REVIEW

A focus on the role of Pax4 in mature pancreatic islet β-cell expansion and survival in health and disease

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Abstract

Blood glucose homeostasis is achieved by the regulation of insulin and glucagon secretion from the pancreatic islet β- and α-cells. Diabetes mellitus, which comprises a heterogeneous group of hyperglycaemic disorders, results mainly from inadequate mass and function of islet β-cells. Autoimmune destruction of β-cells causes type 1 diabetes, while type 2 is characterized by impaired insulin secretion and is often associated with diminished insulin action on its target tissues. Interestingly, similar to type 1 diabetes, a gradual loss of β-cell mass is observed in type 2 diabetes often requiring insulin therapy. Understanding the molecular mechanism that governs β-cell mass plasticity may provide a means to develop strategies to counteract β-cell death while increasing replication. Of particular interest is the islet-specific transcription factor paired box4 (Pax4) that was previously shown to be indispensable for the establishment of the β-cell lineage during development. However, recent accumulating evidence now suggest that Pax4 is also crucial for mature β-cell expansion and survival in response to physiological cues and that mutations or polymorphisms are associated with both type 1 and type 2 diabetes. In contrast, aberrant expression of Pax4 confers protection against apoptosis to insulinomas, whereas it promotes cell growth in lymphocytes. This review summarizes promising new published results supporting the important function of Pax4 in mature islet β-cell physiology and its contribution to pathophysiology when deregulated.

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Introduction: diabetes, obesity and pancreatic islet plasticity

Plasma glucose levels are regulated by the action of insulin, a hormone that is produced and secreted by the pancreatic islet β-cells in response to nutrients. Diabetes mellitus, which comprises a heterogeneous group of hyperglycaemic disorders, results from inadequate mass and function of β-cells (Prentki & Nolan 2006). In 2007, over 200 million people were diagnosed with diabetes mellitus and it is now considered one of the most common non-communicable diseases in the world causing 5% of all deaths per year (www.eatlas.idf.org). Alarmingly, the World Health Organization predicts that this will reach epidemic proportions with at least 366 million people afflicted with the disease by 2030 (www.who.int/dietphysical-activity/publications/facts/diabetes/en). Diabetes mellitus was recently reclassified into three main entities: 1) type 1 diabetes which is linked to selective autoimmune destruction of pancreatic β-cells, 2) type 2 diabetes which is a severe disease of intermediary metabolism usually caused by both β-cell dysfunction and resistance to the biological actions of insulin on its main target tissues (liver, muscle and adipose) and 3) gestational diabetes mellitus (GDM) which is characterized by glucose intolerance with onset or first recognition during pregnancy (Bell & Polonsky 2001, Saltiel & Kahn 2001, Expert Committee on the Diagnosis and Classification of Diabetes Mellitus 2003, Stumvoll et al. 2005, Shaat & Groop 2007). Long-term complications of diabetes include retinopathy and nephropathy as well as cardiovascular and cerebrovascular symptoms. Current therapy for type 2 diabetes and GDM include modification of lifestyle, such as diet and exercise, and the use of pharmacological agents that stimulate insulin secretion, decrease hepatic glucose production and increase sensitivity of target tissues to insulin. Nonetheless, often type 2 diabetic patients, like type 1 diabetics, require insulin therapy (Donath & Halban 2004).

