Prolactin Receptor Is Required for Normal Glucose Homeostasis and Modulation of β-Cell Mass during Pregnancy

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Increased islet mass is an adaptive mechanism that occurs to combat insulin resistance during pregnancy. Prolactin (PRL) can enhance β-cell proliferation and insulin secretion *in vitro*, yet whether it is PRL or other pregnancy-related factors that mediate these adaptive changes during pregnancy is unknown. The objective of this study was to determine whether prolactin receptor (Prlr) is required for normal maternal glucose homeostasis during pregnancy. An ip glucose tolerance test was performed on timed-pregnant $Prlr^{+/+}$ and heterozygous null $Prlr^{+/-}$ mice on d 0, 15, and 18 of pregnancy. Compared with $Prlr^{+/+}$ mice, $Prlr^{+/-}$ mice had impaired glucose clearance, decreased glucose-stimulated insulin release, higher nonfasted blood glucose, and lower insulin levels during but not before pregnancy. There was no difference in their insulin tolerance. $Prlr^{+/+}$ mice show a significant incremental increase in islet density and β-cell number and mass throughout pregnancy, which was attenuated in the $Prlr^{+/-}$ mice. $Prlr^{+/+}$ mice also had a more robust β-cell proliferation rate during pregnancy, whereas there was no difference in apoptosis rate between the $Prlr^{+/+}$ and $Prlr^{+/-}$ mice before, during, or after pregnancy. Interestingly, genotype of the mothers had a significant impact on the offspring's phenotype, such that daughters derived from $Prlr^{+/-}$ mothers had a more severe phenotype than those derived from $Prlr^{+/+}$ mothers. In conclusion, this is the first *in vivo* demonstration that the action of pregnancy hormones, acting through Prlr, is required for normal maternal glucose tolerance during pregnancy by increasing β-cell mass. **(*Endocrinology* 150: 1618–1626, 2009)**

Until recently, it was believed that in adults, β-cells are terminally differentiated and had little or no proliferative capacity. However, examination of pancreas from patients with longstanding type 1 diabetes has uncovered evidence of β-cell apoptosis and persistent inflammation decades after the onset of diabetes, a time point where no evidence of β-cell or active inflammation is expected. This suggests an ongoing supply of β-cells in adult pancreas and that β-cell proliferation and perhaps neogenesis exists in the adult human pancreas (1).

Pregnancy provides a unique model to study mechanisms that regulate islet mass and β-cell function because significant increases in islet mass and insulin secretion occur in the mother during pregnancy, a process that quickly reverses at parturition (2, 3). In animal studies, pregnancy-induced β-cell adaptation consists of 1) a lowered threshold for glucose-stimulated insulin secretion, 2) increased insulin biosynthesis, and 3) an increase in β-cell proliferation (4–8).

In rodents, several lines of evidence suggest that prolactin (PRL) and/or placental lactogens (PLs) are responsible for the pregnancy-associated changes in β-cell mass and function. First, during pregnancy, the rise in PRL and PL levels parallels the increases in β-cell mass and glucose-stimulated insulin hypersecretion (9). PRL receptor (Prlr), the receptor for both PRL and PLs is present on insulin-secreting cell lines (10) and pancreatic β-cells (11), and expression increases during pregnancy (12). *In vitro* exposure of isolated islets to PRL/PLs increases insulin secretion and β-cell proliferation and number (10, 13) and lowers the threshold of glucose-stimulated insulin secretion (14), mim-

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Abbreviations: AUC, Area under the curve; BrdU, bromodeoxyuridine; G15, gestation d 15; IPGTT, ip glucose tolerance test; ITT, insulin tolerance test; P5, d 5 postpartum; PL, placental lactogen; PRL, prolactin; Prlr, PRL receptor; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

icking the effects of pregnancy on β-cells. Overexpression of PL in β-cells leads to fasting and postprandial hypoglycemia, inappropriately elevated serum insulin concentrations, increased islet volume and number, increased islet insulin content, and increased β-cell replication (15). Conversely, deletion of Prlr in mice leads to decreased β-cell mass, islet density, and β-cell fraction as well as a 20–35% reduction in pancreatic insulin content in the nonpregnant state. The Prlr-null ($Prlr^{-/-}$) mice also have blunted insulin secretory response and impaired glucose tolerance in comparison with their wild-type littermates (16). Taken together, these studies strongly support the idea that PRL and PL can enhance β-cell proliferation and function, and are the prime candidates as the mediators of β-cell adaptation to pregnancy.

