Beta cell specific pyruvate dehydrogenase alpha gene deletion results in a reduced islet number and β-cell mass postnatally

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Abstract

The ability of pancreatic β-cells to undertake glucose-stimulated insulin secretion (GSIS) depends on the generation of adenosine triphosphate (ATP) within the mitochondria from pyruvate, a major rate-limiting enzyme being pyruvate dehydrogenase (PDH) complex (PDC). However, glucose metabolism also controls β-cell mass. To examine the role of PDC in the regulation of pancreatic β-cell development and maturation, we generated β-cell-targeted PDHα subunit knock-out male mice (β-PDHKO) and compared these with control males (β-PDHCT) from birth until 6–8 weeks age. Pancreas morphology, transcription factor expression, pancreatic insulin content, and circulating glucose and insulin values were compared. Compared to β-PDHCT male mice, β-PDHKO animals had significantly reduced pancreatic insulin content from birth, a lower serum insulin content from day 15, and relative hyperglycemia from day 30. Isolated islets from β-PDHKO mice demonstrated a reduced GSIS. The number of islets per pancreatic area, mean islet area, and the proportion of islet cells that were β-cells were all reduced in β-PDHKO animals. Similarly the number of insulin-immunopositive, extra-islet small endocrine cell clusters, a possible source of β-cell progenitors, was lower in β-PDHKO mice. Analysis of pancreatic expression of transcription factors responsible for β-cell lineage commitment, proliferation, and maturation, *Pdx1, Neurogenin3*, and *NeuroD1* showed that mRNA abundance was reduced in the β-PDHKO. This demonstrates that PDC is not only required for insulin expression and glucose-stimulated secretion, but also directly influences β-cell growth and maturity, and positions glucose metabolism as a direct regulator of β-cell mass and plasticity.

Keywords: Pyruvate dehydrogenase complex deficiency, insulin secretion, β-cell mass, β-cell maturity, *Pdx1* expression

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Introduction

Glucose-stimulated insulin secretion (GSIS) is essential for regulation of cellular glucose metabolism and maintenance of whole body glucose homeostasis. Glucose derived from dietary carbohydrates primarily regulates insulin secretion by pancreatic β-cells, and insulin in turn regulates glucose uptake and metabolism by peripheral tissues to maintain glucose homeostasis. Enhanced glucose metabolism by β-cells after a mixed meal results in an increase in the cytosolic ATP concentration and increased cellular adenosine diphosphate (ATP/ADP) ratio which causes sequentially the following events in β-cells: closure of K_{ATP} channels, depolarization of the plasma membrane, opening of voltage-sensitive L-type Ca^{2+} channels, influx of Ca^{2+}, and insulin secretion.[1,2] During this process mitochondrial metabolism plays a critical role in providing metabolites generated primarily from glucose-derived pyruvate. Two central enzymes involved in pyruvate metabolism in the mitochondria are the pyruvate dehydrogenase (PDH) complex (PDC), and pyruvate carboxylase, forming acetyl-CoA and oxaloacetate, respectively. Citrate formed from these two compounds and metabolites generated from its further metabolism in the mitochondria (ATP, guanosine triphosphate (GTP), glutamate, etc.) and in the cytosol (malonyl-CoA, nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), etc.) serve as mediators of GSIS.[3–5] The role of pyruvate carboxylase as an anaplerotic enzyme has been extensively documented in insulin secretion by β-cells. Although the contribution of the acetyl moiety from acetyl-CoA is equally important in generating these mediators of insulin secretion, the role PDC plays has not been investigated extensively.[6,7] We recently described a murine model of β-cell-specific PDC deficiency to demonstrate the role of PDC in GSIS in both *in vitro* and *in vivo*. The results from this study clearly demonstrated development of hypoinsulinemia, hyperglycemia, and impairment in GSIS both *in vitro* and *in vivo* in β-PDHKO male mice.[8]

Glucose is not only a major nutrient regulator of insulin secretion but also impacts on gene expression in β-cells.[9,10] Glucose metabolism by β-cells increases not only the binding of three important transcription factors, namely pancreatic and duodenal homeobox gene 1 (Pdx1), musculoaponeurotic fibrosarcoma oncogene homolog A (MafA), and B2/E47[11,12] but also promotes their interactions to enhance preproinsulin gene transcription.[10,12] Glucose also promotes the translocation of PDX1 from the cytosol to the nucleus.[13,14] Additionally, glucose increases stability of preproinsulin mRNA.[15] The above observations clearly indicate the important role of glucose metabolism in preproinsulin gene transcription and related processes, but it is not known how impairment in glucose oxidation by β-cells might affect expression of the preproinsulin and *Pdx1* genes in β-PDHKO male mice.

A number of knock-out (KO) mouse models have allowed investigation of the importance of the insulin signaling pathway for the maintenance of islet mass and glucose homeostasis. For example, β-IRKO mice (IR:insulin receptor) manifest a type 2 diabetes phenotype showing impaired GSIS by islets and progressive glucose intolerance.[16,17] Although β-IRKO mice did not show developmental defects in islets, they did show impairment in the ability to increase β-cell mass with increasing age. Insulin receptor substrate 1 knock-out (IRS1KO) islets (IRS1:IR substrate 1) exhibited impairment in GSIS and a reduction in islet insulin content.[18] β-cell-specific knockout IRS2 (β-IRS2KO) mice had reduced β-cell mass and β-cell proliferation at age 8 weeks, but not earlier at 10 days age.[19,20] Similarly, β-PdkKO (Pdk:3-phosphoinositide-dependent protein kinase 1) mice showed a reduction in both islet number and the number and size of β-cells and developed progressive hyperglycemia.[21] Interestingly, the effect of glucose on pancreatic cell development was documented using an *in vitro* model of rat embryonic pancreas cultured on a filter at the air–liquid interface.[22] These investigators demonstrated that glucose is crucial for both α- and β-cell development in islets by regulating the transition between Neurogenin 3 (*Neurog3*) and Neurogenic differentiation (*NeuroD*). It is of interest to determine possible alterations in gene expression of *Neurog3, NeuroD*, and *Pdx1* in islets during development in the β-PDHKO mice in which glucose metabolism is compromised due to PDC deficiency. It is possible that impairment in preproinsulin and *Pdx1* gene expression, and possibly in energy homeostasis, in β-cells may impact on β-cell development. In the present study, we have investigated gene expression in β-cells and islet architecture during development in a β-PDHKO mouse model. The results show significant changes in β-cell number, transcription factor expression, and insulin expression and content in postnatal life.

