Notch/Rbp-j signaling prevents premature endocrine and ductal cell differentiation in the pancreas

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Summary

To investigate the precise role of Notch/Rbp-j signaling in the pancreas, we inactivated Rbp-j by crossing Rbp-j floxed mice with *Pdx.cre* or *Rip.cre* transgenic mice. The loss of Rbp-j at the initial stage of pancreatic development induced accelerated α and PP cell differentiation and a concomitant decrease in the number of Neurogenin3 (Ngn3)-positive cells at E11.5. Then at E15, elongated tubular structures expressing ductal cell markers were evident; however, differentiation of acinar and all types of endocrine cells were reduced. During later embryonic stages, compensatory acinar cell differentiation was observed. The resultant mice exhibited insulin-deficient diabetes with both endocrine and exocrine pancreatic hypoplasia. In contrast, the loss of Rbp-j specifically in β cells did not affect β cell number and function. Thus, our analyses indicate that Notch/Rbp-j signaling prevents premature differentiation of pancreatic progenitor cells into endocrine and ductal cells during early development of the pancreas.

Introduction

The pancreas plays a key role in the maintenance of nutritional homeostasis through its exocrine and endocrine functions. The acini and ducts form the exocrine pancreas that produces and transports digestive enzymes into the duodenum. Besides, there are five known endocrine cell types in the pancreas: glucagon-producing α cells, insulin-producing β cells, somatostatin-producing δ cells, pancreatic polypeptide (PP)-producing PP cells, and ghrelin-producing ε cells (Heller et al., 2005).

Notch signaling regulates various developmental processes, such as neurogenesis, somitogenesis, angiogenesis, and hematopoiesis (Ishibashi et al., 1995; Hrabé de Angelis et al., 1997; Xue et al., 1999; Han et al., 2002). Interaction of a Notch receptor with its ligand induces cleavage of the receptor's intracellular domain (Notch ICD), which translocates to the nucleus and binds to Rbp-j to induce the expression of Hes family transcriptional repressors (Kageyama and Ohtsuka, 1999). Rbp-j is a key mediator of Notch signaling because it is expressed ubiquitously and associates with all four types of Notch receptors (Kato et al., 1996). Various Notch-related genes are expressed in the developing pancreas (Lammert et al., 2000). However, multiple anomalies and early embryonic lethalities of mice with homozygous deletions of genes such as *Dll1*, *Notch1*, *Notch2*, *Jagged1*, *Rbp-j*, or *Hes1* limits assessment of the importance of Notch/Rbp-j signaling in the pancreas (Swiatek et al., 1994; Ishibashi et al., 1995; Oka et al., 1995; Hrabé de Angelis et al., 1997; Apelqvist et al., 1999; Hamada et al., 1999; Xue et al., 1999; Jensen et al., 2000a). Although excess α cell differentiation in the pancreas has been reported at around E10 in mice with a generalized KO of Dll1 or Hes1 (Apelqvist et al., 1999; Jensen et al., 2000a), because β cells start to expand at around E13 and their differentiation occurs independently of α cells (Jensen et al., 2000b), the influence of Notch signaling on β cells remains to be elucidated. To address this issue, we created mice with developmental stage-specific deletion of *Rbp-j* in the pancreas using the Cre/loxP-mediated DNA recombination system.

Results

Accelerated premature differentiation of α and PP cells but not of β, δ, and ε cells in pancreatic Rbp-j KO (PRKO) mice

By crossing floxed Rbp-j ($Rbp\text{-}j^{f/f}$, designated as F/F) mice with *Pdx.cre* mice, we generated pancreatic Rbp-j KO ($Rbp\text{-}j^{f/f}$ *Pdx.cre*, designated as PRKO) mice (Gu et al., 2002; Han et al., 2002; see the Supplemental Data available with this article online). The *Pdx.cre* mouse begins to recombine loxP sites in the pancreatic epithelium before E9.5 (Figure S1B). Notch signaling negatively regulates proneural basic helix-loop-helix (bHLH) factors through Hes activation (Kageyama and Ohtsuka, 1999). A unique proendocrine bHLH transcription factor, Ngn3, is required for the development of pancreatic endocrine lineages (Gradwohl et al., 2000; Gu et al., 2002). We observed a premature increase in the number of $Ngn3^+$ cells in the pancreatic buds of PRKO mice (Figure S2A). At E11.5, a few scattered α cells among the protruding epithelial cells of F/F mice were observed (Figures 1E and 1E′). In PRKO mice, the number of α and PP cells increased and they surrounded the pancreatic buds (Figures 1F, 1F′, 1J, and 1J′). However, β, δ, and ghrelin-producing cell differentiation was not enhanced in the mutants (Figures 1D, 1D′, 1H, 1H′, and S3B). The number of $Ngn3^+$ cells decreased in PRKO mice compared with control mice (Figures 1M–1N′ and S2B). The number of proliferating cells detected by phosphohistone H3 (pHH3) immunostaining was comparable between control and mutant mice (Figures 1O–1P′). No apoptotic cells were

