuration [7]. Positive cross-regulatory loops like this one (gene A is dependent on B, gene B is dependent on A) typically exhibit bistable behaviour [9, 159, 160]. By this we mean that reciprocal activation between two genes (such as *Hnf1 α* and *Hnf4 α*) can serve to maintain the activity of both genes in a very stable state. However, because they are interdependent, a transient reduction of either gene that is severe enough to fall below a certain threshold can lead to a severe inhibition of the other gene. This results in an alternate stable state, in which the two genes are expressed at a level below that required to activate each other [160]. Reduced gene dosage of *Hnf1 α* or *Hnf4 α* could therefore increase the probability that the transition from the active to the inactive stable state occurs due to the diminished ability of a one-allele system to prevent severe transient reductions of a gene product [7, 161, 162]. In such a scenario, a germ-line mutation that disrupts a single *Hnf1 α* or *Hnf4 α* allele can lead to the stable functional knockdown of all four alleles that compose the circuit. This is expected to occur selectively in cells where the two genes are interdependent, in keeping with the cellular-specificity of MODY.

MODY thus apparently results from the collapse of cell-specific transcriptional networks due to the haplo-insufficiency of key network genes. In fact, the genet-

ic analysis of MODY may have helped to reveal not only unsuspected beta cell transcription factors, but also the keys to understand the functional logic of underlying regulatory networks.

Pancreatic transcription networks as complex systems

As more knowledge is gained about pancreatic transcription networks, it will become theoretically possible to interpret general aspects of their behaviour, and not solely their individual components. Studies in much more manageable models than the mammalian pancreas, such as *Escherichia coli* and *Saccharomyces cerevisiae*, have provided enough data to indicate that transcription factors that control most cellular functions form complex networks [128, 129, 130, 163]. It is thus worth considering some overarching principles governing the behaviour of complex networks.

One interesting notion is that networks as diverse as the Internet, ecological webs or transcription and metabolic networks are generally arranged in a highly non-random manner [130, 164, 165]. A very common non-random configuration is known as the scale-free network. In such a network a few nodes are disproportionately highly connected to other nodes, whereas most nodes have very few links [164]. Such networks are extremely robust insofar as the individual loss or change in behaviour of most nodes have few consequences. However, selective inactivation of the few highly interconnected nodes can be catastrophic [164]. It will be interesting to see to what extent this concept applies to pancreatic networks as well as potentially to the fact that some transcription factor mutations in mice and humans with pancreatic transcription factor loss of function lead to particularly severe pancreatic phenotypes, whereas others appear to be inconsequential.

Another interesting non-random feature of most complex networks is the over-representation of connectivity patterns or “motifs” [128, 129, 130]. Experimental studies have shown that transcription factor networks in particular possess a disproportionately high representation of certain motifs such as feedforward loops, autoregulation, multi-component feedback loops or multi-input motifs [128, 129, 130] (Fig. 5). Of particular interest is a landmark study that used ChIP-on-chip to identify the *in vivo* binding sites in the *S. cerevisiae* genome for 106 of the 141 known *S. cerevisiae* transcription factors [128] (schematic description of ChIP-on-chip, see Fig. 1). This study provided a bird’s eye view of the topology of a transcription network in a eukaryotic cell and showed that transcription factor genes are highly interconnected, with frequent well-defined regulatory motifs such as those shown in Fig. 5, as well as highly connected hub-like nodes [128].

Interestingly, similar motifs have also been encountered in pancreatic networks, as discussed in this review. Experimental and mathematical modelling data suggest that such over-represented motifs fulfil specific regulatory strategies, some of which have been discussed earlier in this review [9, 129, 130, 131, 159]. In some cases, motifs or combinations of motifs form modules whose function can be analysed independently of the remaining network [166]. In fact, it is possible that an understanding of the function of large networks can, to a large extent, be achieved through careful hypothesis-driven analysis of key modules. Resources such as ChIP-on-chip screens could thus provide the “road maps” that describe the global network topology and help identify unsuspected subnetworks.

Concluding remarks

The development of the pancreas is controlled by transient networks that operate during consecutive precursor cell stages. Specific gene networks are also in place to provide long-term maintenance of the differentiated beta cell phenotype. The past few years have seen unprecedented progress in our understanding of pancreatic networks, largely through the testing of focused hypotheses. Further advances are expected in the field as a consequence of large-scale gene expression, ChIP, proteomics and mutation screens. The significance of these studies will be determined by whether they can be applied to the major developing and adult pancreatic cell types, as opposed to transformed cell lines or heterogeneous cellular populations.

From what is already known, it is apparent that most pancreatic transcription networks are not simple lineal hierarchies, but complex networks analogous to those recently described in more simple cellular models. Further complexity is to be expected from the integration of gene networks with other regulatory networks, including those formed by signal transduction pathways and protein–protein interaction webs. We believe that an approach combining the availability of large-scale network maps with more focused hypothesis-driven experimentation will eventually provide an in-depth understanding of the behaviour of pancreatic networks that is concordant with the complex biological reality. This in turn should provide foundations for the development of new therapies for beta cell loss and dysfunction in human diabetes.

Note added in proof. While the manuscript was in press, Odom et al. (Science 303:1378–1381, 2004) have reported a study which analyses the *in vivo* binding sites of *Hnf1 α* , *Hnf4 α* and *Hnf6* in hepatocytes and islet-enriched pancreatic tissue using chromatin immunoprecipitations and a microarray containing the upstream regions of 13 000 human genes.

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