The phenomenal rise in the incidence of diabetes is predominantly attributable to its close association with development of obesity. Indeed, ~60% of all cases of type 2 diabetes are directly linked to overweight due to shifting dietary habits and increasingly sedentary
lifestyles of humans (Yach et al. 2006). Studies in animal models have demonstrated that obesity-associated insulin resistance as well as increased insulin requirements during pregnancy is matched by a corresponding stimulation in insulin output through β-cell hyperplasia and hypertrophy. For instance, rat pancreatic β-cell numbers transiently increase by 50% during pregnancy before returning to normal levels post partum (Scaglia et al. 1995). This β-cell plasticity reaches remarkable levels in Zucker diabetic fatty (fa/fa) rats in which a fourfold increase in islet mass is observed with progression of obesity (Unger 2005). A similar increase in islet volume was shown in ob/ob mice when compared with control animals (Bock et al. 2003). The expansion of β-cell mass is also observed in human obesity as well as in pregnancy, the latter most likely mediated by increased circulating placental lactogen and prolactin (Kloppel et al. 1985, Brelje et al. 1993, Sorensen & Brelje 1997, Butler et al. 2003). Interestingly, up to 20% of obese individuals develop type 2 diabetes probably caused by a defective β-cell adaptation due to increased sensitivity to harmful environmental factors such as free fatty acids combined with predisposing genetic factors (Kashyap et al. 2003). The latter emphasizes that overall β-cell mass adaptation in response to physiological as well as to pathophysiological conditions is not only governed by the generation of new insulin-producing cells but also by susceptibility to cell death. In the extreme such as in type 1 diabetes, autoimmune-mediated cell destruction is greater than the rate of regeneration leading to hyperglycaemia and invariably requiring insulin therapy. However, in rare cases, spontaneous remission characterized by increased C-peptide secretion has been reported in type 1 diabetic patients, clearly indicating the capacity of β-cell regeneration to prevail over autoimmune annihilation (Bonfanti et al. 1998). Fundamental mechanisms governing β-cell replenishment remain to be identified. In this review, we discuss the potential role of the transcription factor paired box4 (Pax4) as a key player orchestrating the gene network governing β-cell mass expansion and survival under both physiological and pathophysiological conditions.

Defining the regenerative unit of the pancreatic islet: the β-cell at centre stage

The search for the regenerative β-cell or precursor pool that responds to metabolic demands has proved more challenging and controversial than anticipated (Bock 2004, Bonner-Weir et al. 2004, Bonner-Weir & Sharma 2006). Indeed, depending on the experimental model and degree of injury inflicted to the pancreas, new β-cells were shown to be generated by neogenesis of ductal epithelium cells (Peshavaria et al. 2006), by transdifferentiation of acinar cells (Lipsett & Finegood 2002, Minami et al. 2005) and from intra-islet nestin-positive precursor cells (Zulewski et al. 2001). Bone marrow stem cells also appear to indirectly contribute to islet regeneration by promoting proliferation of resident islet cells (Ianus et al. 2003, Hasegawa et al. 2007). Alternatively, lineage-tracing studies elegantly demonstrated that pre-existing mouse adult pancreatic β-cells, rather than specialized progenitors, were the major source of new insulin-producing cells during adult life and after pancreatectomy (Dor et al. 2004, Nir et al. 2007, Teta et al. 2007). Furthermore, it was recently reported that all β-cells contributed equally to islet growth and maintenance (Brennand et al. 2007). Thus, similar to other organs such as the liver, all of the aforementioned sources most likely contribute to regeneration according to the severity of the injury (Corcèlle et al. 2006). However, new evidence showing sustained β-cell apoptosis in human patients with longstanding type 1 diabetes along with the demonstration of β-cell mitogenesis in a diagnosed type 1 diabetic patient clearly argues in favour of β-cell replication as the predominant mechanism of in vivo islet regeneration in humans (Meier et al. 2005, 2006b). Consistent with this premise, β-cell proliferation was observed in the vicinity of intrapancreatic gastrinomas in humans (Meier et al. 2006a). Therefore, harnessing key regulatory factors responsible for mature β-cell replication would certainly facilitate development of a regenerative therapy for the treatment of diabetes.

