Mice lacking the homeodomain transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic β cells

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SUMMARY

The endocrine pancreas is organized into clusters of cells called islets of Langerhans comprising four well-defined cell types: α, β, δ and PP cells. While recent genetic studies indicate that islet development depends on the function of an integrated network of transcription factors, the specific roles of these factors in early cell-type specification and differentiation remain elusive. Nkx2.2 is a member of the mammalian NK2 homeobox transcription factor family that is expressed in the ventral CNS and the pancreas. Within the pancreas, we demonstrate that Nkx2.2 is expressed in α, β and PP cells, but not in δ cells. In addition, we show that mice homozygous for a null mutation of Nkx2.2 develop severe hyperglycemia and die shortly after birth.

Immunohistochemical analysis reveals that the mutant embryos lack insulin-producing β cells and have fewer glucagon-producing α cells and PP cells. Remarkably, in the mutants there remains a large population of islet cells that do not produce any of the four endocrine hormones. These cells express some β cell markers, such as islet amyloid polypeptide and Pdx1, but lack other definitive β cell markers including glucose transporter 2 and Nkx6.1. We propose that Nkx2.2 is required for the final differentiation of pancreatic β cells, and in its absence, β cells are trapped in an incompletely differentiated state.

Key words: Nkx2.2, Pancreas, Transcription factors, β cells, Diabetes

INTRODUCTION

The pancreas plays a central role in nutrient regulation through the function of two distinct populations of cells: the exocrine cells secrete digestive enzymes through the duct system into the gut and the endocrine cells secrete hormones into the bloodstream. The hormones produced by the endocrine pancreas regulate nutrient metabolism in mammals; in particular, loss or dysfunction of the insulin-producing β cells causes diabetes mellitus. Development of the endocrine pancreas involves a complex process of cell differentiation that ultimately gives rise to four distinct hormone-producing cell types: α, β, δ and PP cells, precisely organized within the islets of Langerhans (for reviews on pancreas development, see Slack, 1995; Sander and German, 1997). While all the cells of the endocrine pancreas are thought to arise from a common endodermal precursor, the early process of lineage determination and cell-type differentiation within the pancreas remains unclear.

In the mouse, morphologic development of the pancreas first appears at embryonic day (E) 9.5 as a small bud of epithelial cells on the dorsal aspect of the gut at the junction of the foregut and the midgut (Pictet and Rutter, 1972). At this point in development, the first differentiated cells of the pancreas, the glucagon-producing α cells, appear (Herrera et al., 1991; Teitelman et al., 1993; Upchurch et al., 1994). Insulin-producing β cells appear within the next 24 hours. As the dorsal pancreatic bud grows and fuses with a ventral bud, invaginations within the pancreatic epithelium develop into ducts. Endocrine cells continue to differentiate from the ductal epithelium throughout pancreatic development. At approximately E14.0, several events occur simultaneously: the termini of the ducts begin to form acini and differentiate into the exocrine cells of the pancreas; the first somatostatin-producing δ cells appear; the endocrine cells start to form clusters; and the number of endocrine cells and the levels of insulin and glucagon production per cell increase dramatically (Pictet and Rutter, 1972). Finally, at E18.0, shortly before birth in the mouse, the pancreatic polypeptide (pp)-producing PP cells appear, and the endocrine cells begin to form well organized islets (Herrera et al., 1991).

Recently, mice with targeted disruptions of genes encoding transcription factors that are expressed in the pancreas have provided insights into the regulation of islet development. Pdx1, a homeodomain protein, is normally expressed in all cells of the
MATERIALS AND METHODS

Targeting construct
To generate Nkx2.2 mutant mice, three overlapping approx. 14 kb genomic clones containing the entire Nks2.2 gene were isolated from a 129J mouse genomic library (provided by Anton Berns, Amsterdam). The gene was mapped using a combination of Southern analysis and restriction enzyme digests. A targeting construct was generated using pBS KS- (Stratagene) as the cloning vector. The organization of the targeting vector is shown in Fig. 2. Briefly, the 6.0 kb BglII-XhoI 5′ fragment was ligated 5′ to the PGK-neo cassette. The 4.0 kb BamHI 3′ fragment was placed downstream of PGK-neo. Finally, the PGK-thymidine kinase (tk) cassette was inserted 3′ to the 4.0 kb Nkx2.2 fragment.

