RESEARCH COMMUNICATION

β-Cell-specific inactivation of the mouse *Ipf1/Pdx1* gene results in loss of the β-cell phenotype and maturity onset diabetes

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To study the late β-cell-specific function of the homeodomain protein IPF1/PDX1 we have generated mice in which the *Ipf1/Pdx1* gene has been disrupted specifically in β cells. These mice develop diabetes with age, and we show that IPF1/PDX1 is required for maintaining the β cell identity by positively regulating insulin and islet amyloid polypeptide expression and by repressing glucagon expression. We also provide evidence that IPF1/PDX1 regulates the expression of Glut2 in a dosage-dependent manner suggesting that lowered IPF1/PDX1 activity may contribute to the development of type II diabetes by causing impaired expression of both Glut2 and insulin.

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At present, very little is known about the sources and the nature of the inductive molecules that control the development of the pancreas and the generation of individual differentiated cell types. In contrast, genes encoding a number of different transcription factors that exert important functions at different levels during pancreas development have been isolated (Jonsson et al. 1994; Offield et al. 1996; Ahlgren et al. 1997; Sosa-Pineda et al. 1997; St-Onge et al. 1997 and references therein). The endocrine cells, which control multiple homeostatic functions, are organized into islets, and the predominant islet cell type, the β cell, responds to glucose by synthesizing and secreting insulin, thus exerting a feedback control of blood glucose levels. The intrinsic regulatory molecules that establish and maintain hormone production and the glucose-sensing system in β cells are, however, still largely unknown.

The homeodomain factor IPF1/PDX1 is initially expressed in the early mouse pancreatic anlagen (Leonard et al. 1993; Ohlsson et al. 1993; Miller et al. 1994; Guz et al. 1995; Ahlgren et al. 1996), but is later restricted to differentiating β cells (Ohlsson et al. 1993). IPF1/PDX1 has been proposed to regulate the expression of a variety of different pancreatic endocrine genes including insulin, somatostatin, glucokinase, islet amyloid polypepride (IAPP), and glucose transporter type 2 (Glut2) (Leonard et al. 1993; Ohlsson et al. 1993; Serup et al. 1995; Waeber et al. 1996; Watada et al. 1996a,b). Mice lacking IPF1/PDX1 fail to form a pancreas, precluding an analysis of later contribution of IPF1/PDX1 to β-cell development and function (Jonsson et al. 1994; Ahlgren et al. 1996; Offield et al. 1996). To address the role of IPF1/PDX1 in adult differentiated β cells we have generated β-cell-specific *Ipf1/Pdx1* mutant mice using the CRE–Lox system (Lakso et al. 1992; Gu et al. 1993; Kilby et al. 1993; Tsien et al. 1997). In this paper we show that expression of IPF1/PDX1 is critical for maintaining the hormone-producing phenotype and the homeostatic regulation of the glucose-sensing system in β cells.

Results and Discussion

Homozygous *Ipf1/Pdx1–loxP* mice carrying the *loxP* sites flanking exon 2 (Fig. 1A) are indistinguishable from +/+ mice in all respects. By crossing these mice with mice expressing the Cre recombinase under the control of the Rat insulin1-promoter (Rip1) (Dahl et al. 1996), we generated mice that allow CRE-mediated excision of the *loxP*-flanked exon 2 in insulin-expressing β cells (Fig. 1A). The *Rip1/Ipf1*$^{\blacktriangle}$ mutant mice exhibit an apparently normal pancreas that contains islets (Figs. 1 and 3, below; data not shown), and initially such mice were healthy. However, at ~3–5 months of age mutant mice (n = 10) exhibited markedly elevated urine (>55 mM, n = 10) and blood glucose levels (Table 1) and thus were overt diabetic (OD).