Pax4 as a master regulator of islet development

Pax genes encode a family of transcription factors that are key regulators of tissue development and cellular differentiation in embryos acting to promote cell proliferation, migration and survival. Pax proteins comprise nine members divided into four groups based on the specific assembly of three structural domains: the paired domain, the homeodomain and the octapeptide (Lang et al. 2007). DNA binding activity is conferred by either the paired or homeodomain. Pax6 and Pax4, which form subgroup IV characterized by the absence of the octapeptide, are predominantly expressed in the pancreatic islet as well as in the central nervous system for Pax6. Pax4 was shown to be essential for the generation of islet cell progenitors and subsequent β- and somatostatin-producing δ-cell maturation, while Pax6 was found to be crucial for α-cell fate lineage during embryogenesis (Sosa-Pineda et al. 1997, St-Onge et al. 1997, Greenwood et al. 2006). Interestingly, Pax4 expression is predominantly restricted to β- and δ-cells, whereas Pax6 is ubiquitously expressed in all islet cell types (Sosa-Pineda et al. 1997, St-Onge et al. 1997). During development, the
Pax4 transcript is initially detected in the pancreatic bud at embryonic day (E) 9.5, becoming maximal at E13.5–15.5 and thereafter declining to low expression levels. Lineage-tracing studies performed on transgenic mice bearing a pax4 promoter/cre recombinase gene DNA cassette confirmed confinement of the Pax4 expression domain exclusively to endocrine cells of the islets (Greenwood et al. 2006). Consistent with its tissue and cell-specific expression pattern, targeted disruption of the pax4 gene in mice results in the absence of mature β- and δ-cells with a commensurate increase in the α-cells (Sosa-Pineda et al. 1997, Wang et al. 2004). This increase was attributed to the α-cell-specific transcription factor Arx that is repressed by Pax4 during development (Collombat et al. 2003, 2005). Substantiating the latter finding, conditional expression of Arx in either embryonic or adult β-cells was recently found to convert β-cells into α- and pancreatic polypeptide (PP)-producing cells (Collombat et al. 2007). In mouse mutant Pax4 embryos, scattered insulin-staining cells are apparent at early stages of development (E8.5–9), indicating that Pax4 expression is most likely not mandatory for the generation of β-cell precursors. However, the strong induction of pax4 gene expression between E13.5 and E15.5 indicates that Pax4 is critical for sustaining the phenotype as well as the proliferation and/or survival of these early committed insulin-producing cells (Sosa-Pineda et al. 1997). Consistent with this premise, the time interval between E13.5 and E15.5 corresponds to a period of massive proliferation and differentiation of β-cells, the so-called secondary transition phase (Pictet & Rutter 1972).

### Pax4 as a key player coordinating mature islet β-cell plasticity

Expression of Pax4 in mature islets has been a matter of debate nourished by the inability to detect low endogenous transcript levels as well as the lack of specific antibodies (Sosa-Pineda et al. 1997, Smith et al. 1999, 2000). The failure to generate Pax4 antibodies most likely stems from its high homology to other Pax members, rendering it difficult to delineate specific antigenic peptide to Pax4. However, novel approaches in antibody production may provide a means to circumvent this caveat in the close future. Nonetheless, recent studies, including ours, have provided evidence for the expression of the Pax4 transcript in adult human, rat and mouse pancreatic islets (Zhang et al. 2001, Heremans et al. 2002, Kojima et al. 2003, Zalzman et al. 2003, Brun et al. 2004, Theis et al. 2004). Furthermore, several human genetic studies have highlighted the importance of Pax4 on islet physiology (Table 1). Indeed, mutations in the pax4 gene were shown to be associated with type 2 diabetes in the Japanese population as well as in Afro-Americans (Shimajiri et al. 2001, 2003, Kanatsuka et al. 2002, Mauvais-Jarvis et al. 2004, Tokuyama et al. 2006). More recently, two mutations in Pax4 were also linked to a subform of type 2 diabetes, maturity onset diabetes of the young (MODY) in the Thai population (Plengvidhya et al. 2007). In addition, two haplotypes of Pax4 have been correlated with type 1 diabetes in Scandinavian families (Holm et al. 2004). Consistent with the latter, an independent study identified two variants of the pax4 gene that were differentially distributed among normal individuals and type 1 diabetic children in the Swiss and German populations. The Pax4C variant was frequent in type 1 diabetic children (73%) and rare in the control population (32%). In contrast, the heterozygote Pax4A/C combination was prevalent in controls (62%) and in islet antibody-positive subjects (73%-6%) that did not develop diabetes but rare in patients that became diabetic (17.5%; Biason-Lauber et al. 2005). However, no correlation between this polymorphism and type 1 diabetes could be established in Finnish, Hungarian and UK populations (Hermann et al. 2005, Martin et al. 2006). Discrepancies in these studies most likely arise from genetic heterogeneity or population differences in gene–gene as well as gene–environment interactions. As the common denominator between type 1 and type 2 diabetes is a gradual destruction of β-cells, we have recently proposed the working hypothesis that Pax4 is likely critical for the expansion as well as the survival of β-cell mass. Dysfunction in the pax4 gene during