Generation of mutant animals
The generation of recombinant ES clones and Nkx2.2 mutant mice was performed as described extensively in Qi et al. (1995). Southern analysis was used for all genotyping of ES cells and mice. The southern probe is shown in Fig. 2.

Immunohistochemistry
Immunohistochemistry and immunofluorescence assays were performed on paraffin sections as described previously (Sander et al., 1997). Primary antibodies were: guinea pig anti-insulin (Linco) or monoclonal anti-insulin (Sigma); guinea pig anti-glucagon (Linco); rabbit anti-PP (Dako); rabbit anti-somatostatin (Dako); rabbit anti-glucokinase (provided by C. Newgard); rabbit anti-glucose transporter 2 (Chemicon); rabbit anti-IAPP (Advanced Chemtech); rabbit anti-prohormone convertase 1/3 (provided by D. Steiner); rabbit anti-amylose (Sigma); monoclonal anti-synaptophysin (Biogenics); monoclonal anti-Nkx2.2 (provided by T. Jessell); rabbit anti-Pdx1 (provided by H. Edlund); monoclonal anti-Is11 (Developmental Hybridoma Bank); rabbit anti-Pax6 (provided by S. Saule); rabbit anti-Brain4 (provided by M. G. Rosenfeld) and rabbit anti-Nkx6.1 (antibodies were raised against a GST fusion protein containing the carboxy-terminal 70 amino acids of hamster Nkx6.1). Secondary antibodies were used as described previously (Sander et al., 1997). For immunofluorescence assays, we used fluorescein coupled anti-rabbit, fluorescein coupled anti-mouse, rhodamine coupled anti-guinea pig, rhodamine coupled anti-rabbit (Cappel) and marina blue coupled anti-mouse (Molecular Probes). Biotinylated secondary antibodies (Vector) were detected with the ABC Elite immunoperoxidase system (Vector).

Glucose levels
Glucose levels were measured with the One Touch Glucose Monitoring kit (Johnson and Johnson) using 20 μl of peripheral blood from 2- to 3-day old mice.

Quantification of insulin and glucagon concentrations
Protein was extracted as described previously (Sander et al., 1997). The concentrations of insulin and glucagon were determined by radioimmunoassays (RIA) using commercially available kits (Linco).

RESULTS
To precisely determine the temporal and spatial domains of Nkx2.2 expression in the embryonic pancreas, we used a monoclonal antibody directed against chicken Nkx2.2 (Ericson et al., 1997) to localize the protein (Fig. 1). Initial expression of Nkx2.2 on day 9.5 of embryonic development (E9.5) is coincident with the appearance of the dorsal pancreatic bud, which forms near the junction of the foregut and midgut endoderm (Fig. 1b). As the pancreatic epithelium grows and develops into a branched structure at E12.5, Nkx2.2 expression persists in most cells (Fig. 1c). However, by E15.5 when distinct exocrine and endocrine compartments can be identified, expression of Nkx2.2 becomes restricted to most of
Role of Nkx2.2 in pancreas development

