

The Gene Pax4 Is an Essential Regulator of Pancreatic β -Cell Development

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The *Pax*-gene family encodes a group of transcription factors characterized by the presence of a highly conserved DNA-binding motif, the paired domain. Pax proteins are key regulators of vertebrate organogenesis since they play major roles in embryonic pattern formation, cell proliferation and cell differentiation (Chi and Epstein, 2002; Dahl *et al.*, 1997; Dohrman *et al.*, 2000; Epstein *et al.*, 1994). Indeed, mutations in *Pax* genes lead to profound defects in organisms as diverse as flies, mice and humans (Chi and Epstein, 2002; Dahl *et al.*, 1997). To date, nine mammalian *Pax* genes are known and these are grouped into five different subclasses according to their structural similarities. One of these subclasses comprises two close homologues, Pax4 and Pax6, that contain a second DNA-binding domain: the homeodomain (Dahl *et al.*, 1997; Dohrman *et al.*, 2000). Previous studies showed that Pax4 is a crucial regulator of mammalian pancreas development since lack of its activity prevents the formation of mature pancreatic insulin-producing (beta) cells (Dohrman *et al.*, 2000; Sosa-Pineda *et al.*, 1997; Wang *et al.*, 2004). Presently, it is not yet clear how Pax4 is specifically required for the development of beta cells. Nonetheless, evidence gathered from recent studies has begun to unravel important aspects of the molecular function of Pax4 in pancreatic endocrine cells. Here, I will try to summarize the results of different efforts aimed at understanding how Pax4 is required for both, beta cell development and beta cell function.

Keywords: β -Cell; Development; Pancreas; Pax4.

Pax4 expression during mouse pancreas organogenesis

The *Pax4* gene was first identified in mice, and subsequently it was also cloned from humans and rats (Dohrman *et al.*, 2000). To date, no reports are available on the presence of *Pax4* homologues in other vertebrates. *Pax4* is a unique member of the *Pax* family since its expression is largely restricted to embryonic stages (Sosa-Pineda *et al.*, 1997; Wang *et al.*, 2004). In mice, *Pax4*-transcripts are only visible in a few cells of the ventral neural tube, in the posterior stomach, the pancreas and duodenum (Dohrman *et al.*, 2000; Wang *et al.*, 2004). A detailed characterization of Pax4-expressing pancreatic cells has been hampered by the lack of anti-Pax4 antibodies suitable for immunohistochemistry. Nonetheless, to analyze the expression of Pax4 we have used tissues of otherwise normal *Pax4*-heterozygous mice that harbor an insertion of the β -galactosidase (*β -gal*) reporter gene in one of two *Pax4* loci (Sosa-Pineda *et al.*, 1997). Thus, our comparative analysis of the distribution of *Pax4* transcripts (after *in situ* hybridization) and of β -gal proteins (detected with anti- β -gal antibodies) in murine tissues revealed a similar transient expression of Pax4/ β -gal proteins and *Pax4* transcripts during mouse organogenesis (Sosa-Pineda *et al.*, 1997; Wang *et al.*, 2004).

In the pancreas of mouse embryos, the onset of Pax4 expression was observed at around E9.5 of development in a few cells of the dorsal pancreatic rudiment. One day later, Pax4-expressing cells were also detected in the ventral pancreatic bud (Sosa-Pineda *et al.*, 1997). In the following days, the population of Pax4-positive cells expanded rapidly and peaked between E13.5 and E15.5 of development. Towards the end of gestation a gradual decline in the number of Pax4-expressing cells was ob-

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Abbreviations: β -gal, beta galactosidase; bHLH, basic helix-loop-helix; E, embryonal day; Ngn3, neurogenin 3; EMSA, electrophoretic mobility shift assay.

served and shortly after birth, only a few of Pax4-positive cells remained noticeable (Sosa-Pineda *et al.*, 1997; Wang *et al.*, 2004). A transient expression of Pax4 in pancreatic tissues of mice was also reported by Smith *et al.* (1999). In pancreata of adult rodents no expression of Pax4 has been detected; in contrast, in islets of adult humans low levels of Pax4 transcripts continue to be expressed (Dohrman *et al.*, 2000; Heremans *et al.*, 2002; Smith *et al.*, 1999; 2000; Wang *et al.*, 2004).