Materials and methods

Generation of pancreatic β-cell-specific PDH-deficient mice

Animal protocols were approved by the Institutional Animal Care and Use Committee of the State University of New York at Buffalo in accordance with the Guide for the Use and Care of Laboratory Animals. Generation of a mouse line carrying two loxP sites inserted into introns surrounding exon 8 of the X-linked pyruvate dehydrogenase alpha1 gene ($Pdha1^{flox8}$) were reported previously.[23] This mouse colony had a 129J genetic background. In our previous study we bred females (genetic background 129J, $Pdha1^{flox8}/Pdha1^{flox8}$) with Cre^{ins} transgenic males (genetic background C57BL/6J $Pdha1^{wt}$/Y, Cre^{ins}) from The Jackson Laboratory (Bar Harbor, ME).[8] Hence the progeny of this breeding had a mixed genetic background. To overcome the possible contribution of this mixed genotype on the metabolic phenotype of the β-PDHKO male progeny reported previously,[8] floxed females (genotype 129J, $Pdha1^{flox8}/Pdha1^{flox8}$) were back-crossed for 10 generations with wild-type males (B6 genetic background). The progeny of the last breeding were intra-bred to derive a floxed colony with B6 genetic background. In the present study, to generate pancreatic β-cell-specific deficient (β-PDHKO) male mice, the floxed females from this B6-transferred colony were bred with the transgenic males [B6.Cg-Tg(Ins2-cre) 25Mgn/J] harboring a *Cre* transgene driven by the rat insulin gene II promoter. To generate control mice (β-PDHCT), wild-type B6 females ($Pdha1^{wt}/Pdha1^{wt}$) were bred with transgenic Cre^{ins} B6 males. Only Cre^{+} males of this breeding were used as control.

Tail DNA for initial genotyping as well as DNA isolated from other tissues (islets, liver, heart, and skeletal muscle) was genotyped as per manufacturer's protocol (Omniprep™ I, Genotechnology Inc., St. Louis, MO). Genomic DNA was amplified using a *Pdha1* and *Cre* primers mentioned in supplement Table 1. The presence of three *Pdha1* alleles ($Pdha1^{wt}$, $Pdha1^{flox8}$, and $Pdha1^{\Delta ex8}$) and *Cre* transgene was determined by polymerase chain reaction (PCR) analysis.[23,24]

Determination of pancreatic insulin content

Insulin levels in serum and pancreatic tissue were assayed using a commercially available radioimmunoassay kit (Millipore, Billerica, MA) according to the manufacturer's instruction. One-day-, 15-day-, and 60-day-old mice were anaesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and decapitated. The trunk blood was collected from 15-day- and 60-day-old mice and centrifuged to obtain serum and stored at −20°C until used for insulin assay. Pancreatic tissues were weighed, cut into pieces, and the known quantity of tissue was homogenized in acid–ethanol solution (126 mM ethanol, 0.005 N HCl),[25] centrifuged, and the supernatants were stored at −20°C until assayed for insulin. Whole pancreas from 1-day-old male mice (sex determined by genotyping) was processed as described earlier and the insulin content was expressed as the quantity of insulin per whole pancreas.

Islets isolation and studies of insulin secretory response

Pancreatic islets were isolated from 60-day-old mice using collagenase digestion as described.[26] Briefly, mice were anaesthetized with ketamine/xylazine (100 mg/kg/10 mg/kg) and killed by decapitation and pancreas were

carefully dissected, trimmed, and digested with collagenase (Crescent Chemical Co., Islandia, NY) in a 37°C water bath with vigorous agitation for 10–12 min. Tissue digest was allowed to sediment, washed, and islets were manually collected under a stereomicroscope. In order to study the GSIS response, islets were suspended in Krebs–Ringer bicarbonate buffer (16 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 5.5 mM glucose, 0.01% bovine serum albumin, pH 7.4) and preincubated for 30 min at 37°C in a shaking water bath in the presence of 95% O_2 and 5% CO_2. Islets were resuspended in fresh Krebs–Ringer bicarbonate buffer containing 5.5 or 16.7 mM glucose and an aliquot was withdrawn at 60 min for determination of secreted insulin.[8] The results are expressed as nanogram insulin secreted/five islets/60 min.

Quantitative real-time PCR and gene expression

The transcript levels of preproinsulin, *Pdx1, Ngn3*, and *NeuroD1* on postnatal days 1, 15, and 60 were determined using iCycler real-time PCR (Bio-Rad, Hercules, CA). Total RNA was extracted from mouse pancreas using Trizol reagent (Invitrogen, Carlsbad, CA). One microgram of RNA was used for reverse transcription using iScript cDNA kit (Bio-Rad, Hercules, CA) according to the manufacturer's instruction. For real-time PCR, 1 μg of total RNA was converted into cDNA in 20 μL. The cDNA was then diluted fivefold and 2 μL was used in a 25 μL reaction mixture that included SYBR Green PCR master mix (Bio-Rad, Hercules, CA). Primers for real-time PCR were designed using Primer 3 input (http://frodo.wi.mit.edu/primer3/). To avoid the possible contamination by genomic DNA, primers were designed to bridge the exon–intron boundaries within the gene of interest. The sequences of the primers used for assays are presented in supplement Table 1. The quantitative PCRs were run on the Bio-Rad iCycler for SYBR Green according to the manufacturer's recommendations. The resulting PCR cycle time (Ct) values were collected by using the software provided for the iCycler, and the data were then analyzed in Microsoft EXCEL to determine ΔCt (test Ct–18S Ct). The reverse transcription reactions were performed in triplicate with tissue RNA from eight animals and the results were normalized to the 18S mRNA levels by using the 2ΔΔCT method[27] and reported as percent change in β-PDHKO mice compared to age-matched controls as 100%.

Immunohistochemistry

Pancreata from 1-day and 6–8-week-old mice were fixed in 10% formalin and embedded in paraffin. Sections (5 μm) were cut and mounted on SuperFrost® Plus glass slides (Fischer Scientific, Toronto, ON, Canada). Dual staining immunohistochemistry was performed for insulin and glucagon to facilitate measurement of the percent islet contribution of β- and α-cells, respectively, as described previously.[28] Tissues were blocked using Background Sniper (Biocare Medical, Concord, CA). Glucagon was localized using a mouse monoclonal antibody (1:2000; Sigma-Aldrich Inc., St. Louis, MO) with a horse anti-mouse secondary antibody (1:100; Vector Laboratories, Burlingame, CA) and visualized with 3,3′-diaminobenzidine (DAB) (Biogenex, Fremont, CA). Insulin was localized using a guinea pig polyclonal antibody (1:300; Abcam, Cambridge, MA) with a goat anti-guinea pig secondary antibody (1:100; Vector), followed by Vectastain ABC-AP kit and visualized using Vector Red (both from Vector). To establish the specificity of the antibodies, the primary antibodies and secondary antibodies were substituted by antibody diluting solution. Immunostaining was analyzed by Northern Eclipse software (version 7.0; Empix Imaging, Mississauga, ON, Canada). Total pancreas area for each section and area of insulin and glucagon immunoreactivity for each islet were measured. β- and α-cell mass were calculated by dividing the total area positive for each hormone by the total area of the pancreas section and multiplying by the weight of the pancreas. Islets were counted and sized into groups as follows to determine the islet size distribution: extra-islet β-cell clusters ($<500\,\mu m^2$), small ($500–5000\,\mu m^2$), medium ($5000–10{,}000\,\mu m^2$), and large ($>10{,}000\,\mu m^2$).