Several key questions remained unanswered on the mechanism of β-cell expansion during pregnancy. First, is the action of PRL/PLs through Prlr required for the β-cell mass expansion during pregnancy? With the availability of Prlr-null mice, we can directly examine glucose homeostasis of these mice. Second, if PRL/PLs are responsible for β-cell expansion during pregnancy, what is the mechanism? Although the increase in β-cell number during pregnancy has been well described (9), the relative contributions of proliferation of existing islets *vs.* neogenesis and/or reduced apoptosis are not known. Our objective was to use the pregnancy milieu to understand mechanisms that lead to enhanced β-cell mass and function. Prlr heterozygous ($Prlr^{+/-}$) female mice were chosen for this study because $Prlr^{-/-}$ females cannot carry a pregnancy beyond midgestation and therefore are not suitable for studying islet changes associated with pregnancy. In a previous study using $Prlr^{+/-}$ mice, it was found that in comparison with $Prlr^{+/+}$ mice, the pregnant $Prlr^{+/-}$ mice had decreased neurogenesis during pregnancy (17), leading us to surmise that these mice are likely to have an abnormal β-cell phenotype. We show here that $Prlr^{+/-}$ mice have impaired glucose tolerance during pregnancy but not during the nonpregnant state. Furthermore, the increase in β-cell mass associated with pregnancy is attenuated in the $Prlr^{+/-}$ mice, with a decrease in cell proliferation as the responsible mechanism.

Materials and Methods

Mice

Heterozygous Prlr-null mice ($Prlr^{+/-}$) on a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME), and working mouse stock was generated by crossing $Prlr^{+/-}$ with wild-type C57BL/6 ($Prlr^{+/+}$) mice. The pups were genotyped as previously described (18). $Prlr^{+/-}$ mice derived from $Prlr^{+/-}$ mothers (crossed with wild-type fathers) are designated $Prlr^{+/-(+/-)}$, whereas $Prlr^{+/-}$ mice derived from $Prlr^{+/+}$ mothers (crossed with heterozygous mutant fathers) were designated $Prlr^{+/-(+/+)}$. Mice were maintained on 12-h light, 12-h dark cycle with liberal access to food and water. Mice were studied at 3–4 months of age. All experimental procedures were approved by the Animal Use Review Committee at the University of Calgary in accordance with standards of the Canadian Council on Animal Care.

Intraperitoneal glucose tolerance (IPGTT) and insulin tolerance test (ITT)

For the IPGTT, mice were fasted 14–16 h overnight and injected with 2 g/kg body weight of glucose (20% D-glucose solution) ip, followed by blood sampling from tail vein at times 0, 10, 20, 30, 45, 60, and 120 min after the injection. Blood glucose was determined using a glucometer (FastTake). Glucose excursion, measured as area under the curve (AUC) across these time points, was determined in nonpregnant and pregnant mice on gestation d 15 (G15) and G18. Additional blood samples (30 μl) were obtained at times 0 and 30 min for measurement of serum insulin, using an ELISA kit (Linco Research, St. Charles, MO; EZRMI-13K). For the ITT, mice were fasted for 4 h and given an ip injection of 0.75 U/kg insulin (Novolin R). Blood glucose levels were determined at times 0, 15, 30, and 60 min after the injection and expressed as a fraction of the blood glucose at time 0.