In mice, the peak of Pax4 expression (E13.5-E15.5) coincides with a period of considerable endocrine differentiation, including that of β -cell precursors (Pictet and Rutter, 1972). At these stages, we and others observed colocalization of Pax4 with various endocrine markers (i.e., Ngn3, Islet1, Nkx2.2, and Pax6), including insulin and glucagon (albeit only in a few cells) (Smith *et al.*, 2000; Wang *et al.*, 2004). In pancreatic tissues, the activity of the pro-endocrine bHLH transcription factor Ngn3 is both necessary and sufficient to initiate endocrine development (Apelqvist *et al.*, 1999; Gradwohl *et al.*, 2000). The colocalization of Pax4 and Ngn3 in some cells of the embryonic pancreas thus suggests that in these tissues the onset of Pax4 expression occurs concomitant with, or shortly after endocrine specification. This proposal is supported by the results of Smith *et al.* (2000; 2003) indicating that Pax4 is indeed a target of Ngn3 (see below). Moreover, since in developing pancreata Pax4 co-localizes transiently with insulin or with glucagon, it is possible that Ngn3 induces the expression of Pax4 in all endocrine precursors or, at least, in those giving rise to α - or β -cells. Notwithstanding, it appears that once α -cells start to mature, their expression of Pax4 decays rapidly since much fewer Pax4+/Glu+ cells than Pax4+/Ins+ cells are seen (Wang *et al.*, 2004). To undoubtedly identify those precursors that express Pax4 at some point during pancreas development, it will be necessary to irreversibly mark their progeny by using an approach involving Cre-recombinase (Sato *et al.*, 2000).

Phenotypic alterations in pancreata of Pax4-null mice

In mice, *Pax4* was inactivated by homologous recombination (Sosa-Pineda *et al.*, 1997). *Pax4*-homozygous newborn mice were indistinguishable from their littermates, but died 3–5 days after birth as a result of a profound deficiency of pancreatic insulin producing cells. *Pax4*-nullizygous mice also lacked pancreatic somatostatin producing cells, duodenal endocrine cells and somatostatin and serotonin cells of the stomach (Larsson *et al.*, 1998; Sosa-Pineda *et al.*, 1997). Our previous characterization of *Pax4*-deficient embryonic pancreata revealed lack of expression of early markers of β -cell differentiation in these tissues, such as Pdx1, Hlx9 or MafA (Wang *et al.*,

2004; our unpublished data). Notwithstanding, the loss of Pax4 function did not entirely preclude the formation of a small number of insulin-producing cells; nonetheless, these cells did not represent mature β -cells since they lacked expression of the β -cell markers Nkx6.1 or MafA (Wang *et al.*, 2004; our unpublished data). Furthermore, in newborn *Pax4*-null mice we also found that the number of glucagon producing cells was significantly increased and they appeared to be clustered atypically (Dohrman *et al.*, 2000; Sosa-Pineda *et al.*, 1997; Wang *et al.*, 2004). Our initial characterization of *Pax4*-deficient pancreata thus suggested that the lack of Pax4 activity forced endocrine progenitors to adopt an alternative α -cell fate rather than a β/δ fate (Dohrman *et al.*, 2000; Sosa-Pineda *et al.*, 1997).