Statistical analysis

For the histomorphological analysis of islets, nine β-PDHCT and 12 β-PDHKO mice were analyzed at age 1 and 14 days, and 13 and 10, respectively, at 6–8 weeks. Data are presented as mean ± standard error of mean. Differences between mean values were compared statistically using analysis of variance for genome and age followed by a Bonferroni's posttest. Differences were considered statistically significant at $p < 0.05$.

Results

Generation of β-cell-specific PDH-deficient (β-PDHKO) male progeny

We have used Cre-loxP gene targeting to inactivate the *Pdha*1 gene selectively in the β-cells of mouse pancreas to investigate its effects on insulin secretion and function. Two loxP sites inserted into introns of X-linked $Pdha1^{flox8}$ are catalyzed by *Cre* recombinase to generate a null mutation in which exon 8 is deleted. In the previous study, we reported initial characterization of β-cell-specific PDC deficiency in β-PDHKO male mice with a mixed genetic background (a progeny of 129J floxed females with C57BL/6J Cre transgenic males). Furthermore, we compared the PDHKO male progeny with the PDHCT control males which were generated by breeding 129J floxed females with males with B6 genetic background only (and devoid of the presence of *Cre* allele). To avoid possible effects of a mixed genetic background on the expressed phenotype of PDHKO male mice and also their comparison with the PDHCT control male mice (with an absence of Cre allele), we reinvestigated in the present study the phenotype of PDHKO male mice under B6 genotype only, and also compared these with the PDHCT male mice with the same genotype and carrying the *Cre* allele. Many of the phenotypic parameters in the present re-derived mouse line are similar to those reported earlier,[8] and hence pertinent differences in the phenotypes between the two PDHKO mouse

strains with different genotypes are identified below. Additional characterization of gene expression and morphometric analyses of islets in PDHKO male mice are also presented.

In the present study, PDC deficiency in β-cells was induced by mating homozygous female mice (B6 genetic background) carrying $Pdha1^{flox8}/Pdha1^{flox8}$ alleles with transgenic homozygous male mice (B6 genetic background) harboring a *Cre* recombinase transgene driven by rat insulin gene II promoter (genotype: $Pdha1^{wt}$/Y; Cre^{ins}). No embryonic lethality was noticed with this breeding and the litter size was found to be normal (PDHCT: 7.3 ± 0.37; PDHKO: 8.2 ± 0.61; n = 8–10; ns). Genotype analysis showed that β-PDHCT mouse had a 700-bp band for wild-type allele in islets (I), liver (L), heart (H), and skeletal muscle (SM) tissues (supplement Figure 1, upper panel). The lower panel in supplement Figure 1 shows the 240-bp *Cre* band in the corresponding tissues. A representative β-PDHKO mouse showed the presence of an 800-bp band from the $Pdha1^{flox8}$ in all tissues analyzed (islets, liver, heart, and skeletal muscles) (supplement Figure 1, upper panel). The lower panel indicated the presence of the *Cre* gene in all tissues from β-PDHKO mouse. In isolated islets there was a 400-bp band from the $Pdha1^{\Delta ex8}$ in addition to the 800-bp band from the $Pdha1^{flox8}$, indicating the β-cell-specific KO of *Pdha1* gene. A faint 800-bp band from $Pdha1^{flox8}$ and a stronger 400-bp band for $Pdha1^{\Delta ex8}$ indicated that majority of the cell population in the islets tissue were β-cells with Cre-mediated excision. However, a small population of other cells showed the presence of an 800-bp band from the undeleted $Pdha1^{flox8}$.

Blood glucose levels in β-PDHKO and β-PDHCT male mice fed *ad libitum*

Tail blood glucose levels were measured in *ad libitum* fed β-PDHKO and β-PDHCT male mice at different ages in order to determine the effect of PDC deficiency in β-cells on plasma glucose levels during postnatal life. On postnatal day 15 there was no significant difference in blood glucose levels between β-PDHKO and control (β-PDHCT) male mice. However, blood glucose levels were increased significantly in β-PDHKO male mice (mean value ranging from 207 to 224 mg/dL) on postnatal days 30, 45, and 60 as compared to age-matched controls (mean value ranging from 158 to 171 mg/dL) (Figure 1).

Serum insulin levels in β-PDHKO male mice

Serum insulin levels were measured in β-PDHKO male mice and age-matched β-PDHCT animals on postnatal days 15 and 60 in order to determine the effect of PDC deficiency. Serum insulin levels in β-PDHKO mice were significantly decreased on postnatal days 15 and 60 as compared to age-matched β-PDHCT mice. On postnatal day 15 the mean level of insulin was 31 pM in β-PDHKO mice compared to 51 pM in the control mice (40% reduction) (Figure 2(a)). On postnatal day 60, the mean serum insulin level was 103 pM in β-PDHKO compared to 130 pM in β-PDHCT control mice (21% reduction) (Figure 2(b)).