Immunohistochemistry

Pancreas was isolated from mice and fixed with 4% paraformaldehyde/PBS solution at 4 C overnight, embedded in paraffin blocks, and longitudinally sectioned to 7 μm. Every 40th tissue section was stained for insulin to identify β-cells. Therefore, there is at least 280 μm distance between stained sections, minimizing the possibility of sampling the same islet twice. Antigen retrieval was achieved by incubating tissue sections with 1 mg/ml trypsin (Sigma Chemical Co., St. Louis, MO) at 37 C for 20 min. After 1 h blocking with 1% goat serum/PBS at room temperature, tissues were incubated with primary antibody (guinea pig antiinsulin at 1:750 from Dako, Carpinteria, CA; rabbit or guinea pig antiglucagon at 1:500 from Linco; all diluted in 1% goat serum/PBS) overnight at 4 C. This was followed by fluorophore-conjugated secondary antibodies (Cy3-antiguinea pig from Jackson ImmunoResearch Laboratories, West Grove, PA; Alexa-488 goat antirabbit from Molecular Probes, Eugene, OR; both diluted in 1% goat serum/PBS at 1:300) for 1 h at room temperature. Bisbenzimide H 33342 trihydrochloride (1:10,000; Sigma) was added to the secondary antibody for nuclear staining. Sections were mounted using DakoCytomation fluorescent mounting medium.

Proliferation assay

Pregnant mice were injected with bromodeoxyuridine (BrdU) (100 mg/kg body weight) 4 h before pancreas isolation. Paraffin-embedded pancreatic tissue was processed as above. To detect BrdU incorporation into the DNA, antigen retrieval was achieved by treatment with 0.2% Triton X-100 for 5 min at room temperature, microwave in 0.01 M sodium citrate (pH 6) for 10 min, incubation in 1.5 N HCl at 37 C for 20 min, and treatment with 0.25% trypsin (Invitrogen, Carlsbad, CA) at 37 C for 5 min. After blocking in 5% BSA for 1 h at room temperature, tissues were incubated with anti-BrdU antibody (mouse, 1:500; Sigma) and antiinsulin antibody (guinea pig, 1:750; Dako) overnight at 4 C, followed by incubation with fluorophore-conjugated secondary antibodies (Cy3-antiguinea pig from Jackson ImmunoResearch; Alexa-488 goat antimouse from Molecular Probesl both diluted in 1% goat serum/PBS at 1:300) for 1 h at room temperature. Bisbenzimide H 33342 trihydrochloride (1:10,000; Sigma) was added to the secondary antibody for nuclear staining. The percentage of insulin-positive β-cells that is actively incorporating BrdU into their DNA on G15 was counted for all four genotypes. At least 12,000 cells per genotype were counted. The percentage of BrdU-positive β-cells on d 15 of pregnancy was compared between the wild-type and mutant mice by *t* tests.

Apoptosis assay

Pancreas isolated from $Prlr^{+/+}$ and $Prlr^{+/-}$ mice on G0 and G18 and d 5 postpartum (P5) were processed to detect the presence of apoptotic β-cells by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining (DeadEnd Colorimetric TUNEL System; Promega, Madison, WI). Paraffin-embedded mice pancreatic sections were labeled by biotinylated dUTP using the recombinant terminal deoxynucleotidyl transferase. The percentage of β-cells undergoing apoptosis on G0, G18, and P5 between the $Prlr^{+/+}$ and $Prlr^{+/-}$ mice was compared by *t* test.

Islet isolation

Pancreatic islets were isolated from nonfasted wild-type and $Prlr^{+/-}$ adult pregnant female mice. Pancreas was distended using collagenase V (1 mg/ml, 2.5 ml/pancreas; Sigma), surgically removed and then incubated in collagenase (2 ml/pancreas) at 37 C for 15 min under agitation. The digested pancreas was passed through a 500-μm filter and then separated from exocrine tissue by centrifugation on a dextran (1.100/1.085/1.075/1.045 g/ml) gradient. Islets were picked off the 1.085/1.075 and 1.075/1.045 g/ml interface, washed with HBSS (supplemented with 0.25% fraction V BSA) (Invitrogen), and then lysed for protein determination in RIPA buffer.