### Table 1 Mutations and polymorphisms in the paired box 4 (pax4) genes that are associated with diabetes in specific populations

<table>
<thead>
<tr>
<th>Nucleotide change&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Diabetic phenotype</th>
<th>Population</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C492T</td>
<td>Type 2, MODY</td>
<td>Thai</td>
<td>Plengvidhya et al. (2007)</td>
</tr>
<tr>
<td>A1168C</td>
<td>Type 1</td>
<td>Swiss/German</td>
<td>Biason-Lauber et al. (2005)</td>
</tr>
<tr>
<td>Nucleotide 1 (intron 7) G/A</td>
<td>Type 2, MODY</td>
<td>Thai</td>
<td>Plengvidhya et al. (2007)</td>
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<sup>a</sup>The nucleotide position is relative to the transcriptional initiation site defined as +1.
development and/or in mature islets would contribute to increased susceptibility to apoptosis coincident with decreased cell proliferation leading to a gradual loss of β-cells and ultimately to diabetes in adulthood.

To understand the mechanisms that control *pax4* gene expression and thereby impacting its function in mature β-cells, several studies have mapped and characterized the regulatory regions of both the human and mouse *pax4* genes (Smith et al. 2000, Xu & Murphy 2000, Brink et al. 2001). Sequence deletion analysis revealed that the optimal mouse *pax4* gene promoter region required for pancreatic islet expression was found within the first 400 bp upstream of the transcription initiation site. This region shares 88% similarity with the human sequence (Brink et al. 2001, Brink & Gruss 2003). *Pax4* expression was shown to be dependent on the concerted action of the key pancreatic transcription factors Pan1, Beta2/NeuroD, hepatic nuclear factor (HNF)-1α, HNF-4α, pancreatic duodenal homeobox (Pdx)1 and E47/E12 interacting with the proximal promoter (Smith et al. 2000, 2004, Kanno et al. 2006). A further increase in transcription was observed when Beta2/NeuroD was substituted by the early pancreatic committing transcription factor Ngn3 (Smith et al. 2000, 2004). The action of Beta2/NeuroD, HNF-1α, HNF-4α and Pdx1 on *Pax4* gene expression is interesting, as mutations in these transcription factors have been associated with MODY (Hani et al. 1999, Fajans et al. 2001, Love-Gregory et al. 2004). Thus, Pax4 may participate as a downstream target of these diabetes-linked transcription factors and most likely aggravates the MODY phenotype by its inability to promote cell replication and to protect against apoptosis.

Evidence for the implication of Pax4 in β-cell adaptation in response to physiological cues emerges from studies performed with the proinflammatory cytokine interleukin (IL)-1β. Indeed, low concentration of IL-1β stimulates *pax4* gene expression, correlating with increased human β-cell proliferation induced by the cytokine. In contrast, higher concentrations inhibited Pax4 mRNA levels with a concomitant increase in apoptosis (Maedler et al. 2006). This bimodal effect was shown to involve the Fas signalling pathway, which can switch from a mitogenic signal to induction of apoptosis when the FLICE-inhibitory protein (FLIP) is inactive (Maedler et al. 2006). This pathway was also found to be important in sustaining the secretory function of β-cells, as Fas-deficient mice exhibited impaired glucose-induced insulin secretion (Schumann et al. 2007). It would therefore appear that Pax4 is an integrated factor of the Fas/FLIP signal transduction pathway, which relays either the positive or negative effects of IL-1β pending levels of the cytokine. The latter most likely mimics the *in vivo* conditions observed in both type 1 and type 2 diabetic patients (Donath & Halban 2004). More recently, the cytokine ciliary neurotrophic factor was also found to significantly increase Pax4 mRNA levels and to promote the survival of neonatal rat islets (Rezende et al. 2007), substantiating the notion that Pax4 is an important mediator of cytokine signalling pathways and a regulator of β-cell plasticity in mature islets.