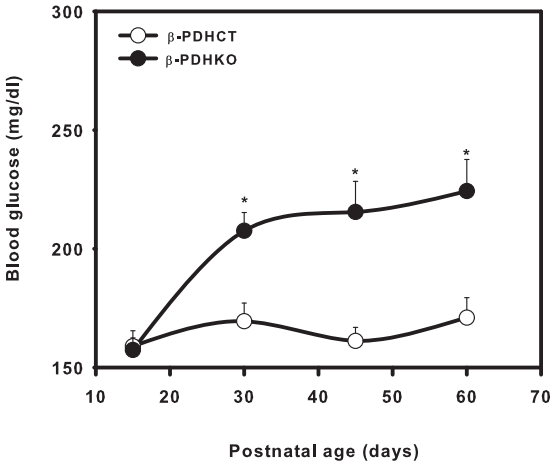

Figure 1 Measurement of tail blood glucose levels in β-PDHCT and β-PDHKO male mice at different ages during the postnatal life as indicated. The results are means ± SEM (n = 21–44). *P < 0.05

Pancreatic insulin content and secretion in β-PDHKO male mice

To determine whether PDC deficiency in β-PDHKO male mice affected the insulin content in pancreatic β-cells, this was measured on postnatal days 1, 15, and 60. Pancreatic insulin content was reduced in β-PDHKO mice as compared to β-PDHCT control animals (Figure 2(c) to (e)). Importantly, the insulin content was reduced by about 30% even in β-PDHKO newborn male pups as compared to age-matched control β-PDHCT mice (Figure 2(c)). On postnatal days 15 and 60, the insulin content was markedly reduced by 55 and 64%, respectively, in β-PDHKO mice as compared to age-matched controls (Figure 2(d) and (e)). To understand the physiological basis of hyperglycemia due to PDC deficiency, GSIS from isolated islets from 60-day-old β-PDHKO mice or control mice was measured in the presence of either 5.5 or 16.7 mM glucose for 60 min (Figure 3). In islets from β-PDHKO mice, insulin secretion was reduced by 41 and 50%, respectively, in the presence of 5.5 and 16.7 mM glucose, as compared to islets from age-matched control mice. The results indicated that β-cells responded poorly to a glucose stimulus for the secretion of insulin when associated with a β-cell-specific PDC deficiency in β-PDHKO mice.

In summary, in agreement with our previously published β-PDHKO model,[8] β-PDHKO mice in the present study became relatively hyperglycemic with age, exhibited reduced circulating insulin levels, a reduced pancreatic insulin content, and impaired GSIS. We therefore subsequently examined whether this was related to an altered islet morphology and expression of transcription factors controlling β-cell development and/or function. Unlike to our previous model,[8] β-PDHKO mice of homogeneous genetic background in the present study demonstrated a substantially altered islet morphology and transcription factor expression as described below.

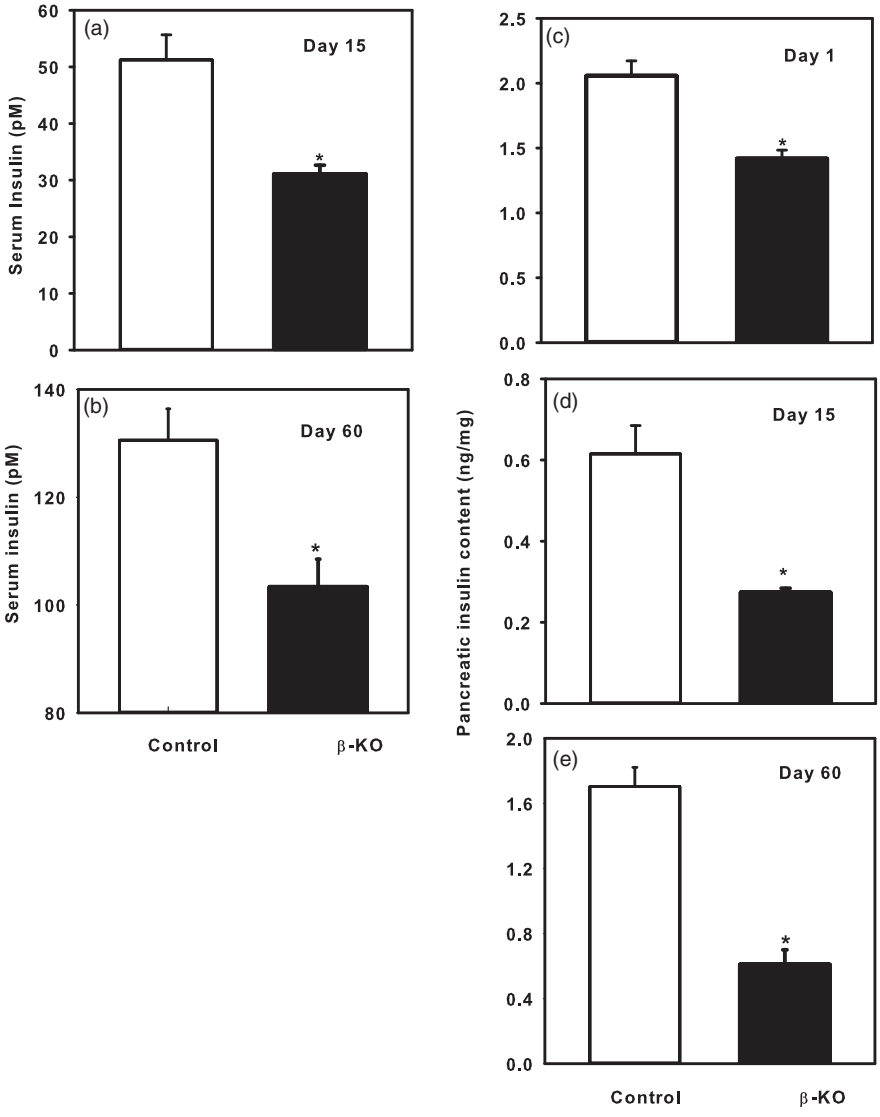

Figure 2 Serum insulin levels and pancreatic insulin contents in β-PDHCT (control) and β-PDHKO (β-KO) male mice. (a and b): Serum insulin levels on postnatal days 15 and 60. (c, d, and e): Pancreatic insulin content on postnatal days 1, 15, and 60. Results are means ± SEM (n as indicated): for serum insulin: day 15 (n = 9–10), day 60 (n = 14–20); for pancreatic insulin content: day 1 (n = 8–11), day 15 (n = 9–11), day 60 (n = 5–9). *P < 0.05

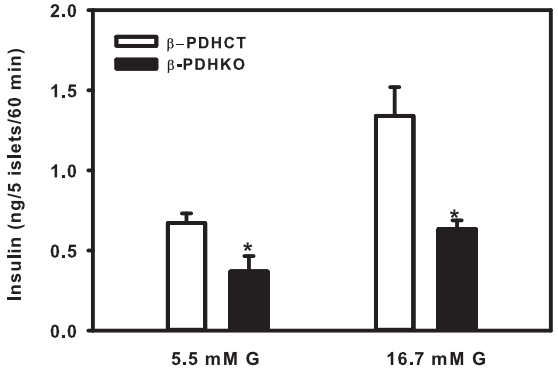

Figure 3 Effects of β-cell-specific PDC deficiency on insulin secretion by pancreatic islets. Pancreatic islets were isolated from 2-month-old β-PDHCT and β-PDHKO male mice. Glucose-stimulated insulin secretion in the presence of 5.5 or 16.7 mM glucose (G) for 60 min is shown. Results are means ± SEM (n = 5–8). *P < 0.05