We examined the onset of *Ipf1/Pdx1* inactivation by performing immunohistochemistry using anti-insulin and anti-IPF1 antibodies on pancreases derived from mice at different developmental stages. Although RT–PCR analysis of Rip1/Cre expression revealed that the Cre transgene is activated during the fetal stage (Fig. 1B), a very limited extent of Ipf1/Pdx1 inactivation was observed in pancreases derived from postnatal day 1 (P1) *Rip1/Ipf1*$^{\blacktriangle}$ mice (Fig. 1C). However, by 3 (Fig. 1D) and 5 weeks (Fig. 1E) of age, inactivation of *Ipf1/Pdx1* becomes more prominent. To determine the cause of diabetes and the role of IPF1/PDX1 in β cell function, we examined the formation of islets and the state of differentiation of β cells in OD *Rip1/Ipf1*$^{\blacktriangle}$ mice. No significant reduction in the number of islets was observed in the mutant mice (data not shown). In normal mice, 82% of pancreatic islet cells are insulin-expressing (Ins^{+}) β cells of which >99% coexpress IPF1/PDX1 (Figs. 1F and 2A; data not shown). In the OD *Rip1/Ipf1*$^{\blacktriangle}$ mutants the number of Ins^{+} cells was reduced by ~40%, and of the remaining Ins^{+} cells only 17% (n = 96/559) coexpressed IPF1/PDX1 (Figs. 1G and 2A), indicating that Cre/*loxP*-mediated recombination of the *Ipf1/Pdx1* gene had occurred in at least 80% of β cells.

[*Key Words: Ipf1/Pdx1;* β-cell-specific mutants; hormone production; glucose-sensing; diabetes]

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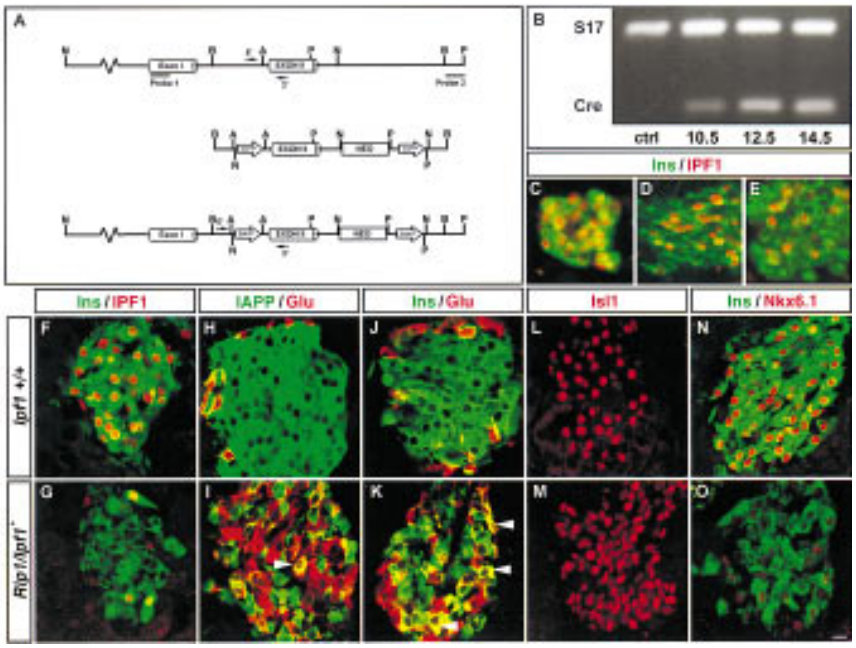

Figure 1. Cre–*loxP*-mediated targeting of the *Ipf1/Pdx1* gene. (*A*) Schematic representation of targeting construct, genomic DNA, and the expected product of Cre/*loxP* recombination. The PCR primers used for genotyping and the probes used for confirmation of correct recombination events are shown. (*B*) RT–PCR analysis using Cre- and rpS17 control primers on total RNA prepared from the alimentary tract of E10.5, E12.5, and E14.5 *Rip1/Ipf1*$^{\blacktriangle}$ and E14.5 *Ipf1*$^{+/+}$ (ctrl) embryos. Pancreatic sections from neonatal (*C*), 3 (*D*)-, and 5 (*E*)-week-old prediabetic *Rip1/Ipf1*$^{\blacktriangle}$ mice stained with anti-Ins and anti-IPF1 antibodies. (*F–O*) β-Cell-specific loss of *Ipf1/Pdx1* affects the β-cell phenotype. Confocal images showing *Ipf1*$^{+/+}$ (*F,H,J,L,N*) and diabetic *Rip1/Ipf1*$^{\blacktriangle}$ (*G,I,K,M,O*) islets from 18-week-old mice. See text for details. (*C–O*) Bar, 10 μm.