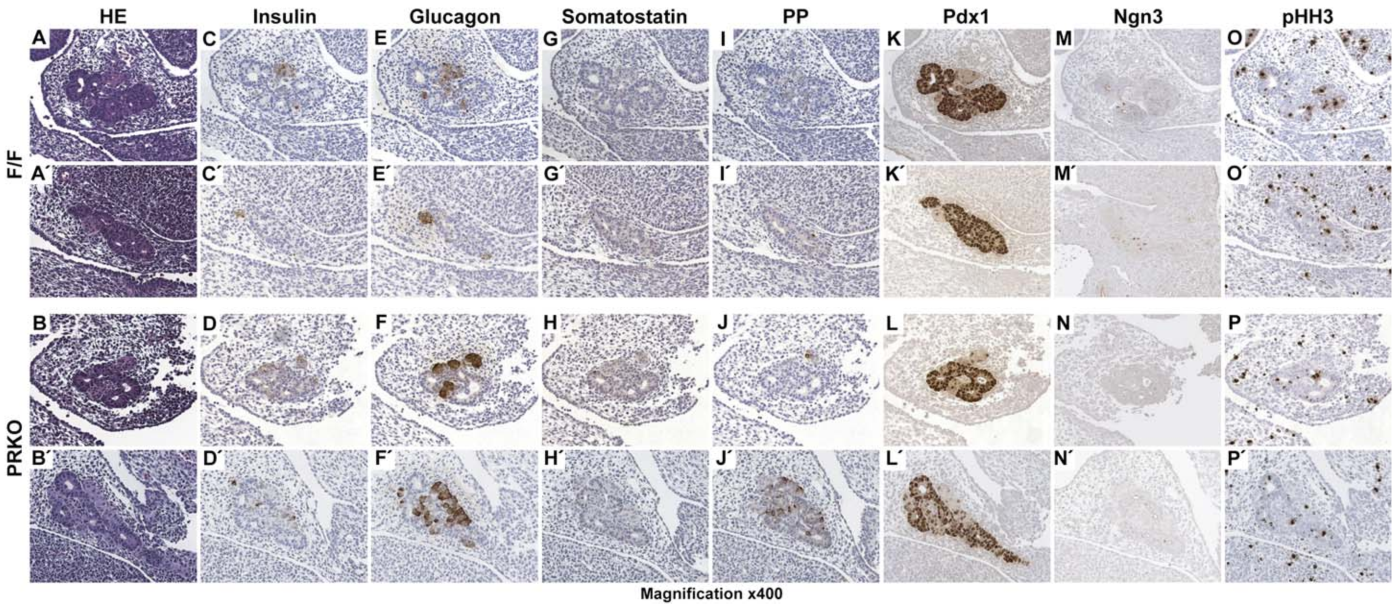

Figure 1. Accelerated premature differentiation of α and PP cells but not β, δ, and ε cells in pancreatic Rbp-j KO (PRKO) mice
A–P′) HE staining (**A–B′**), and immunostaining (**C–P′**) of representative serial pancreatic sections from F/F mice and PRKO mice at E11.5.

detected in the pancreatic epithelia of control or mutant mice (Figures S3C–S3E). These data indicate that earlier commitment to proendocrine (Ngn3$^+$) cells induced by defective Notch signaling results in precocious endocrine cell differentiation and a substantial loss of proendocrine cells during early pancreatic development.

Elongated tubular structures with decreased branching morphogenesis in the pancreas of the PRKO mouse