Expression of Nkx2.2 is maintained within the pancreatic islets into adulthood (Fig. 1d), and is not detectable in the exocrine tissue. The mouse islet comprises four distinct endocrine cell populations: α, β, δ, and PP cells, each of which produces one of the four pancreatic hormones, glucagon, insulin, somatostatin and pancreatic polypeptide (pp), respectively (Slack, 1995; Sander and German, 1997). Glucagon expression begins at E9.5 in the developing pancreatic bud and insulin expression can be detected by E10.5. Somatostatin-producing δ cells arise at E15.5 and PP cells are generated just before birth, at E18.5. To determine which endocrine cell types express Nkx2.2 protein, we performed double immunohistochemistry to analyze the co-expression of Nkx2.2 protein and each of the four pancreatic hormones at E18.5. As shown in Fig. 1, Nkx2.2 protein appears to be present in every insulin-expressing β cell. In addition, double immunofluorescence reveals that Nkx2.2 co-localizes with glucagon in approximately 80% of the α cells and with pancreatic polypeptide in most, but not all, of the PP cells. In contrast, Nkx2.2 is not expressed in somatostatin-producing δ cells.

To address the role of Nkx2.2 in pancreatic development, we generated mice in which the Nkx2.2 gene was deleted by standard gene targeting techniques in ES cells (see Fig. 2 and Materials and Methods). The deletion completely eliminates Nkx2.2 activity since we are unable to detect any Nkx2.2 protein in the CNS and pancreas of homozygous mutant mice by immunohistochemistry. Consistent with a null mutation in Nkx2.2, there is complete penetrance of all phenotypes observed in the homozygous mutant mice. Mice heterozygous for the Nkx2.2 mutation survive to adulthood, are fertile and are indistinguishable from wild-type animals. Homozygous Nkx2.2 mutants survive through gestation and are grossly indistinguishable from their wild-type and heterozygous littermates at birth. The mutant neonates are as active as their littermates and have milk in their stomachs, indicating that they feed normally. By the second day after birth, however, the homozygous mutant animals begin to display severe growth retardation and are diabetic, with blood glucose levels five-fold higher than the control littermates (Table 1). Mutant animals do not survive longer than 6 days postnatally. At the time of death, the gross morphology of the pancreas appears normal. However, pancreas histology reveals...
a general reduction in islet cell mass and the islets display an unusual string-like morphology, whereas the exocrine cells appear unaffected (data not shown).

To study the effect of Nkx2.2 deficiency on the endocrine pancreas, we examined hormone expression from the four endocrine cell types using immunohistochemistry. Throughout all stages of embryonic development examined (E10.5, E12.5 and E18.5), we are unable to detect any insulin-positive cells in the mutant islets, even with increased concentrations of the insulin antibody (Fig. 3e). In addition, the number of glucagon-producing cells and the amount of glucagon expressed per cell was significantly reduced (Fig. 3f). The decrease in glucagon-expressing cells can be detected as early as E10.5, and by the end of embryogenesis (E18.5) there are approximately 20% of the normal number of cells. There appears to be a modest reduction in both the number of PP cells and the intensity of PP staining in the mutant mice (Fig. 3g), while somatostatin expression appears unaltered (Fig. 3h), which correlates with the absence of Nkx2.2 expression in the δ cells (Fig. 1h).

Radioimmunoassays on pancreata from several litters of E18.5 embryos revealed that insulin content in the mutant pancreata is decreased by greater than 150 fold and glucagon content is reduced by approximately 50 fold in comparison to their wild-type and heterozygous littermates (Table 2).

Interestingly, a large proportion of the cells within the mutant islet clusters do not express any of the four pancreatic hormones (Figs 3 and 5d). To determine whether these cells retain general endocrine characteristics, we analyzed them for the expression of several known endocrine and exocrine markers. Glucagon expression is used to delineate the perimeter of the islet in serial sections (Fig. 4c,f). In the Nkx2.2 mutant at E18.5, expression of amylase, an exocrine enzyme, is confined to the exocrine tissue and there is no ectopic expression in the islets (Fig. 4d). At the same developmental timepoint, synaptophysin, a representative endocrine marker (Wiedenmann et al., 1986), is expressed in all cells in the mutant islet clusters (Fig. 4e). Furthermore, all the mutant islet cells at E18.5 express the neuroendocrine cell adhesion factor N-CAM (Cirulli et al., 1994), despite the abnormal string-like morphology of the mutant islets (data not shown).