Thus, although the recombination event commences at the neonatal stage the OD state develops first when ~80% of the Ins^{+} cells have lost IPF1/PDX1. The time required to reach the state of 80% inactivation of both alleles is dependent on the amount of Cre recombinase (Lakso et al. 1992; Tsien et al. 1997). Transgene expression is known to vary between transgenic lines and also between cells in a given transgenic line (Martin and Whitelaw 1996; Kioussis and Festenstein 1997). In addition, islet neogenesis, that is, emergence of new β cells, is known to continue for as long as 3 weeks postnatally (Githens 1993; Sander and German 1997), and this possibly also prolongs the time required for enough β cells to have lost IPF1/ PDX1. Finally, excision of *Ipf1/Pdx1*, that is, an insulin gene transactivator, most likely negatively affects the level of Rip1/Cre transcription, and this is also likely to influence the time required for both *Ipf1/Pdx1* alleles to become inactivated.

β cells also express IAPP (Figs. 1H and 2A) but in the *Rip1/Ipf1*$^{\blacktriangle}$ mutants the number of IAPP^{+} cells was decreased by 40% (Figs. 1I and 2A), and only 43% of all Ins^{+} and/or IAPP^{+} cells coexpressed the two proteins (Fig. 2B). In addition, cells coexpressing IAPP and glucagon were present (Fig. 1I, arrowhead). In normal mice the ratio of Ins^{+} to glucagon-expressing (Glu^{+}) cells is ~5:1 and no islet cells coexpress the hormones (Figs. 1J and 2A,C). In the *Rip1/Ipf1*$^{\blacktriangle}$ mice, however, there was a significant increase in the number of Glu^{+} cells, from 15% in Ipf1$^{+/+}$ mice to 38% in the mutants, resulting in an ~1:1 ratio of Ins^{+}/Glu^{+} cells (Figs. 1J,K and 2A,C). In addition, ~22% of all Ins^{+} and/or Glu^{+} cells coexpressed the two hormones (Figs. 1K, arrowheads, and 2C). The increase in the number of glucagon cells in the mutant mice was also associated with a more homogenous distribution of Glu^{+} cells within the islets as compared to the normal, peripheral location of Glu^{+} cells in wild-type islets (Fig. 1H–K). No significant change in the number of somatostatin-expressing (Som^{+}) cells was observed in *Rip1/Ipf1*$^{\blacktriangle}$ mutants, and no Som^{+} cells coexpressed either insulin or glucagon (data not shown). The expression of the LIM–homeodomain protein ISL1 (Karlsson et al. 1990; Thor et al. 1991) (Fig. 1L,M), which is required for the generation of all islet cell types (Ahlgren et al. 1997), was also unaffected, showing that islets do form and cells within islets still retain endocrine properties in mutant mice.

These results show that IPF1/PDX1 is required for maintaining the hormone-producing phenotype of the β

Table 1. *Pancreatic insulin content and blood glucose levels*

	Blood glucose levels[a]				Pancreatic insulin content[b]	
	nonfasted		fasted			
Ipf1$^{+/+}$	5.9 ± 0.5	(*n* = 8)	3.9 ± 0.1	(*n* = 8)	10.0 ± 0.6	(*n* = 8)
Ipf1$^{+/-}$	7.2 ± 0.7	(*n* = 8)	5.0 ± 0.5	(*n* = 8)	9.9 ± 0.6	(*n* = 8)
ND *Rip1/Ipf1*$^{\blacktriangle}$	8.1 ± 1.2	(*n* = 3)	3.9 ± 0.6	(*n* = 3)	3.1 ± 0.5	(*n* = 4)
OD *Rip1/Ipf1*$^{\blacktriangle}$	>27.8	(*n* = 4)	11.4 ± 1.7	(*n* = 8)	1.1 ± 0.2	(*n* = 4)

Results are mean ± S.E.M. of (*n*) animals in each group. ND *Rip1/Ipf1*$^{\blacktriangle}$ mice were 8–12 weeks of age, *Ipf1*$^{+/+}$, *Ipf1*$^{+/-}$ and OD *Rip1/Ipf1*$^{\blacktriangle}$ were 17–19 weeks of age.

[a]Blood glucose levels are expressed as mmoles/liter from nonfasted and over night-fasted animals, respectively.