To identify putative targets of Pax4, we have compared RNAs from pancreata of E14.5 wild-type or *Pax4*-nullizygous embryos using microarrays (Prado *et al.*, 2004; our unpublished results). The results of this screening revealed that in E14.5 *Pax4*-deficient pancreata a significant accumulation of *ghrelin* transcripts occurred. Ghrelin is a potent orexigenic (appetite-stimulating) peptide primarily expressed in the oxyntic (acid-producing) mucosa of the stomach (Brogio *et al.*, 2003; Nakazato *et al.*, 2001). In rodents and humans, a small number of ghrelin-expressing cells have also been detected in the pancreas, small intestine, and colon (Brogio *et al.*, 2003; Prado *et al.*, 2004; Wierup *et al.*, 2002). In a recent study, Prado *et al.* (2004) characterized the reduced population of ghrelin-expressing cells that is normally present in the pancreatic islets of wild-type mice. This analysis revealed that occasionally, ghrelin and glucagon co-localize in the same cell; however, since most ghrelin-positive cells did not express any other hormone the authors proposed that these represent a new islet cell type: ϵ -cells (Prado *et al.*, 2004). Remarkably, in the pancreata of *Pax4*-deficient mice we observed a considerable expansion of cells expressing *ghrelin*-transcripts or ghrelin-protein at all stages analyzed (i.e., between E11.5 and P2) (Prado *et al.*, 2004; our unpublished results). These results thus showed that in *Pax4*-nullizygous pancreata, two important, parallel alterations had occurred: a severe depletion of insulin-producing (β) and somatostatin-producing (δ) cells, and a significant expansion of glucagon-producing (α) and ghrelin-producing (ϵ) cells (Fig. 1) (Prado *et al.*, 2004; Sosa-Pineda *et al.*, 1997). Hence, it is possible that during normal pancreas organogenesis Pax4 promotes the β and δ cell fate of pancreatic endocrine progenitors and conversely, that the lack of Pax4 activity forces these progenitors to adopt an alternative α or ϵ cell fate. Interestingly, in *Pax4*-deficient pancreatic tissues we noticed that almost all the cells expressing ghrelin also co-express glucagon (Elghazi *et al.*, unpublished results). In fact, these cells resemble a population of endocrine cells that is normally present in the pancreas of early wild-type mouse

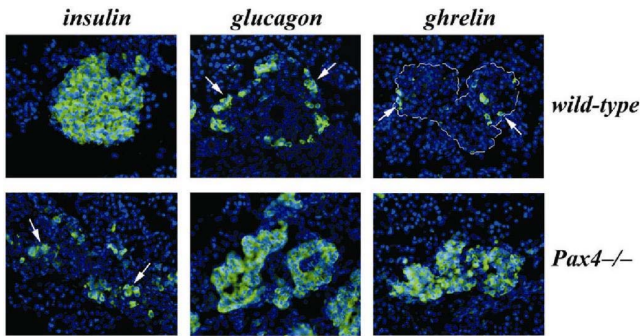


Fig. 1. In pancreatic islets of newborn mice, insulin-producing cells are predominant. These cells aggregate to form the islet's core and are surrounded by a sheet of cells that produce glucagon or ghrelin (upper panel). In contrast, Pax4-deficient newborn pancreata only have a few dispersed insulin-producing cells and large cell-aggregates synthesizing glucagon or ghrelin (lower panel).

embryos (E10.5-E11.5) (Prado *et al.*, 2004; our unpublished results). Thus, an alternative explanation for our observations is that in the absence of Pax4 the population of early ghrelin/glucagon double-positive cells became considerably expanded or, that the lack of Pax4 activity promoted an alternative 'early cell-fate' of differentiation to most pancreatic endocrine progenitors. Following the fate of pancreatic Pax4-deficient progeny by indelibly marking these cells will be necessary to assign cell-autonomous or non-cell autonomous function to this gene.

To gain further insight into the role of Pax4 in β -cell development, we have sought to identify possible genetic interactions between Pax4 and other genes that also control β -cell differentiation. One candidate gene is *Nkx2.2*, since previous studies showed that the activity of its encoded product is crucial for the formation of mature β -cells (Sussel *et al.*, 1998). Thus, we compared the pancreatic alterations in mice that were deficient in Pax4 (Pax4^{-/-}) or *Nkx2.2* (Nkx2.2^{-/-}), with those of mice that were double-nullizygous for Pax4 and *Nkx2.2* (Pax4^{-/-} Nkx2.2^{-/-}). Our analysis of gene expression at a stage when β -cell differentiation normally commences showed remarkable similarities between pancreata deficient in Pax4 and those deficient in *Nkx2.2* (Wang *et al.*, 2004). The pancreatic defects, however, were more profound in the *Nkx2.2*-null background possibly because the lack of *Nkx2.2* activity reduces the expression of *Pax6* dramatically (Prado *et al.*, 2004; Wang *et al.*, 2004). Remarkably, a recent study also showed excess of ghrelin-producing cells in the pancreata of *Nkx2.2*-deficient mice, with more than 90% of the islet cells expressing ghrelin in these mutant tissues (Prado *et al.*, 2004). Overall, our results and those of Prado *et al.* (2004) indicate that in developing pancreata both *Nkx2.2* and Pax4 are independently required to specify or maintain the islet β -cell fate, and to prevent the expression of ghrelin (Fig. 2) (Prado *et al.*, 2004; Wang *et al.*, 2004).