At E15, pancreatic Pdx1$^+$ epithelium of control mice exhibited complex and ramified networks (Figure 2B). However, in the mutant, branching of Pdx1$^+$ epithelium was severely impaired, and dilated tubular structures were prominent (Figure 2B′). The decreased epithelial branching was not associated with increased cell death or decreased proliferation, because these cells were not apoptotic (Figure 2D′), but exhibited active division (Figure 2M′). Moreover, acinar and β cells were scarcely differentiated (Figures 2C′ and 2H′), and aggregated α cells existed around the columnar tubular epithelium (Figure 2G′). The cells lining the lumens of tubular structures showed positive staining with cytokeratin (CK) and Dolichos biflorus agglutinin (DBA) lectin (Dor et al., 2004) (Figures 2K–2L′). The glucose transporter 2 (Glut2), expressed on the surface of differentiated β cells (Figure 2N), is also thought to be a marker of early pancreatic progenitor cells (Pang et al., 1994); however, Glut2 was not expressed in the tubular epithelium (Figure 2N′). In the control pancreas, *Hes1* expression was not detected (Figure 2O), but scattered Ngn3$^+$ cells were evident at this stage (Figure 2P). In the mutant pancreas, expression of *Hes1* and Ngn3 was virtually absent (Figures 2O′ and 2P′). ISL1 is a LIM homeodomain protein whose expression is initiated after Ngn3 extinction but before hormone production (Ahlgren et al., 1997). ISL1 expression was not detected in the tubular epithelium (Figure 2Q′). This analysis of various differentiation markers shows that the cells lining these tubular structures are not early progenitors, nor are they on the endocrine lineage. The tubular morphologies and high columnar epithelium resembling that of the large pancreatic duct rather suggest that the cells positive for ductal markers are duct cells. We confirmed that all of these cells were derived from Rbp-j-deficient cells by lineage tracing (Figure S4).

At later embryonic stages of PRKO mice, the acinar cell area was much smaller and CK$^+$ ductal cells occupied a larger area compared with F/F mice (Figures S5A–S5F). Although compensatory acinar growth was observed (Figures S5E–S5H), the interval sections revealed a much smaller pancreas in the mutant than in the control mouse (Figure S5I).

PRKO mice are born with pancreatic hypoplasia and exhibit insulin-deficient diabetes

The adult PRKO mouse exhibited a small pancreas (Figure 3A). The absolute pancreatic weight (PRKO, 209 ± 73 mg versus F/F, 685 ± 81 mg; $p = 0.0038$; Figure 3B) and the ratio of pancreatic weight to total body weight (data not shown) were lower in PRKO mice than in F/F mice. In pancreatic sections from PRKO mice, the number of islets per pancreas area was reduced (PRKO, 0.15 ± 0.06 versus F/F, 0.62 ± 0.13 islets/mm^2; $p = 0.015$; Figures 3C and 3D), and the size of the islets was smaller compared with F/F mice (Figure 3C). The relative endocrine cell mass was quantified by estimating the hormone-positive area per total pancreatic area in multiple pancreatic sections. The β cell mass of the PRKO mice was markedly reduced to about 25% of the β cell mass of F/F mice (PRKO, 0.21 ± 0.08% versus F/F, 1.13 ± 0.04%; $p < 0.001$; Figure 3E), and the absolute α cell mass was also significantly reduced to about 50% of that of the F/F mice (PRKO, 0.11 ± 0.02% versus F/F, 0.24 ± 0.02%; $p < 0.001$; Figure 3E). Total pancreatic insulin content (expressed per mg of pancreas weight) estimated from an acid-ethanol extract of the whole pancreas. PRKO mice had much lower insulin contents