[b]Insulin is expressed as μg/mg pancreas protein from nonfasted animals.

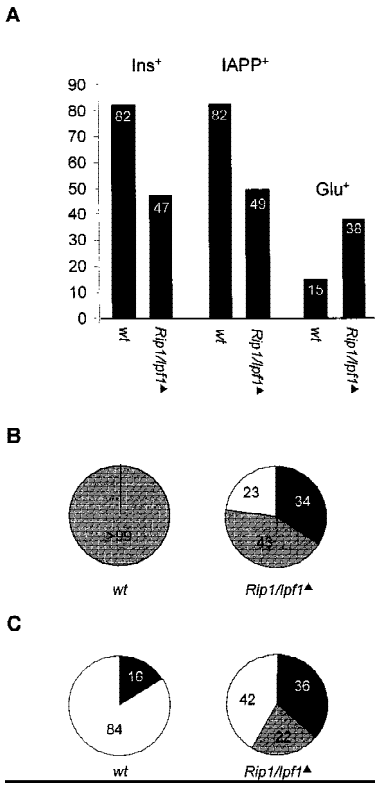

Figure 2. *Rip1/Ipf1*▲ mice show abnormal islet composition. (*A*) The distribution of insulin (Ins^+)-, IAPP ($IAPP^+$)-, or glucagon (Glu^+)-expressing cells per total number of islet cells in wild-type and *Rip1/Ipf1* mice. (*B*) Ratio of Ins^+ to $IAPP^+$ cells in islets from wild-type and *Rip1/Ipf1* mice. (Open area) Ins^+ only; (solid area) $IAPP^+$ only; (shaded area) Ins^+ and $IAPP^+$ coexpressed. (*C*) Ratio of Ins^+ to Glu^+ cells in islets from wild-type and *Rip1/Ipf1* mice. (Open area) Ins^+ only; (solid area) Glu^+ only; (shaded area) Ins^+ and Glu^+ coexpressed. (*A–C*) n = 600–1050 cells.

cell by positively regulating insulin and IAPP expression and by repressing Glu^+ in β cells. The homeodomain protein Nkx6.1 is expressed in β cells (Fig. 1N; Jensen et al. 1996), and virtually no $Nkx6.1^+$ cells were detected in *Rip1/Ipf1*▲ mutants (Fig 1O). Interestingly, Nkx6.1 is homologous to the homeodomain protein Gtx which can act as a transcriptional repressor (Komuro et al. 1993). The loss of Nkx6.1 in the mutants may therefore suggest that IPF1/PDX1 positively regulates the expression of Nkx6.1, which in turn might repress glucagon expression in β cells.

The pancreas of the OD *Rip1/Ipf1*▲ mutant mice contained ~60% of the normal number of β cells (Fig. 2A) and ~10% of the normal total pancreatic insulin content (Table 1). This suggests that a progressive decrease in insulin expression may be the causative agent underlying the development of diabetes in the *Rip1/Ipf1*▲ mice. The diabetic mutants responded to insulin administration by transiently reverting to normoglycemia (data not shown), excluding insulin resistance as a cause of diabetes. IPF1/PDX1 has, however, been suggested to regulate the expression of the Glut2 and glucokinase genes (Waeber et al. 1996; Watada et al. 1996a), two key components of the β cell glucose-sensing system. *Glut2*-deficient mice show an impaired glucose-induced insulin secretion and develop diabetes (Guillam et al. 1997), and mutation and/or inactivation of the glucokinase gene results in impaired insulin secretion and development of diabetes (Frougel et al. 1992; Bali et al. 1995; Grupe et al. 1995) both in mouse and man. Thus, an impaired expression of these genes could also possibly contribute to the diabetic phenotype of *Rip1/Ipf1*▲ mutant mice.