The quantity and distribution of the atypical endocrine cells in the mutant, combined with a complete absence of insulin expression, suggested that the non-hormone producing cells in the

**Table 1. Blood glucose levels in Nkx2.2 wild-type, homozygous and heterozygous neonatal animals**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of animals</th>
<th>Blood glucose levels (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>4</td>
<td>81.25±3.45</td>
</tr>
<tr>
<td>+/–</td>
<td>9</td>
<td>74.0±2.0</td>
</tr>
<tr>
<td>–/–</td>
<td>7</td>
<td>424.4±11.86</td>
</tr>
</tbody>
</table>

Blood glucose levels were determined from peripheral blood taken from neonatal animals (see Materials and Methods). The values shown represent the mean ± s.e.m. value from the number (No.) of samples assayed.

**Table 2. Quantification of hormone levels in pancreata taken from E18.5 embryos carrying the Nkx2.2 null allele and their wild-type and heterozygous littermates**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of animals</th>
<th>µg insulin/ mg protein</th>
<th>µg glucagon/ mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>4</td>
<td>14.25±1.58</td>
<td>1.58±0.58</td>
</tr>
<tr>
<td>+/–</td>
<td>6</td>
<td>14.38±1.29</td>
<td>1.02±0.13</td>
</tr>
<tr>
<td>–/–</td>
<td>5</td>
<td>0.09±0.0017</td>
<td>0.03±0.009</td>
</tr>
</tbody>
</table>

Hormone concentrations were determined in protein extracts from individual pancreata taken from wild-type, heterozygous and homozygous E18.5 embryos (see Materials and Methods). The values shown represent the mean ± s.e.m. value from the number (No.) of samples assayed.
the mutant islets may represent immature or partially differentiated β cells. To test the hypothesis, we examined the expression of several β cell-specific proteins in the Nkx2.2 mutant islets. Islet amyloid polypeptide (IAPP) is a β cell peptide hormone that is co-secreted with insulin (Cooper et al., 1989), and prohormone convertase 1/3 is a proinsulin processing enzyme that is restricted to β cells within the pancreas (Marcinkiewicz et al., 1990). In the mutant islets, both of these β cell markers are expressed at normal levels, suggesting that the unidentified cells are incompletely differentiated β cells (Fig. 5e,i). However, two additional β cell-specific proteins that regulate glucose catabolism, glucose transporter 2 and glucokinase (Matschinsky, 1990; German, 1993; Pang et al., 1994) are undetectable in the mutant islet cells by immunohistochemistry (Fig. 5f,j). These characteristics, coupled with the absence of insulin production, suggest that the Nkx2.2 mutant islets contain the normal proportion of β cells, but that these cells have failed to differentiate completely and are defective in β cell function. Although it is possible, we cannot assume that these cells define a normal stage of β cell differentiation.

To elucidate the relationship between Nkx2.2 and other transcription factors involved in pancreas development, the expression of the homeodomain factors islet-1 (Isl1), Pax6, Brain 4 (Brn4), Pdx1, and Nkx6.1 was examined in the Nkx2.2 mutants (Fig. 6). Isl1 is a LIM homeodomain protein involved in the early formation of the pancreas (Ahlgren et al., 1997). It is expressed in all post-mitotic islet cells and plays a key role in endocrine development. Interestingly, expression of Isl1 is unaffected in the Nkx2.2 mutant pancreas (Fig. 6g). Normal Isl1 expression, in addition to normal levels of proliferation in the mutant pancreas (data not shown), suggest that all the cells of the islet, including the immature β cells are able to exit mitosis normally. We next examined the expression of the homeodomain protein Pdx1, which is required for the growth and differentiation of the entire pancreas beyond the initial bud stage (Jonsson et al., 1994; Offield et al., 1996). Normally, Pdx1 is uniformly expressed in the early pancreatic bud and becomes restricted to β cells later in development. In the Nkx2.2 mutants, early expression of Pdx1 appears normal and Pdx1 does become restricted to the defective β cells. However, by E18.5, the level of Pdx1 expression within these cells appears to be reduced (Fig. 6f).