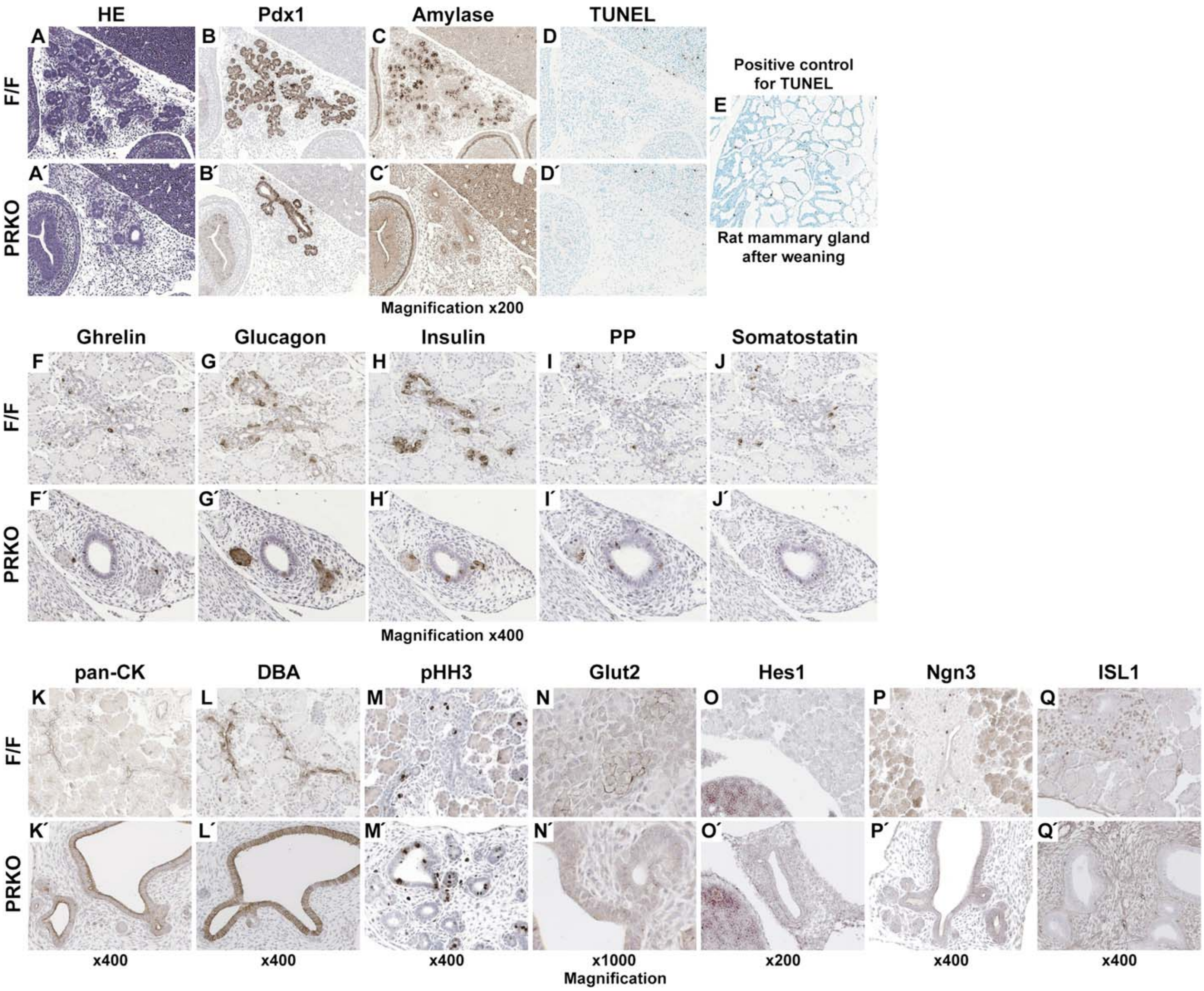

Figure 2. Elongated tubular structures with decreased branching in PRKO mice at E15

A–J′) Dilated and elongated duct-like structures in PRKO mice. HE staining (**A and A**′), immunostaining (**B–C**′ **and F–J**′), and TUNEL assay (**D and D**′) of serial pancreatic sections from F/F mice and PRKO mice at E15. The mammary gland of a postlactating female Wistar rat was used as a positive control for apoptosis (**E**).

K–Q′) Characterization of duct-like cells in PRKO mice. Immunostaining (**K–N**′, **P–Q**′) and *Hes1* in situ hybridization (**O and O**′) of pancreatic sections from F/F and PRKO mice at E15.

than the F/F mice (PRKO, 0.9 ± 0.2 pg/mg pancreas versus F/F, 96.4 ± 9.9 pg/mg pancreas; $p < 0.001$; Figure 3F). In addition to the scarcity of islets, histological analysis of the adult pancreas in PRKO mice revealed that the endocrine cells were frequently observed in association with distended pancreatic ducts (Figures 3J, 3L, 3N, 3P, and 3R). The relative ductal hyperplasia observed during the embryonic stages of PRKO mice (Figure S5D) became obscured in adult PRKO mice (Figure 3C).

The growth of PRKO mice and F/F mice fed normal chow was observed for four months. PRKO mice had a leaner phenotype than F/F mice and exhibited no further weight gain (Figures 3S and 3T). At eight weeks of age, PRKO mice developed significant hyperglycemia during fasting and feeding (fasting—PRKO, 348 ± 61 mg/dl versus F/F, 98 ± 6 mg/dl; $p < 0.001$; morning fed—PRKO, 524 ± 59 mg/dl versus F/F, 124 ± 12 mg/dl; $p < 0.001$; Figure 3U), which was accompanied by notably decreased plasma insulin concentrations (fasting—PRKO, below detection limit versus F/F, 0.48 ± 0.07 ng/ml; $p < 0.001$; morning fed—PRKO 0.04 ± 0.03 ng/ml versus F/F, 1.35 ± 0.25 ng/ml; $p = 0.0013$; Figure 3V). At this age, the mutant mice showed polyuria and polydipsia, and some appeared lethargic. Daily food intake increased in PRKO mice compared with control mice (PRKO, 8.3 ± 0.6 g/24 hr versus F/F, 4.1 ± 0.3 g/24 hr; $p < 0.001$; Figure 3W), which corresponded to diabetic hyperphagia. Thus, PRKO mice exhibited characteristics typical of diabetes with defective insulin secretion. Furthermore, PRKO mice had lower serum amylase activities than F/F mice (PRKO, 711 ± 58 U/dl versus F/F, 1121 ± 67 U/dl; $p = 0.0015$; Figure 3X), presumably due to pancreatic hypoplasia and severe diabetes.