In OD *Rip1/Ipf1*▲ mice Glut2 expression was virtually extinct (Fig. 3A), whereas the expression of glucokinase appears unaffected (data not shown). In nondiabetic (ND) *Rip1/Ipf1*▲ 11-week mice the pancreatic insulin content is ~30% of the normal level (Table 1), the number of $Glut2^+$ cells is reduced, and the level of Glut2 expression is highly variable (Fig. 3B). Thus, excision of the *Ipf1/Pdx1* gene in β cells leads to an early loss of Glut2 expression (Fig. 3A–C,E–G), which via impaired glucose-stimulated insulin release probably contributes to the development of hyperglycemia. The combined, progressive loss of both Glut2 and insulin expression as a result of *Ipf1/Pdx1* inactivation would then lead to the subsequent development of OD in the *Rip1/Ipf1*▲ mice. Interestingly, *Glut2*-deficient mice also show an increased number of α versus β cells (Guillam et al. 1997) but, unlike in *Rip1/Ipf1*▲ mice, the α cells still remain located at the periphery of the islets (B. Thorens, pers. comm.). This suggests that the change in the ratio of Glu^+ versus Ins^+ cells in *Rip1/Ipf1*▲ mice primarily is mediated by the loss of IPF1/PDX1 in β cells, resulting in a change in the pattern of hormone production.

A strong family history of early onset type II diabetes is associated with heterozygosity for a point mutation in the human *Ipf1* gene (Stoffers et al. 1997a,b). Our results raise the possibility that a dosage-dependent regulation of Glut2 and insulin expression by IPF1/PDX1 could be

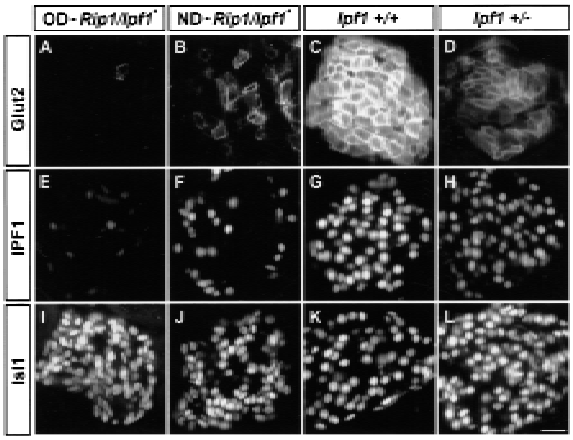

Figure 3. Glut2 expression is dependent on high levels of IPF1/PDX1 expression. Analysis of Glut2 expression in correlation to IPF1 expression by immunohistochemistry on pancreas from 18-week diabetic *Rip1/Ipf1*▲ (*A,E,I*), 11-week ND *Rip1/Ipf1*▲ (*B,F,J*), 18-week $Ipf1^{+/+}$ (*C,G,K*), and 18-week $Ipf1^{+/-}$ mice (*D,H,L*) (Jonsson et al. 1994). Images are representative of at least 10 different islets. Bar, 20 μm.

causative. Mice heterozygous for the original *Ipf1/Pdx1* null mutation are ND and show blood glucose levels in the upper range of normoglycemia but were found to be glucose intolerant. In $Ipf1^{+/+}$ mice intraperitoneal (IP) injections of glucose (1 gram/kg body weight) resulted in an increase in blood glucose levels to 12–13 mM 15–20 min postinjection (Fig. 4). In $Ipf1/Pdx1^{+/-}$ mice, the blood glucose levels increased for 40–50 min, reaching 22–23 mM, and did not return to basal levels 180 min after challenge (Fig. 4). The glucose intolerance could reflect either impaired insulin, Glut2 expression, or both. The *Ipf1/Pdx1*-null (+/−) mice, however, showed a normal total pancreatic insulin content and islet architecture (Fig. 3D,H,L; Table 1; data not shown), and the pattern and levels of expression of ISL1 (Fig. 3I–L), insulin, glucagon, IAPP, and Nkx6.1 appeared normal (data not shown). In contrast, Glut2 was expressed at homogeneous but markedly reduced levels and IPF1/PDX1 at decreased levels (Fig. 3C,D,G,H) providing evidence that IPF1/PDX1 exerts a dosage-dependent regulation of Glut2 expression.