Pax6 is a paired domain homeobox gene that is expressed in all pancreatic endocrine cells. In Pax6 mutant mice, there is a reduction in all four islet cell types, a decrease in hormone expression, and abnormal islet morphology (Sander et al., 1997; St.-Onge et al., 1997). Pax6 protein is detectable at wild-type levels in the islet cells of the Nkx2.2 mutants (Fig. 6h). We also looked at the expression of the POU homeodomain protein, Brn4, which is localized to the α cells (J. K. and M. G., unpublished observations; Hussain et al., 1997). Consistent with the reduction of α cells in the mutant islet, there are fewer cells expressing Brn4. However, the remaining α cells appear to express Brn4 at levels comparable to wild-type (Fig. 6i).

Finally, we looked at Nkx6.1, a homeodomain protein that is β cell-specific in the mature islets (Jensen et al., 1996). Early in the development of both mutant and wild-type littermates (E10.5 to E12.5), Nkx6.1 is widely expressed in the pancreatic epithelium (data not shown). However, at E18.5, by which time Nkx6.1 expression is normally restricted to the β cells, Nkx6.1 protein is undetectable in the Nkx2.2 mutants (Fig. 6i).

**DISCUSSION**

**Role of Nkx2.2 in islet cell differentiation**

Development and differentiation of the pancreas requires the coordinate activation of unique sets of genes. Key regulators of these processes are transcription factors, and recent mouse genetic studies have demonstrated that several transcription factors...
factors are required for normal pancreatic development (Jonsson et al., 1994; Ahlgren et al., 1997; Offield et al., 1996; Sander et al., 1997; Sosa-Pineda et al., 1997; St.-Onge et al., 1997). Among these factors, Nkx2.2 plays a unique role in endocrine pancreas development. Without Nkx2.2, there is a complete arrest of β cell terminal differentiation leading to the accumulation of a large group of cells that display endocrine characteristics and express a subset of β cell markers, but lack insulin. Furthermore, the phenotype of Nkx2.2 null mice demonstrates that the specification of islet cell types and their subsequent differentiation can be independent processes: Nkx2.2 does not appear to be required for β cell specification, but is essential for the differentiation of insulin-producing β cells. In addition, Nkx2.2 is not essential for the maintenance of these incompletely differentiated β cells, since there is a large stable pool of these cells at E18.5.

![Image](image-url)
Despite the presence of incompletely differentiated β cells, insulin expression cannot be detected at any stage of development in the Nkx2.2 null animals. This finding suggests that Nkx2.2 may directly activate insulin gene transcription. Nkx2.2 could function by binding directly to the insulin promoter, by physically associating with an insulin gene-activating complex, or by inducing the expression of other proteins that regulate insulin gene transcription. The consensus DNA binding site for Nkx2.2 is not known, however the consensus binding site for the closely related Nkx2.1 protein has been well characterized (Guazzi et al., 1990). Since Nkx2.1 and Nkx2.2 are nearly identical in their DNA-binding homeodomain regions and Nkx2.2 can bind to the Nkx2.1 consensus site (H. Ee and M. German, unpublished results), we scanned the insulin promoter sequence for close sequence matches to the Nkx2.1 consensus and identified a putative Nkx2.1/Nkx2.2 binding site at the C1 regulatory element (Hwang et al., 1990). However, preliminary experiments suggest that Nkx2.2 is not a binding factor for the C1 element and, in addition, does not bind other homeobox sites present within the insulin gene regulatory region (H. Ee and M. German, unpublished results). Therefore, we cannot yet determine whether Nkx2.2 is directly involved in insulin transcription or whether the lack of insulin production in the mutant islets is secondary to the defect in β cell differentiation.