Protein expression analysis

Isolated islets were lysed in RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS in PBS plus protease inhibitors and phosphatase inhibitors) (200 islets/30 μl), sonicated three times for 20 sec, followed by protein concentration determination using the Bradford methods. Protein (30 μg) was separated by SDS-PAGE and transferred onto polyvinylidene difluoride filters, blocked in 3% BSA for 1 h in room temperature, and then incubation with primary antibody (mouse anti-Prlr antibody, U5, at 1:500 from Affinity BioReagents, Golden, CO; goat anti-actin antibody at 1:5000 from Santa Cruz Biotechnology, Santa Cruz, CA) at 4 C overnight, followed by a 1-h incubation with horseradish peroxidase-conjugated secondary antibody (sheep antimouse antibody at 1:10,000 from Amersham, Piscataway, NJ; donkey antigoat at 1:5000 from Santa Cruz). Protein was visualized by the enhanced chemiluminescence method and scanned within the linear range using ImageJ software.

Islet morphometry

For each pancreas section, consecutive images of adjacent nonoverlapping areas of the entire pancreas section were acquired using a Leica fluorescence microscope and captured with a CoolSnap digital camera (19). Images were analyzed by ImageJ software to measure the insulin-positive area as well as the area of the entire pancreas section. β-Cell fraction was calculated as a ratio of insulin-positive cell area to total pancreatic tissue area on the entire section (16). The number of very small (<30 μm in diameter or fewer than five cells), small (30–50 μm in diameter or fewer than 15 cells), medium (50–100 and 101–150 μm in diameter), and large (151–200 and >200 μm in diameter) islets as well as insulin-positive single/doublet β-cells were quantified and expressed per square millimeter of pancreas area. Results represent the average of four to five tissue sections per animal from four to six animals from each genotype.

Results

Prlr-deficient female mice have impaired glucose tolerance during pregnancy

To determine whether PRL/PL are required to maintain normal maternal glucose homeostasis during pregnancy, we performed IPGTT in both wild-type and heterozygous Prlr-null ($Prlr^{+/-}$) female mice on G0, G15, and G18. Days 15 and 18 of pregnancy represent the time points when peak and declining β-cell proliferation rates, respectively, were previously observed (9). It is important to note that there is no difference in body weight between the wild-type and the $Prlr^{+/-}$ mutant mice, and both genotypes had similar fertility rates and produced a similar number of live pups (data not shown). The reduction in Prlr protein expression in the islets of $Prlr^{+/-}$ mice was confirmed by Western immunoblot analysis (supplemental Fig. 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). IPGTT was performed after overnight fast. Glucose excursion, measured as integrated area under the curve (AUC) across the time points, was comparable between the nonpregnant wild-type and mutant mice (Fig. 1A). During pregnancy, glucose tolerance was impaired in the $Prlr^{+/-}$ mice in comparison with the $Prlr^{+/+}$ mice (Fig. 1, B–D). On d 15 of gestation, the AUC of the $Prlr^{+/-}$ mice was 1946 ± 134 mM · min (n = 14) compared with 1535 ± 99 mM · min in $Prlr^{+/+}$ mice (n = 13; $P = 0.02$). By d 18 of gestation, the AUC of the $Prlr^{+/-}$ mice was 1552 ± 56.5 mM · min (n = 11), whereas that of the $Prlr^{+/+}$ mice was 1271 ± 91 mM · min (n = 7; $P = 0.01$) (Fig. 1B). In addition to their impaired ability to clear a glucose load, the mutant mice had higher nonfasted serum glucose than the wild-type mice during pregnancy ($Prlr^{+/-}$: 9.30 ± 0.30 mM, n = 26; $Prlr^{+/+}$: 8.19 ± 0.26 mM, n = 27; $P = 0.01$) (Fig. 2A).