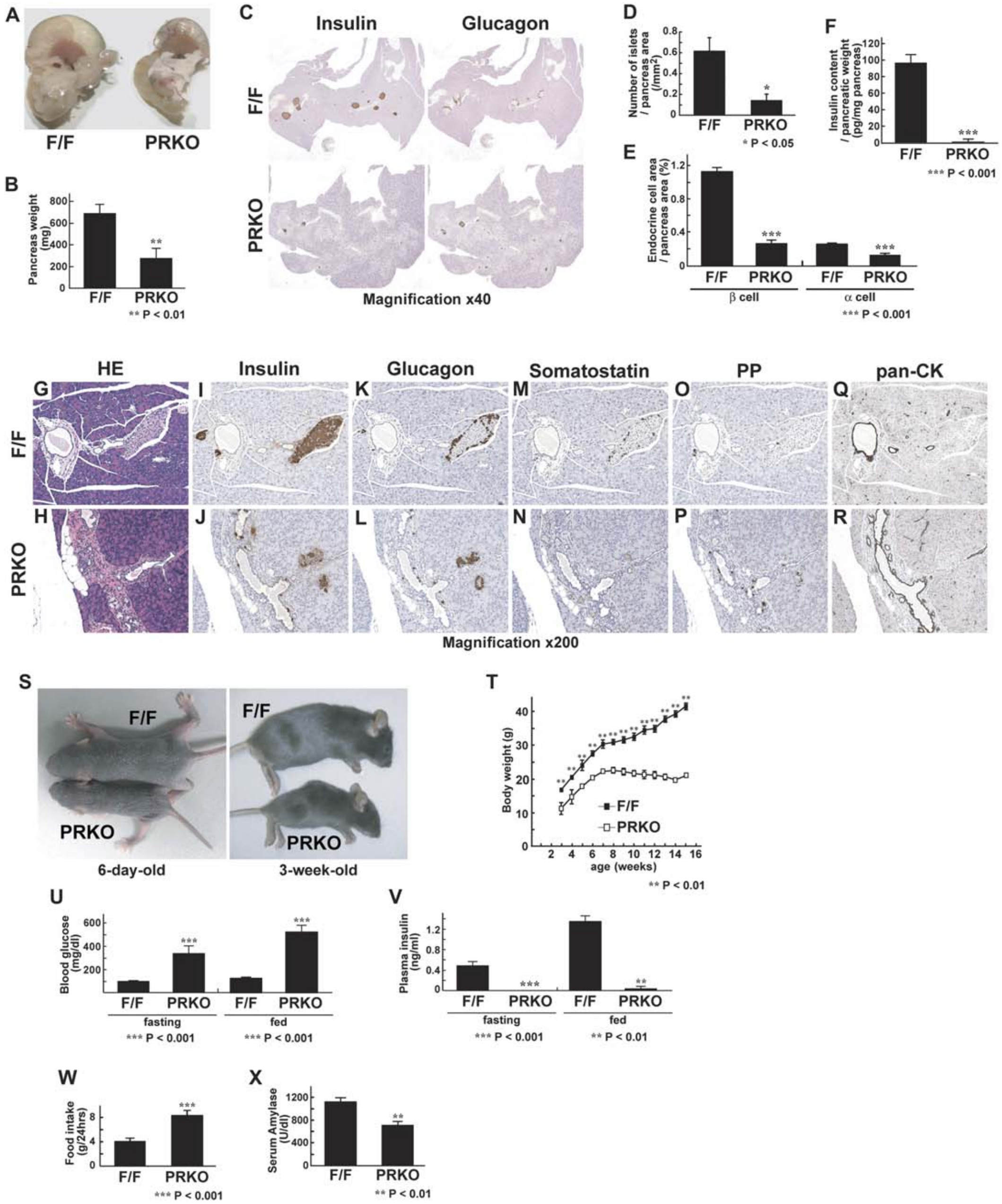

Figure 3. The adult PRKO mouse has a small pancreas, few islets, and low insulin content, which result in overt diabetes

A and B) Small pancreas in an adult PRKO mouse. Gut regions were dissected (**A**), and pancreatic weights were measured at 12 weeks of age (**B**).

C–E) Fewer islets and reduced endocrine cell mass in PRKO mice. Pancreatic sections from 10-week-old F/F mice and PRKO mice were immunostained for insulin and glucagon (**C**) to determine the number of islets (**D**), β cell area (**E**), and α cell area (**E**), which were normalized to total pancreatic area.

F) Lower pancreatic insulin content in PRKO mice. Pancreatic insulin content was measured in acid-ethanol extracts from 6-week-old F/F mice and PRKO mice.