Expression of preproinsulin, Pdx1, Ngn3, and NeuroD1 in β-PDHKO mice

Transcription factors such as pancreatic *Pdx1, Ngn3,* and *NeuroD1* play an important role in β-cell differentiation, development, and maturation. Preproinsulin gene transcription is regulated by the coordinated interactions of these and several other transcription factors.[29] Transcription of preproinsulin is required to maintain normal levels of preproinsulin mRNA levels for insulin biosynthesis. In order to determine whether the expression levels of preproinsulin, *Pdx1, Ngn3,* and *NeuroD1* are affected by PDC deficiency in pancreatic islets of β-PDHKO mice, we quantified the mRNA levels of these genes in the pancreas on postnatal days 1, 15, and 60 using quantitative real-time PCR (Figure 4). No change in the expression level of preproinsulin between the two groups of mice was observed on postnatal day 1

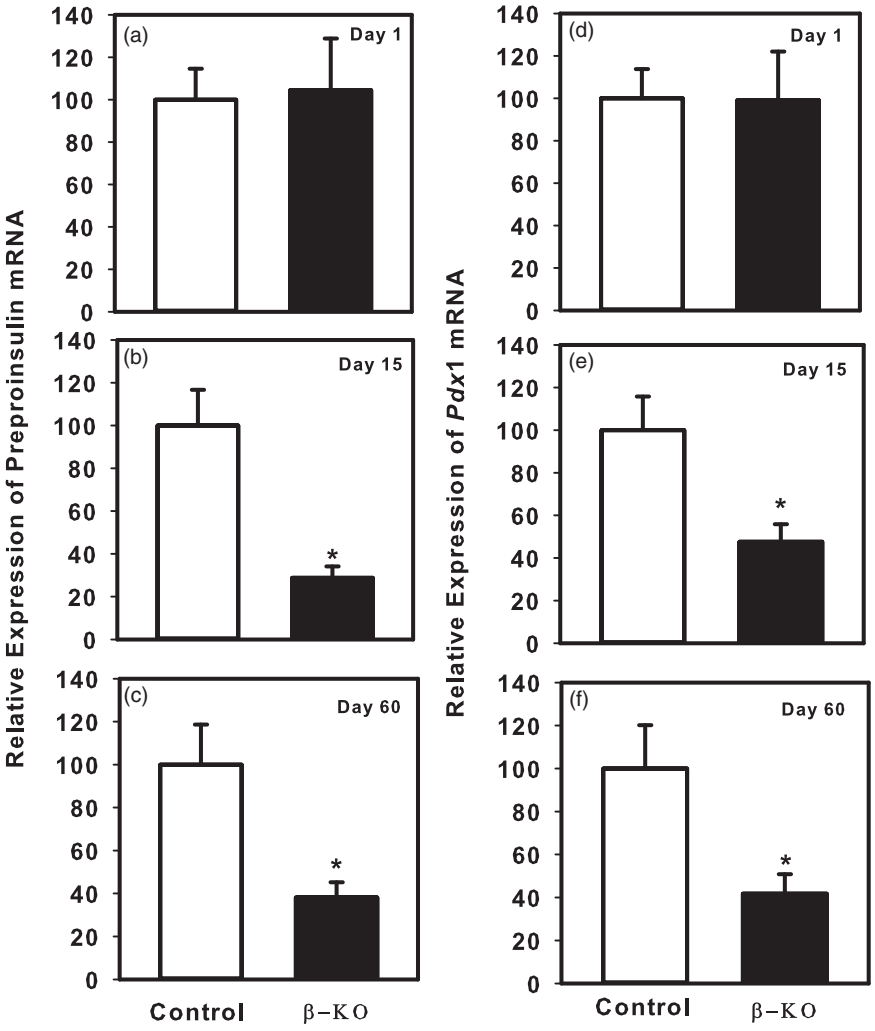

Figure 4 Quantitative real-time PCR analysis of preproinsulin gene (a, b, c) and *Pdx1* gene (d, e, f) on postnatal days 1, 15, and 60, respectively. Total RNA was extracted from the pancreas of β-PDHCT (control) and β-PDHKO (β-KO) male mice, reversed transcribed, and was used to quantify the expression level of the gene. Expression levels were normalized using 18S RNA as an internal standard and fold difference was calculated using comparative ΔΔCT method. Individual samples were run in triplicate and data are means ± SEM (n = 8). *$P < 0.05$

(Figure 4(a)). However, on postnatal days 15 and 60, the mRNA levels of preproinsulin in β-PDHKO mice were reduced by 71 and 58%, respectively, as compared to control mice (Figure 4(b) and (c)). A similar pattern of gene expression was also observed for *Pdx1* on postnatal days 1, 15, and 60 (Figure 4). No significant difference was observed in the *Pdx1* expression between the two groups of mice on postnatal day 1 (Figure 4(d)), but *Pdx1* mRNA levels were reduced by 62 and 52%, respectively, in β-PDHKO mice on postnatal days 15 and 60 as compared to age-matched β-PDHCT control animals (Figure 4(e) and (f)). mRNA levels of *Ngn3* and *NeuroD1* did not show any significant difference between β-PDHKO and control animals on postnatal day 1 (Figure 5(a) and (c)). However, the mRNA levels of *Ngn3* and *NeuroD1* on postnatal day 15 were reduced by 34 and 53%, respectively, in β-PDHKO mice as compared to the age-matched controls (Figure 5(b) and (d)).