The genotype of the mother influences glucose metabolism during pregnancy in her daughters

The glucose tolerance test results were somewhat variable. Because $Prlr^{+/-}$ mice could have been derived from either a $Prlr^{+/+}$ or a $Prlr^{+/-}$ mother, we decided to determine whether the genotype of the mother had any effect on glucose homeostasis in her daughters. In virgin mice (G0), there is no difference in IPGTT between the four genotypes [AUC: $Prlr^{+/+(+/+)}$ = 1115.83 ± 152.05 mM · min; $Prlr^{+/+(+/-)}$ = 1040.58 ± 62.41 mM · min; $Prlr^{+/-(+/+)}$ = 1179.6 ± 160.58 mM · min; and $Prlr^{+/-(+/-)}$ = 1115.83 ± 152.05 mM · min; n = 6–7]. During pregnancy, however, the difference in glucose tolerance became more pronounced when the genotype of the mothers of the experimental mice was considered. For example, when we compared glucose tolerance tests of pregnant $Prlr^{+/+}$ mice derived from a $Prlr^{+/+}$ mother [$Prlr^{+/+(+/+)}$] to that of the pregnant $Prlr^{+/-}$ mice derived from a $Prlr^{+/-}$ mother [$Prlr^{+/-(+/-)}$], we found a 46% increase in glucose excursion in the mutant mice (Fig. 1C) and higher serum glucose throughout the IPGTT (Fig. 1D). $Prlr^{+/-(+/+)}$ and $Prlr^{+/+(+/-)}$ females had intermediate levels. Similar to the IPGTT, the nonfasted blood glucose was lowest in $Prlr^{+/+}$ mice derived from a $Prlr^{+/+}$ mother [$Prlr^{+/+(+/+)}$] (7.81 ± 0.33 mM, n = 16), whereas the others had higher blood glucose levels [$Prlr^{+/+(+/-)}$: 8.76 ± 0.36 mM, n = 11; $Prlr^{+/-(+/+)}$: 9.06 ± 0.40 mM, n = 13; $Prlr^{+/-(+/-)}$: 9.19 ± 0.35 mM; n = 13] (Fig. 2A). Interestingly, the steady-state serum insulin levels were lowest in the pregnant $Prlr^{+/-(+/-)}$ mutant mice and highest in the wild-type $Prlr^{+/+(+/+)}$ mice, whereas the $Prlr^{+/+(+/-)}$ and $Prlr^{+/-(+/+)}$ mice had intermediate insulin levels (Fig. 2B). Furthermore, the pregnant $Prlr^{+/-(+/-)}$ mutant mice secreted less insulin during the first 30 min of an IPGTT (Fig. 2C).

Prlr-deficient female mice have normal insulin tolerance

To examine whether the difference in insulin sensitivities could account for the impaired glucose tolerance in the Prlr-deficient mice, we performed insulin tolerance tests. At time 0 of the ITT, all four genotypes had similar fasting blood glucose levels on both d 15 [$Prlr^{+/+(+/+)}$: 7.65 ± 0.5 mM; $Prlr^{+/-(+/+)}$: 7.96 ± 0.63 mM; $Prlr^{+/+(+/-)}$: 7.15 ± 0.52 mM; $Prlr^{+/-(+/-)}$: 7.23 ± 0.52 mM] and d 18 [$Prlr^{+/+(+/+)}$: 6.63 ± 0.72 mM; $Prlr^{+/-(+/+)}$: 6.13 ± 0.4 mM; $Prlr^{+/+(+/-)}$: 7.2 ± 0.44 mM; $Prlr^{+/-(+/-)}$: 7.15 ± 0.96 mM] of pregnancy. We found no difference in insulin tolerance, expressed as percentage drop in blood glucose, between the wild-