G–R) Duct-associated endocrine cells in PRKO mice. HE staining (**G and H**) and immunostaining (**I–R**) of serial pancreatic sections from 8-week-old F/F and PRKO mice. The number of β cells was markedly reduced (**J**). Endocrine cells are located close to ductal structures (**J**, **L**, **N, and P**).

S and T) Growth retardation in PRKO mice. Gross appearances (**S**) of 6-day-old (left) and 3-week-old (right) PRKO mice (bottom) and control littermates (top). Growth curve (**T**) for litters obtained from mating F/F mice and PRKO mice.

U–W) Insulin-deficient diabetes in PRKO mice. Blood glucose concentrations (**U**) and plasma insulin concentrations (**V**) from fasted and random-fed 8-week-old male F/F mice and PRKO mice. Food intake (**W**) was measured for 24 hr from 16-week-old male F/F mice and PRKO mice.

X) Exocrine pancreatic insufficiency in F/F *Rip.cre* mice. Amylase activity was measured in serum from F/F mice and PRKO mice at 10 weeks of age.

Bars represent means ± SE of n = 4–8 mice. Levels of significance (Student's t test) are shown (*p < 0.05; **p < 0.01; ***p< 0.001).

β cell-specific Rbp-j KO (βRKO) mice have normal β cell number and function

By crossing F/F mice with *Rip.cre* mice, we next generated β cell-specific Rbp-j KO (*Rbp-j$^{f/f}$ Rip.cre*, designated as βRKO) mice (Figure S6). βRKO mice had normal body weight (βRKO, 35.2 ± 2.6 mg/dl versus *Rip.cre*, 37.0 ± 2.5 mg/dl; p = 0.62; Figure 4A). No significant differences were detected in the levels of blood glucose (βRKO, 160 ± 17 mg/dl versus *Rip.cre*,

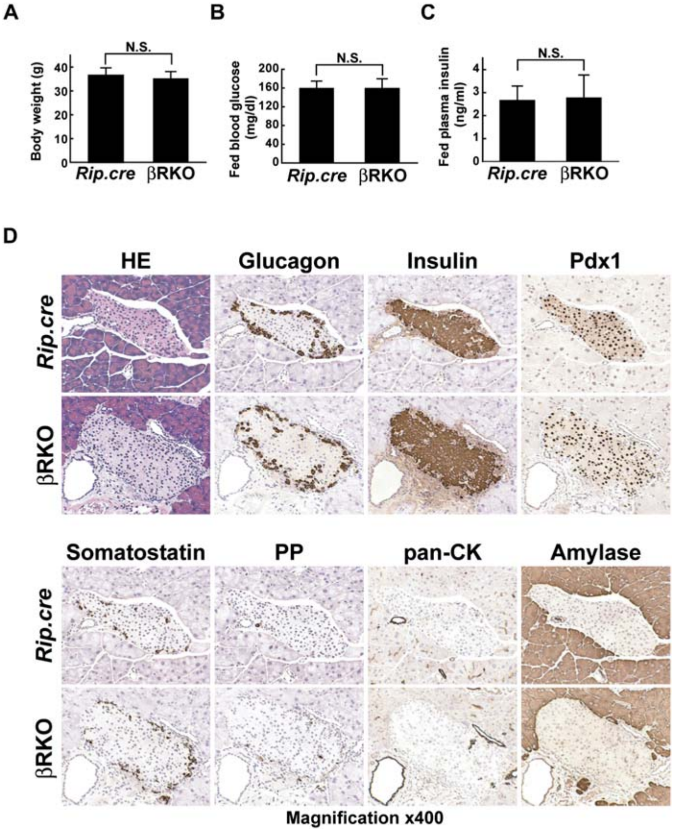

Figure 4. Absence of abnormalities in β cell-specific *Rbp-j* KO mice

A–C) Metabolic parameters of βRKO mice. Body weight (**B**), random-fed blood glucose concentrations (**C**), and random-fed plasma insulin concentrations (**D**) of 19-week-old male *Rip.cre* mice and βRKO mice.

D) Absence of morphological changes in the pancreas of the βRKO mouse. Immunohistochemistry of serial pancreatic sections of 20-week-old *Rip.cre* mice and βRKO mice.

Bars represent means ± SE of n = 7–8 mice. Levels of significance (Student's t test) are shown (NS, not significant).

159 ± 15 mg/dl; p = 0.95; Figure 4B) or plasma insulin (βRKO, 2.78 ± 1.10 ng/ml versus *Rip.cre*, 2.67 ± 0.55 ng/ml; p = 0.92; Figure 4C) in the fed state. Hematoxylin-eosin (HE) staining and immunohistochemical studies with glucagon, insulin, Pdx1, somatostatin, PP, pan-CK, and amylase revealed no abnormalities (Figure 4D). These data indicate that Rbp-j is not required for maintaining β cell function or β cell mass.