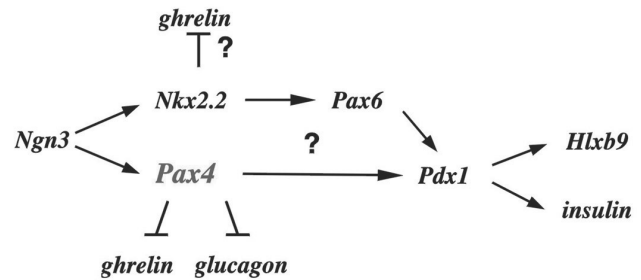


Fig. 2. Proposed model of β -cell development. In β -cell progenitors, Ngn3 activates the expression of both Pax4 and Nkx2.2. In these precursors, Pax4 binds to the promoters of *ghrelin* and *glucagon* thereby repressing their expression. Pax4 activity also increases the levels of Pdx1 proteins throughout a currently unknown mechanism. Consequently, the expression of Hlxb9 is activated and insulin expression ensues. The parallel activity of Nkx2.2 enables β -cell differentiation by increasing or maintaining the expression of Pax6 and by (perhaps indirectly) repressing the *ghrelin* gene. In β -cell precursors, Pax6 directly activates the expression of insulin and Pdx1.

Our future work (and certainly that of others) will focus on elucidating the intricate regulatory network that conveys the β -cell fate of pancreatic progenitors and, in particular, the specific role(s) played by Pax4 in this process. In this regard, our most recent data have shown that in colon carcinoma-derived cells, the ectopic expression of Pax4 significantly reduces the endogenous levels of ghrelin expression. Furthermore, we also confirmed binding of Pax4 to the upstream region of *ghrelin* in intact chromatin of these cells (Elghazi *et al.*, unpublished results). Hence, based on the above data, we propose that in pancreatic endocrine progenitors, one main function of Pax4 is to maintain the repression of the *ghrelin* gene.

Of note, recent data from Collombat *et al.* (2003) showed that the pancreata of mice deficient in the homeodomain-transcription factor *Arx* not only lack mature α -cells but also have an increase of β - and δ -cells. Remarkably, the authors also found that in Pax4-null pancreata, *Arx* transcripts were increased, and conversely, that in *Arx*-null pancreata, the Pax4 transcripts were also increased. Overall, these results suggest not only that *Arx* and Pax4 have opposing actions on the specification of endocrine subtype destiny, but also that in endocrine progenitors, they antagonize each other's expression.

Regulation of Pax4 expression

Smith *et al.* (2000) analyzed human and murine Pax4 transcripts by 5'-RACE and showed similar transcription initiation sites for Pax4 RNAs isolated from adult human islets or from mouse fetal pancreas. These Pax4 transcripts contained two small additional untranslated exons