Whereas Nkx2.2 is involved in the terminal differentiation of β cells, it appears to play a different role in the development of the other islet cell types. In the Nkx2.2 null animals, glucagon- and PP-expressing cells are easily detectable, although at reduced numbers. This suggests there is a defect in the specification or maintenance of these cell types, but not a block in differentiation, as is the case for the β cells. The reduction in α cell number is first evident at E9.5, when glucagon can initially be detected, providing additional evidence for a defect in α cell formation. Furthermore, these cells do not appear to be present in an immature state, since most, if not all, of the incompletely differentiated cells in mutant islets express the β cell markers IAPP, PC1/3, and Pdx1, but do not express the non-β cell marker Brn4. However, since there are fewer markers for the α and PP cell types, we cannot rule out the possibility that a small subset of these cells are also trapped in a partially differentiated state.

From our studies, it is clear that the functional development of the majority of α and PP cells require Nkx-2.2. However, the fact that some α and PP-expressing cells remain in the mutant islet clusters, although at reduced numbers, suggests that there may be another regulatory factor that can partially compensate for the function of Nkx2.2 in these cells. Alternatively, since Nkx2.2 is not expressed in all α and PP cells in wild-type animals, there may exist a small, but distinct class of glucagon- or PP-expressing cells which form independently of Nkx2.2. With our current limited understanding of islet cell determination and lineage, and the lack of additional α and PP cell molecular markers, it is difficult to distinguish between these two possibilities.

It is not surprising that somatostatin expression is normal in the Nkx2.2 null animals considering that Nkx2.2 is not expressed in δ cells. Yet, since δ cells arise at E14.0 when Nkx2.2 expression usually becomes restricted within the pancreas, it is possible that exclusion of Nkx2.2 from δ cells is a necessary step for their differentiation. However, in the mutant embryos, the loss of Nkx2.2 does not appear to result in a shift to the δ cell lineage, therefore the absence of Nkx2.2 alone cannot commit a cell to the δ cell lineage. It is likely that the formation of a δ cell may require other, presumably positive, signals in addition to the absence of Nkx2.2.

Endocrine cell lineage

There is now reasonably good evidence that exocrine and endocrine cells arise from a common endodermal precursor that expresses Pdx1 (Guz et al., 1995). Subsequently, these two cell groups diverge and progress through distinct pathways of
differentiation. It is generally assumed that all islet cell types develop from a common endocrine precursor and then pass through intermediate precursors that give rise to subsets of endocrine cell types, and several lineage models have been proposed (Alpert et al., 1988; Guz et al., 1995; St.-Onge et al., 1997). However, to date, no direct lineage tracing has been performed in the pancreas, and the identification of precursor cells and their descendants has been inferred through expression studies of hormones and transcription factors. As more expression patterns are analyzed, no unifying hypothesis emerges, suggesting there is a greater degree of complexity in lineage determination than expected. For example, our studies of Nkx2.2 could be interpreted as suggesting that the δ cell and β cell lineages are not closely linked, in direct contrast to the conclusions drawn from studies of Pax4 (Sosa-Pineda et al., 1997) and Pdx1 (Guz et al., 1995).

It seems likely that a more complicated mechanism is responsible for the determination and differentiation of each islet cell type. Each cell type population is specified by a transcription factor code, where the presence of a particular set of transcription factors determines cellular fate and the final cellular phenotype. In this regard, it is interesting that Nkx2.2, Pdx1 and Nkx6.1 are all initially expressed globally in the pancreatic bud, but later become restricted to specific endocrine cell types. The inactivation of the genes encoding these factors clearly plays as important a role in pancreatic cell type differentiation as their activation. The sequential activation and inactivation of transcription factors may occur in the different cell types as they progress through a common lineage pathway, or as they differentiate along distinct, but parallel, pathways. Furthermore, the cell type restriction of these regulatory factors between E12.5 and E15.5 may be analogous to the mechanism of lateral inhibition that restricts neural cell differentiation during development of the nervous system. Determining the lineage relationships among the endocrine cell types will require conventional lineage tracing studies, while careful analysis of transcription factor expression patterns and function will reveal the mechanisms that control these lineage patterns.