Changes in pancreatic islet morphology and endocrine cell mass in β-PDHKO mice

The mean islet size was significantly reduced in the pancreata from β-PDHKO mice at 1 day of age compared with β-PDHCT control animals, but not so at 6–8 weeks (Figure 6(a)). However, analysis of the number of islets per area of pancreas showed this to be unchanged at day 1, but significantly reduced in β-PDHKO mice at 6–8 weeks (Figure 6(b)). The islet density had also decreased substantially with age regardless of genome. When the cellular composition of islets was analyzed there were no differences in the mean percentage area of islet occupied by β-cells or in the β-cell mass between groups on day 1, but both were significantly reduced in β-PDHKO mice after 6–8 weeks, with a 60% reduction in β-cell mass (Figure 6(c) and (d)). The mean β-cell mass had increased approximately 18-fold in control animals between 1 day and 6–8 weeks of age. No corresponding, significant changes in the mean percentage area of islet occupied by α-cells or in the

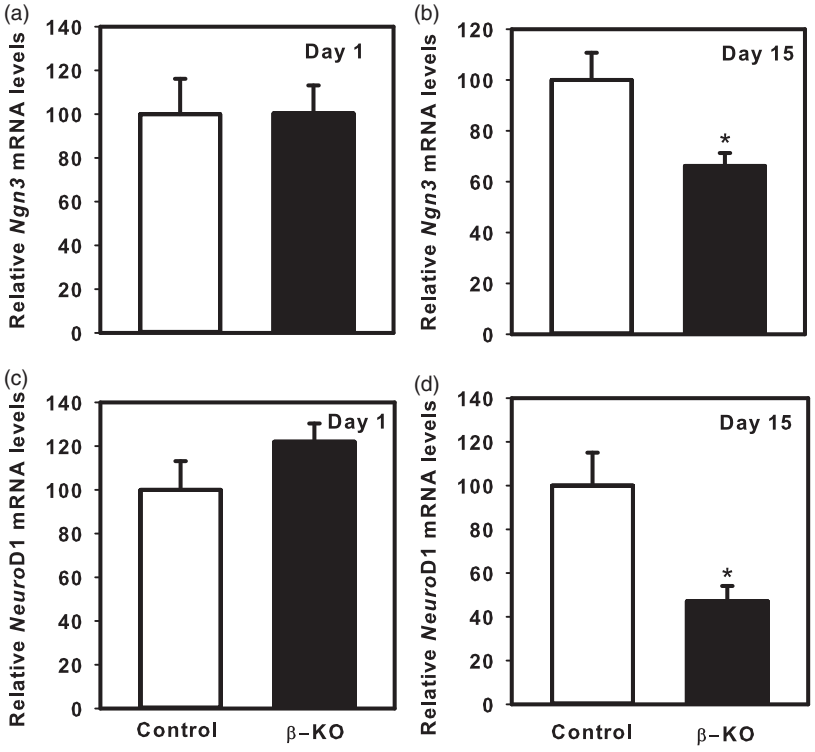

Figure 5 Gene expression of *Ngn3* (a, b) and *NeuroD1* (c, d) in the pancreas of 1-day and 15-day-old β-PDHCT (control) and β-PDHKO (β-KO) male mice. Total RNA was used to quantify the mRNA expression levels of the genes. Expression levels were normalized using 18S RNA as an internal standard and fold difference was calculated using ΔΔCT method. Individual samples were run in triplicate and data are means ± SEM (n = 8). *$P < 0.05$

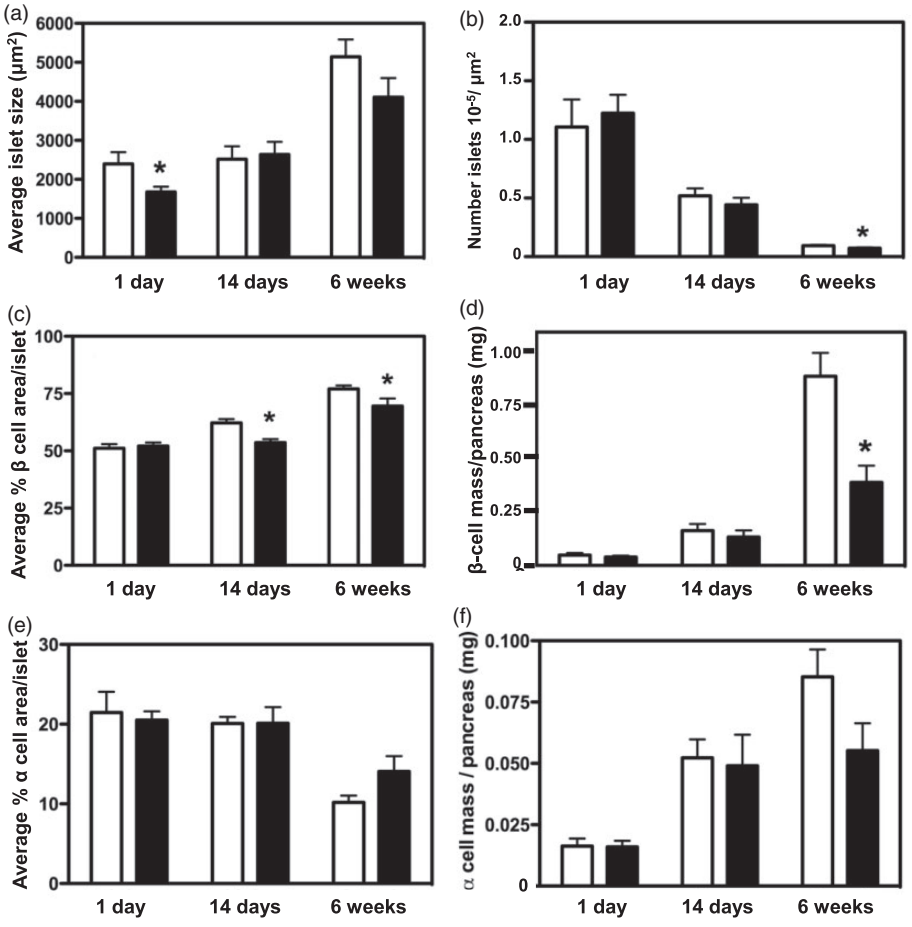

Figure 6 Average islet area (a), number of islets per area of pancreas (b), average percent β-cell area per islet (c), β-cell mass (d), average percent α-cell area per islet (e), and α-cell mass (f) in pancreata from β-PDHKO (closed bars) or PDHCT control mice (open bars) at age 1 day, 14 days, and 6–8 weeks. Results are means ± SEM (n = 9–13) *$P < 0.05$

Table 1 The number of small, medium, and large sized islets per μm^2 pancreatic tissue at 1 day or 6–8 weeks of age for β-PDHKO (PDHKO) and β-PDHCT (Control) mice

	Day 1		6–8 weeks	
Islet size	**Control**	**PDHKO**	**Control**	**PDHKO**
Small islets (500–5000 μm^2)	$9.66 \times 10^{-6} \pm 2.06 \times 10^{-6}$	$1.17 \times 10^{-5} \pm 1.55 \times 10^{-6}$	$7.00 \times 10^{-7} \pm 4.06 \times 10^{-8}$	$5.50 \times 10^{-7} \pm 5.32 \times 10^{-8}$*
Medium islets (5000–10,000 μm^2)	$1.17 \times 10^{-6} \pm 4.43 \times 10^{-7}$	$4.66 \times 10^{-7} \pm 1.38 \times 10^{-7}$	$1.24 \times 10^{-7} \pm 1.90 \times 10^{-8}$	$1.02 \times 10^{-7} \pm 2.66 \times 10^{-8}$*
Large islets (>10,000 μm^2)	$2.31 \times 10^{-7} \pm 1.20 \times 10^{-7}$	$5.76 \times 10^{-8} \pm 5.26 \times 10^{-8}$	$1.28 \times 10^{-7} \pm 1.67 \times 10^{-8}$	$6.59 \times 10^{-8} \pm 1.47 \times 10^{-8}$*

Results are means ± SEM (n = 9–13). *p < 0.05 vs. control.