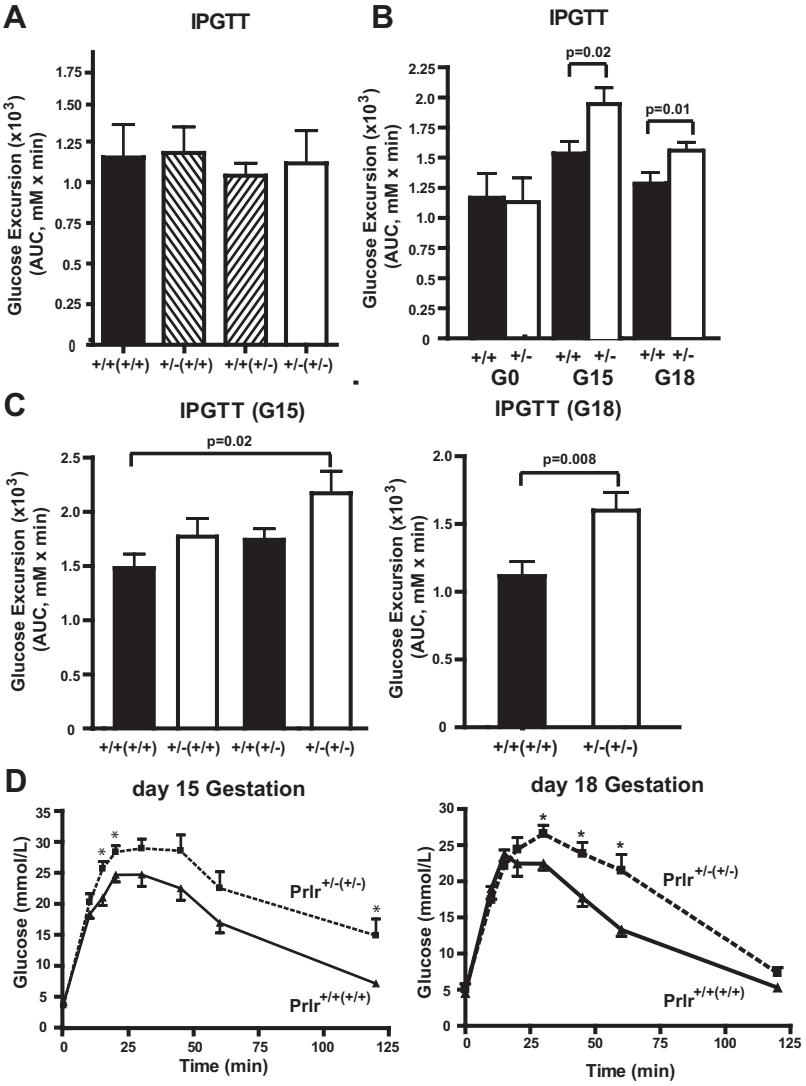

FIG. 1. Effects of heterozygous Prlr deletion on glucose homeostasis during pregnancy. A–C, IPGTT was performed on G0 (A), G15, and G18. Glucose excursions were measured as AUC (millimolar glucose × minutes) and expressed as mean ± SE; n = 7–14 mice from each group. Glucose excursion was compared between the $Prlr^{+/+}$ and the $Prlr^{+/-}$ mice with (A and C) and without (B) considering the genotype of the female parent that gave rise to the experimental pregnant mice. Note that genotype of the female parent that gave rise to the experimental pregnant mice is in brackets. D, Glucose excursion during IPGTT during pregnancy. n = 8–11. *, $P < 0.05$ in comparison with the wild-type mice at the corresponding time point. All experimental mice are 3–4 months old.

type and the Prlr-deficient mice at either G15 or G18 (Fig. 3), and there was no difference in insulin tolerance between the wild-type and the mutant mice before pregnancy (data not shown).

Prlr-deficient female mice have decreased islet mass

Next, we examined whether the impaired glucose tolerance of Prlr-deficient mice was due to a failure to increase β-cell mass during pregnancy. We compared islet density (number of islets/mm^2 of pancreatic tissue), β-cell fraction (the ratio of insulin-positive cells to total pancreas area), and islet mass (β-cell fraction × pancreas mass) between the wild-type and the mutant mice. An islet was considered to be a collection of cells that were positive for insulin and glucagon. Insulin-positive single cells, doublets, and triplets were common and were also counted, and they form part of the very small islet group, *i.e.* fewer than five cells and less than 30 μm in diameter. Glucagon-positive single cells were very rare, and glucagon-positive doublets were almost never observed (Fig. 4A).

In the wild-type mice, the islet density increased between d 0 and 15 of pregnancy, with no further increase between d 15 and 18 (Fig. 4B). When we compared the nonpregnant $Prlr^{+/+}$ and $Prlr^{+/-}$ mice, they had comparable islet density ($Prlr^{+/+}$: 2.36 ± 0.2 islets/mm^2 pancreas area; $Prlr^{+/-}$: 2.69 ± 0.39 islets/mm^2 pancreas area). During pregnancy, however, the $Prlr^{+/-}$ mice had lower islet density (Fig. 4C).