Hierarchy of transcription factors

Genetic studies are beginning to outline the hierarchy of transcription factors involved in β cell development. For example, in Nkx2.2 mutant embryos, early expression of the β cell specific transcription factor Nkx6.1 is unaffected. However, between E12.5 and E18.5, when there are major changes taking place within the pancreas (differentiation of exocrine tissue; β cell proliferation; δ and PP cell formation) Nkx6.1 expression disappears. The data are consistent with a model where Nkx2.2 is required for the maintenance of Nkx6.1 expression as β cells differentiate, and that continued expression of Nkx6.1 is necessary for complete β cell differentiation.

The genetic relationship between Nkx2.2 and Pdx1 is more complicated. Early in development, Pdx1 is expressed in all cells of the pancreatic bud, and is required for expansion of the bud (Guz et al., 1995; Jonsson et al., 1994; Offield et al., 1996). However, later in embryonic development, Pdx1 becomes progressively restricted to β cells (and some δ cells); and at approximately E14.5, Pdx1 becomes upregulated in β cells (Ohlsson et al., 1993) suggesting it plays a role in β cell differentiation. In the Nkx2.2 mutant, early expression of Pdx1 is not affected. The later β cell restriction of Pdx1 expression also occurs, but is quantitatively reduced in comparison to wild-type β cells (see Fig. 6). Therefore, Nkx2.2 may be required for inducing high level expression of Pdx1 in β cells, and the up-regulation of Pdx1 may be a necessary step in the final differentiation of the β cell.

In contrast to Nkx6.1 or Pdx1 expression, the expression of Isl1, Pax6 and Brn4 during embryogenesis does not appear to require Nkx2.2. These genes may therefore either lie upstream or in different pathways relative to the Nkx genes. Furthermore, since Isl1 is expressed in islet cells soon after they exit the cell cycle (Ahlgren et al., 1997), normal expression of Isl1 in the Nkx2.2 mutant suggests that all the islet cells are able to normally exit a proliferating state and proceed with a program of differentiation. This result supports the hypothesis that the immature β cells are able to initiate β cell development and it is subsequent steps of terminal differentiation that are blocked. Additionally, immunohistochemistry using antibody directed against proliferating cell nuclear antigen (PCNA) reveals a normal number of proliferating cells in the mutant islet (data not shown). Therefore, the incompletely differentiated β cells do not result from the accumulation of an undifferentiated, dividing precursor.

Given the importance of the islet transcription factor genes in the development and maintenance of functional β cells, it is not surprising that mutations in these genes have been associated with the development of human diabetes. Maturity-onset diabetes of the young (MODY) 1 and 3 are types of non-insulin-dependent diabetes mellitus (NIDDM) caused by mutations in hepatocyte nuclear factor-4α (HNF-4α) and HNF-1α, respectively (Yamagata et al., 1996a,b). MODY 4 is linked to the human IPF1 (Pdx1) gene (Stoffers et al., 1997). Analogously, partial defects in Nkx2.2, or in other islet transcription factor genes could contribute to the etiology of the more common late onset form of NIDDM (Type 2 diabetes). In contrast to human MODY and Type 2 diabetes, Nkx2.2 null animals develop diabetes immediately after birth due to the lack of insulin production, and homozygous mutations of the islet transcription factors neuroD/BETA2 and Pdx1 cause similar syndromes in mice (Naya et al., 1997; Jonsson et al., 1994; Offield et al., 1996). Therefore these genes and others involved in β cell differentiation should also be considered candidate genes for the analogous human syndrome, neonatal diabetes, a rare but devastating disease of newborns (von Muhlendahl and Herkenhoff, 1995).

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