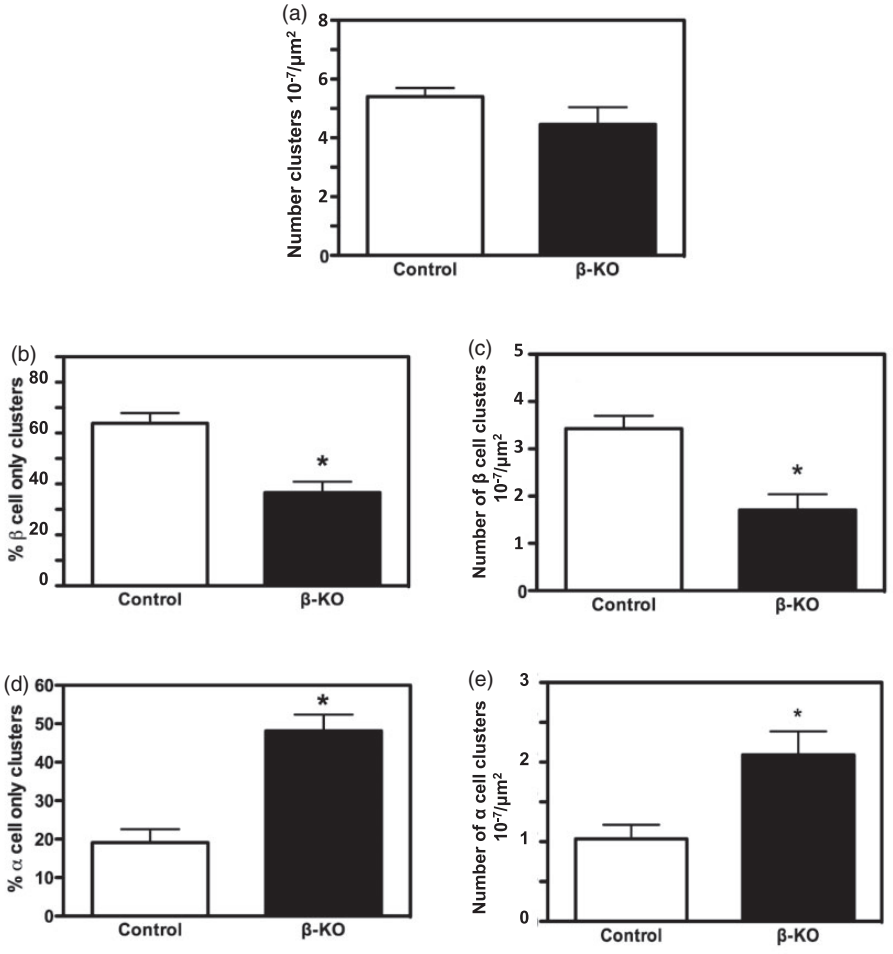

Figure 7 Number of extra-islet endocrine cell clusters per area of pancreas at 6–8 weeks age for β-PDHKO (β-KO) and β-PDHCT (Control) mice (a). The percent of clusters that contained only β-cells is shown in (b) and the number of β-cell only clusters per area of pancreas in (c). The percent of clusters that contained only α-cells is shown in (d) and the number of α-cell only clusters per area of pancreas in (e). Results are means ± SEM (n = 9–13) *$P < 0.01$

α-cell mass were observed, indicating that the changes in β-cell presence observed in β-PDHKO mice were not generalized to all endocrine cells (Figure 6(e) and (f)).

Analysis of the mean islet size distribution showed that the relative numbers of small, medium, and large islets per area of pancreas were each significantly reduced in β-PDHKO mice at 6–8 weeks of age, but not so at day 1 (Table 1). When combined with the reduced β-cell mass this would suggest that there is a relative failure both to generate new β-cells within islets and for these β-cells to proliferate to form larger islets prior to 6 weeks of age in β-PDHKO mice. We were particularly interested in the morphology of small extra-islet endocrine cell clusters which may develop into true islets as the mice age. The number of such clusters per area of pancreas declined between 1 day and 6–8 weeks of age (not shown), but the total number of clusters did not differ between β-PDHKO and β-PDHCT mice (Figure 7(a)). The abundance of clusters containing only β-cells or only α-cells did not differ between β-PDHKO and β-PDHCT mice at birth, but by 6–8 weeks the β-PDHKO animals contained significantly less β-cell-only clusters and more α-cell-only clusters (Figure 7(b) to (e)).

This suggests that following birth progenitor islet endocrine cells in β-PDHKO animals may be less effective at entering a β-cell lineage and more likely to form α-cells.

Discussion

Following the uptake of glucose by β-cells, facilitated by the glucose-carbon transporter 2, and the generation of glucose 6-phosphate, catalyzed by glucokinase, glucose can enter three separate pathways. The major glycolytic pathway results in the generation of pyruvate and the conversion of much of this to acetyl-CoA driven by the mitochondrial multienzyme PDC.[30] This results in a generation of ATP facilitating glucose-sensitive insulin release. A second pathway results in the hexosamine biosynthetic pathway,[31] and a third the pentose phosphate pathway. The latter generates NADPH which can then act as a cofactor to facilitate glucose-stimulated insulin release.[32] GSIS depends on generation of several metabolites (ATP, GTP, glutamate, malonyl-CoA, NADPH, etc.) in the mitochondria or the cytosol via the initial products (acetyl-CoA and oxaloacetate) generated by pyruvate metabolism via PDC and pyruvate carboxylase in the mitochondria. Citrate formation from acetyl-CoA and oxaloacetate in the mitochondria and its further metabolism both in the mitochondria and the cytosol result in the production of the metabolites in support of GSIS. It should be emphasized that acetyl-CoA generated from pyruvate by action of PDC is an equal partner in generating metabolites required for GSIS. Our results clearly demonstrate that impairment in pyruvate metabolism at the level of PDC in the β-cell reduces its capacity to secrete insulin in response to a glucose load.