Hence, these results offer a plausible explanation for the development of maturity onset diabetes in *Ipf1/Pdx1* haploinsufficient individuals and identify *Ipf1/Pdx1*-null (+/−) mice as a relevant animal model for studying this disease. These results also strengthen the idea that an early loss of Glut2 expression is involved in the development of hyperglycemia in *Ipf1/Pdx1* β-cell-specific mutants and that the combined loss of Glut2 and gradual decrease of insulin expression together leads to the manifestation of diabetes. Thus, lowered IPF1/PDX1 expression, or activity, resulting in impaired expression of both Glut2 and insulin could be a more general cause of development of hyperglycemia that in turn may progress to type II diabetes. In conclusion, the results presented here provide direct evidence that IPF1/PDX1 exhibits dual functions during pancreas development. Loss of the early function results in an inability to form a pancreas (Jonsson et al. 1994; Ahlgren et al. 1996; Offield et al. 1996; Stoffers et al. 1997a), whereas the later β-cell expression is required to maintain the β-cell pattern of hormone production, Glut2 expression, and normoglycemia.

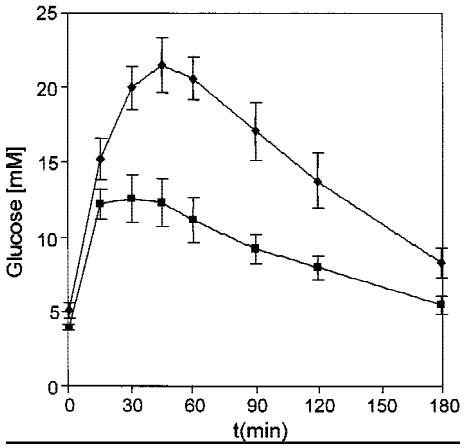

Figure 4. Heterozygosity for the *Ipf1/Pdx1* null mutation results in impaired glucose tolerance. Glucose challenge of 18-week-old $Ipf1^{+/+}$ (■) and $Ipf1^{+/-}$ (♦) (Jonsson et al. 1994) mice. Blood glucose levels are shown at indicated time points after IP administration of glucose. Results are mean ± S.E.M. of eight animals in each group.

Materials and methods

Preparation of the Ipf1/Pdx1-loxP *gene targeting construct*

The two *loxP* sites (Kilby et al. 1993) (5′ and 3′ of exon 2 in *Ipf1/Pdx1;* see Fig. 1A) were constructed using single-stranded oligonucleotides. The upstream *loxP* recognition and core sequence contains *Apa*I sites at its 5′ and 3′ ends and an internal *Nco*I site. Similarly, the downstream *loxP* recognition and core sequence has *Nco*I sites at its ends and an internal *Pst*I site. The internal sites were used to verify homologous recombination. A *Bam*HI–*Xho*I fragment carrying the gene for neomycin resistance (neo^R) (Jonsson et al. 1994) was cloned into the *Bgl*II and *Xho*I sites of the downstream *loxP* site (Fig. 1A). The downstream neo^R–*loxP* construct was inserted into the *Nco*I site 3′ of exon 2A of the genomic mouse *Ipf1/Pdx1* DNA (Fig. 1A; Jonsson et al. 1994). The upstream *loxP* site was subsequently inserted in the *Apa*I site 5′ of exon 2 in the *Ipf1/Pdx1* gene. The functionality of the targeting construct was verified by incubating the final 8.7-kb *Ipf1/Pdx1–neo*^{R}*loxP* fragment with Cre enzyme (NEN–DuPont, Inc.). Bands corresponding to 8.7 and 3.7 kb and the supercoiled circular *loxP* product were identified on an 8% polyacrylamide gel.

Generation of Ipf1/Pdx1–loxP *mice*

E14-1 ES cells (Jonsson et al. 1994) were cultured and electroporated using standard procedures as described previously (Hogan et al. 1994). ES cells carrying the *Ipf1/Pdx1–loxP* mutation were injected into blastocysts of C57BL/6 mice (Hogan et al. 1994), and the resulting male chimeras were subsequently backcrossed to C57BL/6 mice to generate heterozygous mutants and wild-type mice.

RIP1/CRE transgenic mouse production

The Cre expression vector pRip1/Cre was constructed by inserting the 0.9-kb *Xba*I–*Mlu*I CRE fragment, derived from phCMV/Cre (Sauer and Henderson 1990; Baubonis and Sauer 1993), and blunted at the *Mlu*I site, into *Xba*I–*Hin*dIII-digested pRip1ΔEcad (Dahl et al. 1996), blunted at the *Hin*dIII site. A 2.6-kb linearized DNA *Aat*II–*Sal*I fragment containing the Rip1/Cre construct was used to generate transgenic founders by pronuclear injection of the linearized DNA into F_2 hybrid oocytes from B6/CBA parents as described (Hogan et al. 1994). The Cre founders were backcrossed into the B6 background for production of transgenic offspring and then subsequently also crossed with the *Ipf1/Pdx1–loxP* mice to generate homozygous *Ipf1/Pdx1–loxP* mice carrying the Rip1/Cre transgene.