The β-cell fraction and islet mass increased between d 0 and 15 of pregnancy in the wild-type mice, with a more modest increase between d 15 and 18 (Fig. 4, D and E). There was no significant difference in pancreatic weight, β-cell fraction, or islet mass between the $Prlr^{+/+}$ and $Prlr^{+/-}$ mice before pregnancy. But during pregnancy, the $Prlr^{+/-}$ mice had a lower β-cell fraction and reduced islet mass in comparison with the wild-type mice (Fig. 4, D and E). Again, maternal genotype affected the phenotype of the pregnant offspring such that during pregnancy, $Prlr^{+/+(+/+)}$ mice had the highest (3.57 ± 0.45 mg) and $Prlr^{+/-(+/-)}$ mice had the lowest islet mass (1.87 ± 0.18 mg), whereas $Prlr^{+/-(+/+)}$ and $Prlr^{+/+(+/-)}$ mice had intermediate islet mass (2.24 ± 0.73 and 2.42 ± 0.57 mg, respectively).

The numbers of β- but not α-cells are decreased in Prlr-deficient pregnant mice

To determine whether PRL/PLs mediate the increase in β-cell number during pregnancy, we compared the number and size of β- and α-cells between the wild-type and mutant mice during pregnancy.

In the wild-type mice, there is a significant increase in β-cell numbers between d 0 and 15 of pregnancy, with more attenuated increase between d 15 and 18 (Fig. 5A). In contrast, there was only a modest 30% increase in α-cell numbers between d 0 and 15 (Fig. 5C). When we compared $Prlr^{+/+}$ to $Prlr^{+/-}$ mice, there was no difference in the number of β-cells per pancreas area before pregnancy, but during pregnancy, the mutant mice had fewer β-cells (Fig. 5A). Again, maternal genotype affects the daughter's phenotype such that pregnant mice derived from $Prlr^{+/-}$ mothers had reduced β-cell number in comparison with mice derived from $Prlr^{+/+}$ mothers [$Prlr^{+/-(+/-)}$: 59.46 ± 6.67; $Prlr^{+/+(+/-)}$: 78.7 ± 18.59; $Prlr^{+/-(+/+)}$: 70.01 ± 12.27; $Prlr^{+/+(+/+)}$: 88.32 ± 5.14 cells/mm^2 pancreas]. No significant difference in the number of α-cells was observed between the wild-type and the mutant mice during pregnancy (Fig. 5C). This is consistent with the absence of Prlr on α-cells (20). Interestingly, we did not observe a significant increase in cross-sectional area of each β-cell, *i.e.* β-cell size, from d 0–15 of pregnancy. As well, β-cell size was not different between the mutant and wild-type