Consistent with this premise, we previously demonstrated that Pax4 is indeed a key regulator of β-cell mass (Brun et al. 2004). We found that activin A and betacellulin stimulated *pax4* gene expression with a concomitant increase in rat islet β-cell replication. Wortmannin suppressed betacellulin-induced Pax4 expression, implicating the PI3 kinase signalling pathway. Our results corroborated previous studies demonstrating that activin A could induce *pax4* gene expression in pancreatic cell lines (Ueda 2000) and stimulate growth and differentiation of human foetal pancreatic cells in combination with betacellulin (Demetrcio et al. 2000). Furthermore, endogenous Pax4 mRNA levels were also induced in human islets by glucose, activin A and betacellulin. In addition, the incretin glucagon-like peptide (GLP)-1, a new therapeutic agent for the treatment of diabetes, which has been shown to increase β-cell mass in mouse and rat pancreas (Xu et al. 1999, Stoffers et al. 2000), also induced Pax4 expression in human islets in the presence of glucose (Brun et al. In press). Overexpression of mouse Pax4 (mPax4) in rat islets resulted in the induction of the c-myc/Id2 proliferation pathway and of the anti apoptotic gene *bcl-xl*. Two independent studies have demonstrated that forced expression of c-myc requires concomitant induction of Bcl-XL to promote β-cell proliferation rather than apoptosis (Pelengaris et al. 2002, Cheung et al. 2004). Taken together, these studies suggest that Pax4 coordinates the activation of both genes in order to promote cell survival and mitogenesis (Fig. 1). Overexpression of mPax4 in human islets also induced proliferation and conferred protection against cytokine-mediated apoptosis, whereas the diabetes-linked mutant identified in the Japanese population (R121W; Shimajiri et al. 2001) was less efficient (Brun et al. 2004). An elegant study recently demonstrated that the mouse Pax4 protein could permeate islet cells as well as Min6 cells through a novel protein transduction domain located in the paired domain and confer protection against tumour necrosis factorα-induced apoptosis. Furthermore, both c-myc and Bcl-XL expression were increased in transduced cells, indicating that similar proliferative and survival pathways are implicated in both rodent and human islets (Lu et al. 2007). These studies pave the way to exciting new therapeutic strategies using Pax4 transduction to improve islet cell survival in culture prior to transplantation in type 1 diabetic patients (Shapiro et al. 2006). However, an immediate challenge is to identify additional downstream Pax4 target genes that may also prove useful for therapeutics.
Pax4 as an oncogene