in comparison to a *Pax4* RNA previously isolated from human placenta (Tao *et al.*, 1998). Independent studies performed by Brink and Gruss (2001) and Smith and colleagues (2000) showed that a fragment containing approximately 2 kb of the upstream region of *Pax4* was both necessary and sufficient to drive pancreas-specific expression. The analysis of this region of human and mouse *Pax4* also revealed binding sites for the transcription factors HNF4 α , HNF1 α , Pdx1 and NeuroD1. Interestingly, mutations in any of these genes result in a hereditary form of diabetes called Mature Onset Diabetes of the Young (MODY) (Brink and Gruss, 2001; Smith *et al.*, 2000; 2003). By performing EMSA assays with nuclear extracts of α TC3 cells and specific antibodies to supershift the complexes, Smith *et al.* (2000) further confirmed binding of HNF4 α , HNF1 α , Pdx1, and NeuroD1 to specific elements located within the -2.0 kb region of *Pax4*. In this work, the authors also identified possible Pax4-binding sites approximately 4.0 kb upstream of the transcription initiation site of *Pax4*, thus suggesting that Pax4 is capable of repressing its own expression (Smith *et al.*, 2000). Interestingly, the regulation of Pax4 expression by Neurogenin3 appears to require a physical interaction between Ngn3 and HNF1 α (Smith *et al.*, 2003).

Transcriptional properties of Pax4

Pax proteins are defined by the presence of a 128-amino acid DNA-binding domain, the paired domain (PD), which makes sequence-specific contacts with DNA (Epstein *et al.*, 1994). In addition to the paired domain, some Pax proteins (Pax4 and Pax6 included) also have a homeodomain. Pax6 was shown to bind to a cis-acting element called PISCES (pancreatic islet cell enhancer sequence) present in the promoters of *glucagon*, *insulin* and *somatostatin*, and this interaction is necessary to activate the gene's expression (Sander *et al.*, 1997). In separate works, Fujitani, Kaulosova, and Smith reported the optimal DNA-binding sequences for Pax4 and found that the core motif had resemblance to the PISCES sequence (Fujitani *et al.*, 1999; Kaulosova *et al.*, 1999; Smith *et al.*, 1999). The reports of Fujitani *et al.* (1999) and of Smith *et al.* (1999) also showed that Pax4 could bind to the PISCES elements of *insulin*, *glucagon* and *somatostatin*, albeit with a substantially lower affinity than Pax6. In a different study by Ritz-Laser *et al.* (2002), upon modifying the *in vitro* binding conditions the affinity of full length Pax4 to these binding sites was further improved. Thus, it is possible that post-translational modifications (like phosphorylation) or the interaction of other proteins with Pax4 could modify the DNA binding properties of this transcription factor *in vivo*, as it was shown for other Pax proteins (Epstein *et al.*, 1994). A separate study by Petersen *et al.* (2000) also showed that in a glucagon-

producing cell line, both the endogenous expression of glucagon and the activity of a *glucagon*-promoter-controlled reporter gene were considerably reduced upon exogenous expression of Pax4. Similar studies using an *insulin*-promoter-controlled reporter also showed strong activation in the presence of Pax6, but not when Pax4 was cotransfected with Pax6 (Campbell *et al.*, 1999). These results are not completely unexpected, since a strong repressor domain was previously identified in the carboxy-terminal region of Pax4 that was active regardless of the cell type (Fujitani *et al.*, 1999; Smith *et al.*, 1999). Taken together, the results of these studies raise the possibility that in β -cell precursors, Pax4 could inhibit *glucagon* transcription by competing with Pax6 for DNA binding sites; in turn, this would allow Pax6 to bind to the *insulin* promoter and to activate its transcription. Notwithstanding, since a transactivation domain was also identified in the carboxy-terminal region of Pax4 (although this domain seems functional only in the presence of adenoviral E1A protein), it is also possible that Pax4 activates gene transcription in certain cellular contexts (Fujitani *et al.*, 1999). The identification of Pax4-target genes and of possible Pax4-interacting proteins that could modify its transcriptional properties thus appears essential to unveil the molecular mechanism by which Pax4 promotes β -cell development.

Is Pax4 sufficient to specify pancreatic β -cell fate?