Genotyping and RT–PCR

The genotypes of all offspring were performed on genomic DNA isolated from ES cells or the tail tip of 2- to 4-week-old mice by Southern blot or PCR (Hogan et al. 1994). The 5′ and 3′ primers for the Rip1/Cre transgene (~700 bp amplified) were 5′-GGTGCTTTGGACTATAAAGC-3′ and 5′-GTCAGTACGTGAGATATCTTTA-3′ under the following conditions: 35 cycles of 94°C for 30 sec, 50°C for 1 min, 72°C for 1 min, followed by 1 cycle of 72°C for 7 min. The 5′ and 3′ primers for the *Ipf1/Pdx1* gene were 5′-TCAACAGCTGCGATCAGTA-3′ and 5′-AACATCACTGC-CAGCTCCACC-3′ under the following conditions: 1 cycle of 96°C for 5 min, 55°C for 2 min, 72°C for 3 min, followed by 29 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 3 min, and, finally, 1 cycle of 72°C for 7 min, which results in a ~300-bp amplified fragment for the wild-type *Ipf1/Pdx1* allele and a ~240-bp amplified fragment for the *Ipf1/Pdx1–loxP* allele. For *Ipf1/Pdx1* Southern blot analysis, DNA was digested with *Nco*I or *Pst*I and hybridized with probes 1 and 2, respectively. RT–PCR analysis were performed on total RNA prepared from embryonic tissues essentially as described (Tanabe et al. 1995). The primers used for RT–PCR were Cre-primers, 5′-GGACATGTTCAGGGATCGCCAG-GCG-3′ and 5′-GCATAACCAGTGAAACAGCATTGCTG-3′ (Lakso et al. 1992); mouse ribosomal protein S17, 5′-TCGCACCAAGACTGT-GAAGAAGGC-3′ and 5′-TGGCATAACAGATTAAACAGCTCCACG-3′. The conditions used were 35 cycles of 94°C for 30 sec, 60°C for 1 min,

72°C for 1 min, followed by 1 cycle of 72°C for 7 min. Sizes of the resulting PCR products are 269 bp for Cre and 398 bp for S17.

Glucose challenge and insulin measurements

Overnight-fasted wild-type and *Ipf1/Pdx1*-null (+/−) mice were injected IP with 1 gram of glucose per kg of body weight. Blood samples were obtained from the tail vein, and glucose levels were measured immediately before and 15, 30, 45, 60, 90, 120, and 180 min after injection using a Glucometer Elite (Bayer, Inc.). Pancreatic insulin was measured using a commercially available radioimmuno assay for rat insulin (Linco), and total pancreatic protein concentration was determined using the Bio-Rad protein assay.

Immunohistochemistry and confocal image analysis

Immunohistochemistry and confocal image analysis was performed essentially as described previously (Ahlgren et al. 1996). Primary antibodies used were rabbit anti-IPF1/PDX1 (Ohlsson et al. 1993), rabbit anti-ISL1 (Thor et al. 1991), guinea pig anti-insulin C-peptide (Linco), guinea pig anti-glucagon (Linco), rabbit anti-glucagon (Linco), rabbit anti-somatostatin (DAKO), rabbit anti-IAPP/amylin (Euro Diagnostics), sheep anti-glucokinase (H.J. Seitz, University of Hamburg, Germany), rabbit anti-Glut2, kindly provided by Dr. B. Thorens (Institute of Phamacology and Toxicology, Lausanne, Switzerland), and rabbit anti-Nkx6.1 (Jensen et al. 1996), kind gift from Dr. P. Serup. Secondary antibodies used were Cy3-conjugated goat anti-rabbit IgG (Jackson) and Fluorescein (DTAF)-conjugated goat anti-GP IgG (Jackson). For multiple labelling a blocking step using swine anti-Rabbit IgG (DAKO) was included.

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