Pax gene family members have also been associated with cancer development (Robson et al. 2006). Consistent with the latter, elevated expression levels of Pax4 are found in both human and rat insulinomas when compared with mature β-cells (Miyamoto et al. 2001, Brun et al. 2007). Furthermore, a novel Pax4 spliced variant, lacking the carboxy-terminal (C-terminal) end of the protein involved in mediating repression of gene transcription, was also identified in human insulinomas (Miyamoto et al. 2001). Ablation of this C-terminal end may therefore confer tumorigenicity. In contrast, its presence may result in the interaction with cofactors and/or post-translational modifications of Pax4 that limit cell proliferation. Precedents for this premise were demonstrated for other Pax proteins such as Pax3, 5 and 8 that interact with the tumour suppressor pRb and regulate cell cycle (Buckingham & Relaix 2007). To address the potential oncogenic function of Pax4, the latter was suppressed by RNA interference in the insulinoma INS-1E cell line. Inhibition of Pax4 provoked spontaneous apoptosis and further sensitized cells to cytokine-mediated cell death without altering cell proliferation, suggesting that the transcription factor acts as a survival gene rather than promoting replication in this cell line (Brun et al. 2007). The latter study highlights potential pitfalls of using transformed cell lines to delineate the functional role of factors implicated in the regulation of cell replication and apoptosis. Further evidence for the potential mitogenic function of Pax4 in β-cells was provided by the recent discovery that primary lymphoma and haematologic malignancies expressed high levels of Pax4 that correlated with tumorigenesis. Aberrant expression of the transcription factor in these cells which normally do not express Pax4 was caused by the demethylation of CpG islands with subsequent promoter activation (Li et al. 2006). Accordingly, we have recently demonstrated that similar epigenetic modifications are imposed on the pax4 gene in mature pancreatic islets, thus refraining high expression levels when compared with INS-1E cells (Brun et al. In press). Perturbation in the expression of other closely related members of the Pax family can also lead to cancer (Robson et al. 2006). Increased levels of Pax3 were observed in human tumours of neural crest origin, while Pax2 expression was shown to be dispensable for survival of ovarian and bladder cancer cell lines (Muratovska et al. 2003, Parker et al. 2004). Pax5 was also identified as a key factor for the maintenance of the tumorigenic phenotype of neuroblastoma (Baumann Kubetzko et al. 2004). Similar to Pax4, suppression of Pax2 or Pax7 in tumour cell lines resulted in programmed cell death (Margue et al. 2000, Muratovska et al. 2003). Pax4 may thus qualify as a potential oncogene joining other Pax family members as an important transcriptional regulator involved in development and cancer (Robson et al. 2006).

Conclusion: Pax4 a gene of all trades in islet plasticity

The alarming spread of obesity worldwide has entailed a dramatic increase in type 2 diabetes. The mechanism is considered to involve inadequate insulin secretion to meet increasing insulin requirements due to obesity-related resistance to the hormone in the subgroup of individual with inherited susceptibility for type 2 diabetes. The genetic defects are thought to include inappropriate β-cell proliferative capacity and increased susceptibility to apoptosis. Insufficient regeneration of destroyed β-cells in the autoimmune process of type 1 diabetes may also be determined by genetic predisposition. The transcription factor Pax4 could be a common denominator of β-cell growth and survival in the two diabetic conditions. Accordingly, we would like to propose the working model outlined in Fig. 2 to integrate the various functions that Pax4 may have on β-cell physiology and pathophysiology (Fig. 2). Under physiological conditions in which β-cell mass is called upon to compensate for increasing insulin demands such as in pregnancy or obesity, Pax4 acts as an adaptive gene that permits β-cell replication. In contrast,
mutations and polymorphisms that weaken Pax4 transcriptional activity would initially contribute to reduction in β-cell survival and/or proliferation during pancreatic development. Subsequently, the inability of β-cells to be replenished in mature islets coincident with unabated apoptosis would result in the gradual loss of insulin-producing cells. This will cause relative insulin deficiency, hyperglycaemia and ultimately diabetes. Therefore, Pax4 would qualify as a survival gene. In contrast, gain-of-function mutations conferring resistance against apoptosis would provide a selective advantage for tumour development. In these circumstances, Pax4 would be considered an oncostasis gene. Validation of this working model will ultimately require the generation of transgenic mice that can specifically and conditionally express either Pax4 wild-type or diabetes-associated variants in islet β-cells.

Figure 2 Proposed model of Pax4 action in mature β-cell health and disease. Under physiological conditions, transient stimulation of Pax4 by mitogens such as activin A and betacellulin or by low concentrations of cytokines evokes a burst in β-cell proliferation (depicted as black circles) as well as increasing survival. The net result is the increased insulin output to compensate for demands (upper panel). However, chronic exposure to cytokines or potentially to hyperglycaemia or hyperlipidaemia results in the inhibition of pax4 gene expression with a concomitant decrease in cell replication and increase susceptibility to apoptosis (lower left panel). Dysfunction in Pax4 due to mutations or polymorphisms in the gene would have similar consequences. The gradual decline in β-cell mass (represented by open circles) over time would ultimately lead to either type 1 or type 2 diabetes. In contrast, alterations in Pax4 that increase activity confer resistance to apoptosis in insulinomas or promote mitogenesis in lymphomas, resulting in overall augmentation in cell mass (lower right panel; black circles, new cells).
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