Overall, the phenotypic alterations observed in pancreata of mice deficient in Pax4 indicate that the activity of Pax4 is essential for the formation not only of mature β -cells, but also of δ -cells. In addition, since the lack of Pax4 appears to convert β/δ cell precursors into α/ϵ cells, it is possible that in unspecified precursors of the endoderm, Pax4 promotes the β/δ cell-fate. A study by Heremans *et al.* (2002) reported that the infection of adult human pancreatic ductal cells with adenoviruses expressing Ngn3 induced the expression of Pax4 approximately one day later. This result further supports the proposal that *Pax4* is a target of Ngn3. Interestingly, the ectopic expression of both Pax4 and Ngn3 in 'transdifferentiated' ductal cells induced several other endocrine genes, but neither *glucagon* nor *insulin*. The authors suggested that the sustained expression of Pax4 observed in these cells probably accounted for the inhibition of *insulin*- and *glucagon*-gene expression (thus, in 'transdifferentiated' ductal cells, the *Pax4* gene was not subject to auto-regulation). Grappin-Botton *et al.* (2001), used *in vivo* electroporation of chick embryos to test whether transcription factors known to be necessary for pancreatic development are also sufficient to drive a program of pancreatic organogenesis. In these experiments, the ectopic expression of Ngn3 promoted the

differentiation of glucagon- or somatostatin-producing cells (but not of insulin-producing cells) in areas located between the esophagus and the yolk stalk, but outside the pancreatic region. The simultaneous expression of Pax4 and Ngn3 was also not sufficient to elicit the transformation of gut cells into β -cells. Overall, the results of these studies indicate that besides Ngn3 and Pax4, gut endocrine cell sub-type specification (β -cells included) also requires the participation of additional, local gene functions that are probably activated in a Ngn3-independent manner (Grappin-Botton *et al.*, 2001). A different study by Blyszczuk *et al.* (2002) showed that histotypic cultivation of Pax4-expressing embryonic stem (ES) cells, that were previously selected for nestin expression, produced cells with a higher content of insulin than those lacking Pax4 and that these cells were able to secrete insulin in response to glucose or tolbutamide. Most importantly, when Pax4-expressing ES-cells were transplanted under the renal capsule or the spleen of streptozotocin-treated mice, their normoglycemia was restored. These results thus indicate that Pax4 may play a significant role in directing undifferentiated ES cells into insulin-producing cells. Of note, the observation that in Pax4+/nestin-selected cells the production of insulin increased significantly upon their aggregation into spheroids also suggested that the maturation of insulin-producing cells is favored by cell-cell interactions (Blyszczuk *et al.*, 2002).

Pax4 mutations associated with Type 2 diabetes

Type 2 diabetes mellitus results from an inadequate mass of functional β -cells due to the lack of compensation to overcome insulin resistance or from an intrinsic β -cell defect. Thus, mutations in genes encoding crucial regulators of β -cell development, β -cell function or both could predispose to the development of diabetes mellitus (Bonner-Weir, 2001). In the Japanese population with Type 2 diabetes, a missense mutation in the *Pax4* gene (R121W) was found with a frequency of approximately 2.0%. This mutation decreased the binding of Pax4 to its target sequences and rendered this protein unable to effectively inhibit the transcription activity induced by Pax6 (Shimajiri *et al.*, 2001). More importantly, patients carrying the *Pax4*^{R121W} homozygous mutation showed severe defects in the first-phase of insulin secretion, suggesting that this genetic alteration resulted in β -cell dysfunction (Kanatuka *et al.*, 2002). In people of West African ancestry, Mauvais-Jarvis *et al.* (2004) identified two other *Pax4* mutations (R133W or R37W) that predispose them to ketosis-prone diabetes (KPD), a rare form of Type 2 diabetes. The homozygous *Pax4*^{R133W} or heterozygous *Pax4*^{R37W} individuals had decreased repression of target genes, and patients with these mutations showed severely altered

insulin secretion during a glucose-tolerance test. Overall, these studies suggest that specific mutations disrupting the normal function of Pax4 in combination with environmental and other genetic factors result in a reduced pool of β -cells or lack of responsiveness of these cells upon increased demands of insulin. Thus, in some ethnic groups, mutations in the *Pax4* gene increase their predisposition to Type 2 diabetes.

In summary, the results of the aforementioned studies support the proposal that Pax4 is a key component of the molecular machinery responsible for initiating pancreatic β -cell differentiation. Unveiling the molecular mechanisms through which Pax4 regulates early β -cell development should prove valuable for studies aimed at generating functional β -cells from pancreatic or embryonic stem cells that could be used for therapeutic purposes.

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