Discussion

Using the stage-specific conditional gene targeting approach, we documented the effects of Notch/Rbp-j signaling on pancreatic development and function.

Normally, Ngn3 expression peaks between E13.5 and E15.5 (Apelqvist et al., 1999). In PRKO mice, Ngn3 expression peaked at E10.5 and then declined at E11.5 (Figure 1), which suggests that termination of Notch signaling results in earlier commitment to endocrine cell lineages and earlier loss of endocrine progenitor cells. Before E12.5, the majority of endocrine cells formed are α and PP cells, and a wave of β and δ cell generation occurs after E13 (Pictet and Rutter, 1972; Murtaugh and Melton, 2003). In PRKO mice, α and PP cell differentiation was enhanced at E11.5, but β and δ cell differentiation was not enhanced. It was recently reported that Ngn3 protein transduction to E11.5 pancreatic cells resulted in α cell differentiation, but the transduction to E15 cells resulted in β cell differentiation (Dominguez-Bendala et al., 2005). In agreement with that report, our findings suggest that E11 proendocrine cells may lack some factor that contributes to β or δ cell differentiation.

Tubular structures with CK^+ DBA^+ cells dominated in the pancreas of the PRKO mouse at later embryonic stages (Figure 2). Thus, residual $Pdx1^+$ epithelial cells that have not undergone endocrine cell differentiation have a tendency to differentiate into ductal cells. A study demonstrated that genes that participate in the Notch pathway are upregulated in the metaplastic ductal epithelium of pancreatic premalignant legions (Miyamoto et al., 2003). Lineage-tracing studies show that ductal lineage is separated from $Pdx1^+$ $Ngn3^-$ common pancreatic progenitor cells between E9.5 and E12.5 (Gu et al., 2002); those are the times when the disruption of *Rbp-j* genes in PRKO mice occurs. These findings suggest that the appropriate downregulation of Notch signaling is necessary for pancreatic duct cell identity.

In the mutant mouse, the number of Pdx1-positive cells clearly decreased before E15 and the pancreas was small thereafter (Figures 2B′, 3A, and 3B). If the role of Notch signaling is simply to regulate cell fate, hypoplasia of certain types of cell should be accompanied by hyperplasia of other types of cell. For instance, in the determination of T and B lymphocytes, loss of function of Notch1 resulted in blockade of T cell development and enhancement of B cell production, while overexpression of Notch1 resulted in blockade of B cell lymphopoiesis and the generation of T cells (Pui et al., 1999; Wilson et al., 2001). The small pancreas and altered pancreatic cell composition in our mutant mouse suggest that defective Notch signaling allows premature differentiation of α, PP, and duct cells at the expense of later differentiating β, δ, and acinar cells. This mode of regulation is reminiscent of neuronal differentiation, in which Notch/Rbp-j signaling acts as a gatekeeper between self-renewal and commitment (Ishibashi et al., 1995).

In PRKO mice, though inadequate, the differentiation and growth of acinar cells occurred after E15. Persistent Notch ICD expression in pancreatic epithelium has been shown to inhibit acinar cell differentiation (Hald et al., 2003; Esni et al., 2004), and generalized Hes1 KO mice showed increased acinar cell growth (Jensen et al., 2000a). These results also suggest that Notch signaling inhibits acinar cell differentiation and proliferation during later embryonic stages.

The role of Notch signaling in terminally differentiated cells is unknown, although it was speculated that Notch might confer some degree of plasticity on postmitotic neurons (Ahmad et al., 1995). We detected the expression of Notch2 and Dll1 in endocrine cells of adult mice (data not shown). Furthermore, $Ngn3^+$ endocrine progenitor cells were shown to reside within the pancreatic islets (Gu et al., 2002); however, we found no difference between βRKO mice and control mice (Figure 4). It was reported that the β cells in adult islets are mainly formed by self-duplication of preexisting β cells and that the forced expression of Notch1 ICD in the adult pancreas does not perturb mature endocrine cells (Murtaugh et al., 2003; Dor et al., 2004). Together with the results of PRKO mice, Notch signaling may be indispensable only during the early developmental stages of the pancreas.

Our data show that Rbp-j is a key molecule in the propagation of pancreatic progenitor cells and is essential for proper differentiation into mature pancreatic cells.