As we reported previously, a β-cell-specific KO of PDC activity expression by approximately 50% resulted in significantly decreased circulating insulin levels and pancreatic insulin content from birth, and relative hyperglycemia in males from postnatal day 15.[8] In the present study, the development hyperglycemia was first seen at the age of 30 days. This could be due to different genotypes of PDHKO male mice between the present study and our previous report.[8] Alternatively, during the suckling period, pups received maternal milk which represents a high fat/low carbohydrate diet and are dependent on gluconeogenesis to maintain blood glucose homeostasis. Upon weaning, mice received a high carbohydrate chow diet where the major energy source was glucose. Under this dietary condition, hyperglycemia would be expected to appear by day 30. Glucose-stimulated insulin release from isolated islets was impaired by day 60, but was not measured at earlier ages, and the overall metabolic dysfunctional phenotype became more pronounced with advancing age. It was surprising that while insulin levels and glucose-stimulated release were impaired in this previous model, the only morphological change seen in the islets was a relative hypertrophy of individual β-cells.[8] However, that model utilized β-PDHKO male mice of a mixed genetic background. In the present study, the β-cell-specific PDHKO mice were backcrossed on to a common (B6) genetic background resulting in a uniform genetic background for development of a phenotype in β-PDHKO mice. In addition to the expected deficits in pancreatic insulin content, basal and glucose-stimulated insulin release, these animals now demonstrate a reduction in mean islet number, β-cell mass, and a lower expression of key transcription factors needed for β-cell generation and maturation such as *Pdx1, Neurogen3*, and *NeuroD1*.

The β-PDHKO mice demonstrated a transient relative reduction in mean islet size on the day of birth, but not thereafter up to 6–8 weeks of age, compared to β-PDHCT animals. However, the number of islets, regardless of relative size was reduced postnatally in β-PDHKO animals, with a smaller proportion of each islet being occupied by β-cells. While expression of *Pdx1, Neurog3*, and *NeuroD1* in pancreas was unaltered on the day of birth, all three mRNA species declined in abundance at postnatal age 15 compared to control animals. This suggests that the β-PDHKO mice experienced a relative failure to generate new β-cells postnatally within islets, and that the neogenesis of islets was likely to have been impaired from before birth. The absence of major disruption to islet morphology in β-PDHKO at birth is consistent with the relative immaturity of most β-cells in the rodent pancreas at this time, robust glucose-sensitive insulin release not being developed until the time of weaning.[33] PDC is, however, expressed in several tissues from late gestation.[34]

During development of the pancreas *in utero*, new islets are generated from progenitor endocrine cells located behind the tips of the pancreatic ductal tree within the pancreatic ductal epithelium.[35] However, postnatally the pancreatic ducts cease to be a source of new islets within the rodent pancreas, and new β-cells arise for the expansion of β-cell mass by proliferation of existing cells within the islets or by the differentiation of resident islet endocrine progenitors.[36,37] The insulin promoter is functional only in β-cells and hence the expression of *Cre* is initiated only in differentiated β-cells. Consequently, the impairment of PDC activity is not initiated until β-cells are differentiated. It is possible that the development of the pancreatic endocrine progenitor cells in PDHKO fetuses may not be altered, as observed in 1-day-old PDHKO male mice (Figure 6). Whilst *Pdx1* is essential for β-cell neogenesis prior to birth, it is not essential for the generation of new β-cells postnatally, although it is important for their functional maturation and for maintaining a β-cell fate, which will determine the percent of β-cells within an islet.[9] One possible source of new β-cells postnatally are the numerous extra-islet endocrine clusters, which can contain either entirely insulin-positive or glucagon-positive cells.[38] In the immediate postnatal period, there is a rapid development of the endocrine pancreas involving both neogenesis and proliferation of islets and it is possible that the latter process is affected by impairment in glucose oxidation in affected β-cells. This possibility is supported by decreased levels of gene expression as well as the number of β-cells in PDHKO male mice. The insulin-positive cells contained within such clusters are largely immature and lack expression of the glucose transporter 2.[39] However, clusters can give rise to mature islets, at least in young animals. β-PDHKO mice showed a decreased abundance of insulin-positive, but an

increase in glucagon-positive clusters in comparison to control animals, suggesting that the pool of β-cell progenitors, as well as the lineage fate of endocrine cells, is influenced directly by glucose metabolism through PDC action. The smaller relative islet size and reduced proportional content of β-cells in β-PDHKO mice are consistent with the reduced expression of *Pdx1* and other transcription factors, and demonstrates that PDC action is not only required for insulin expression and glucose-stimulated release, but also directly influences β-cell growth and maturity.

The importance of PDC action for islet and β-cell growth and maturation is consistent with other experimental approaches that have linked elements of the glucose metabolic pathways in rodents to β-cell plasticity. A targeted deletion of the glucokinase gene in β-cells of mice not only abolished GSIS but also prevented β-cell proliferation and was associated with a reduced β-cell survival.[40] Similarly hormonal inhibition of the pentose phosphate pathway in rat islets with dehydroepiandrosterone reduced insulin secretion.[41] The pentose phosphate pathway generates NADPH. An intermediate pentose sugar, xylulose 5-phosphate activates type 2A protein phosphatase, which subsequently activates through dephosphorylation of the transcription factor carbohydrate responsive element binding protein (ChREBP).[42] ChREBP is expressed within the pancreatic endocrine cell lineage, and disruption of its action through the use of specific inhibitors or a knockdown of gene expression resulted in a decrease in the differentiation of β-cells.[43] FoxO1 is an important transcription factor for the maintenance of β-cell mass, and its targeted ablation in β-cells resulted in a β-cell dedifferentiation.[44] Peroxisome proliferator activated receptor (PPARγ) and its target genes are downstream effectors of FoxO1 action in β-cells.[45] FoxO1 and ChREBP were shown to compete in the inhibition of thioredoxin-interacting protein expression, a factor that promotes β-cell apoptosis.[46] The hexosamine biosynthetic pathway of glucose metabolism has also been demonstrated to mediate changes in β-cell proliferation and differentiation status.[47]

Thus, it appears that disruption of glucose metabolism within each of the major biosynthetic pathways within β-cells, including the impact of PDC through the glycolytic pathway as demonstrated by us in the present study, results in altered β-cell generation, mass, and functional status, reinforcing the hypothesis advanced by Porat *et al*.[40] that glucose is a direct and major regulator of β-cell mass and plasticity, in addition to the regulation of insulin biosynthesis and release.

Author contributions: MSP and DJH designed the experiments and prepared the initial draft. MS and SM performed animal studies and measurements of the biochemical and molecular biological parameters. BS performed immunohistochemistry analyses. All authors read the final draft and approved its content.

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