Experimental procedures

Generation of pancreas- or β cell-specific Rbp-j KO mice

The generations of mice bearing a floxed allele of *Rbp-j* have been described previously (Han et al., 2002). *Pdx.cre* mice in which Cre recombinase is under the transcriptional control of the mouse Pdx1 promoter were gifts from Dr. Douglas A. Melton (Gu et al., 2002). *Rip.cre* mice in which Cre recombinase is under the control of the rat insulin II promoter were purchased from the Jackson Laboratory. Mice homozygous for the floxed *Rbp-j* allele (F/F) were crossed with *Pdx.cre* or *Rip.cre* transgenic mice. The resultant double-heterozygous mice were then crossed with $Rbp\text{-}j^{f/+}$ mice, resulting in $Rbp\text{-}j^{f/f}$ *Pdx.cre* (PRKO) or $Rbp\text{-}j^{f/f}$ *Rip.cre* (βRKO) mice and their control littermates. Genotyping and assessment of deletion efficiency were performed by Southern blot analyses on genomic DNA obtained from tails or other tissues.

Histological analyses

Whole embryos or excised pancreas were fixed with 4% paraformaldehyde in PBS for overnight at 4ºC then paraffin embedded, and 5 μm sections were cut and mounted on glass slides. Slides were dewaxed, rehydrated, and, in some instances, subjected to antigen retrieval by autoclaving at 121ºC for five minutes with 10 mM citrate buffer. Endogenous peroxidase was inactivated with 0.3% H_2O_2 in methanol for 30 min. The slides were blocked for 1 hr with a reagent containing casein (DAKO Protein Block Serum-Free Solution; Dako), then stained overnight with the following primary antibodies (Abs): rabbit anti-Pdx1 (Guz et al., 1995), rabbit anti-Hes1 (Jensen et al., 2000a), rabbit anti-Ngn3 (Schwitzgebel et al., 2000), guinea pig anti-Insulin (Dako), rabbit anti-Glucagon (Dako), rabbit anti-Somatostatin (Dako), rabbit anti-PP (Dako), rabbit anti-Ghrelin (Kojima et al., 1999), rabbit anti-pHH3 (Cell Signaling Technology), rabbit anti-pan-CK (Santa Cruz), rabbit anti-Amylase (Sigma-Aldrich), rabbit anti-Glut2 (Thorens et al., 1992), mouse anti-ISL1 (Developmental Studies Hybridoma Bank). The slides were washed with PBS the following day and incubated for 2 hr with the following secondary antibodies: biotinylated goat anti-guinea pig IgG; biotinylated goat anti-rabbit IgG, and biotinylated rabbit anti-goat IgG (all from Vector). The slides were then incubated with avidin-biotin complex (ABC) reagent (Vectastain Elite ABC Kit; Vector) for 50 min followed by the addition of diaminobenzidine tetrahydrochloride (DAB) (Dako) as a substrate-chromogen solution. After hematoxylin counterstaining and dehydration, slides were mounted in mounting medium (MGK-S; Matsunami) and pictures were taken using an Axioskop Microscope (Carl Zeiss). Morphometric analyses of pancreas were carried out using the Scion Image analysis program (Scion). The number of islets was calculated, with the definition of an islet as a group of endocrine cells containing at least five visible nuclei. The endocrine cell mass was calculated as the ratio of each hormone-positive cell area to the total area of the pancreas section. An In situ Apoptosis Detection Kit (Takara) was used for TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) assays, and tissue taken from the involuting mammary gland of a post-lactating female Wistar rat was used as a positive control for apoptosis. Counterstaining of these sections was performed with methyl green. For E15 embryos, in situ hybridization of *Hes1* was carried out using digoxigenin-labeled cRNA probes according to the reported protocol (Tomita et al., 2000).

Analysis of metabolic parameters

Blood glucose values were determined from whole venous blood taken from mouse tails using an automatic glucometer (Glutest Ace, Sanwa Kagaku) or an enzyme colorimetric assay kit (Glucose CII test, Wako). Blood for insulin and amylase was taken by retroorbital bleeds. Plasma insulin levels were measured using an ELISA kit (Morinaga). For glucagon, blood samples were collected into tubes containing EDTA (1 mg/ml blood) and aprotinin (500 KIE/ml blood). For measurements of pancreatic insulin contents, pancreas were quickly dissected, weighed, and frozen in liquid nitrogen. Insulin was extracted by mechanical homogenization in iced acid ethanol. After 24 hr at 4ºC, samples were centrifuged, and the supernatant was collected and stored at −20ºC. Insulin concentrations were determined by ELISA. Amylase activity was measured according to the Caraway method using a kit (Amylase-Test Wako, Wako). All values are expressed as mean ± standard error.

Supplemental data

Supplemental Data include six figures, Supplemental Results, and Supplemental Experimental Procedures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/3/1/59/DC1/.

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