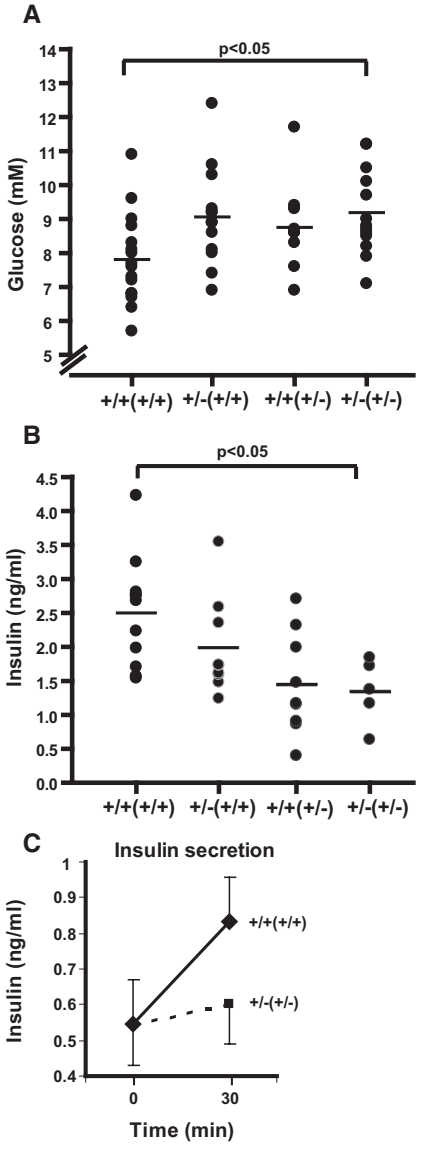

FIG. 2. Effects of heterozygous Prlr deletion on serum glucose and insulin levels during pregnancy. Serum glucose (millimolar) (A) and insulin (nanograms per milliliter) (B) were measured at 0800 h on nonfasted mice on d 15 of pregnancy. Results are expressed as mean ± SE; n = 10–12. All experimental mice are 3–4 months old. C, Serum insulin concentrations of $Prlr^{+/+(+/+)}$ and $Prlr^{+/-(+/-)}$ mice at times 0 and 30 min of the IPGTT on d 15 of pregnancy.

mice at d 15 [$Prlr^{+/-(+/-)}$: 153.5 ± 12.7 μm^2; $Prlr^{+/-(+/+)}$: 160.9 ± 23.7 μm^2; $Prlr^{+/+(+/-)}$: 147.0 ± 7.12 μm^2; $Prlr^{+/+(+/+)}$: 155.7 ± 18.97 μm^2]. By d 18 of pregnancy, however, β-cell size is significantly larger than that on d 0, and there is a trend toward larger β-cell cross-sectional area in the $Prlr^{+/+}$ than in the $Prlr^{+/-}$ mice (Fig. 5B). These results suggest that from d 0–15 of pregnancy, increase in β-cell number accounts for most of the increase in islet mass, whereas between d 15 and 18, cellular hypertrophy becomes a more significant contributor to β-cell mass.

β-Cell proliferation is blunted in Prlr-deficient pregnant mice

To understand the mechanisms that determine the change in β-cell mass during pregnancy, we measured β-cell proliferation and apoptosis rates.

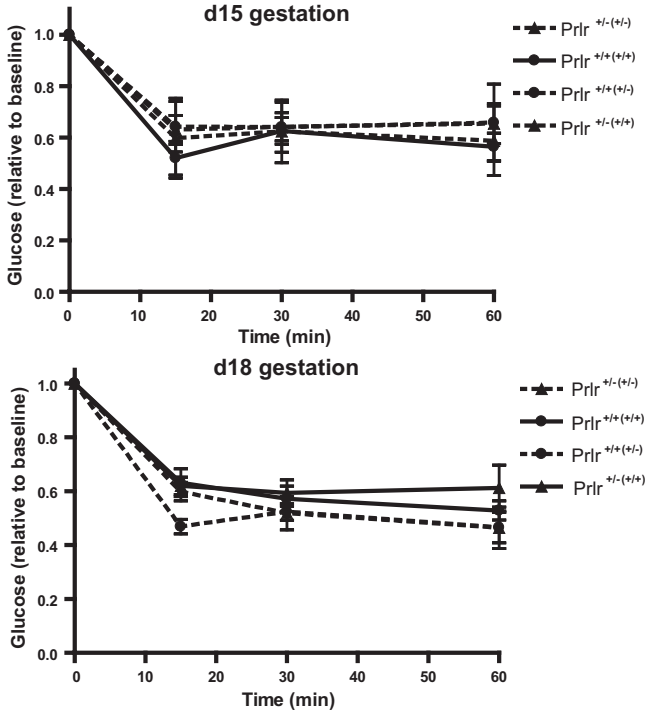

FIG. 3. Insulin tolerance of $Prlr^{+/+}$ *vs.* $Prlr^{+/-}$ mice during pregnancy. Intraperitoneal ITT was performed on 4-h-fasted mice during mid (G15) and late (G18) gestation. Blood glucose at time 0 (just before insulin injection) was set at 1, and subsequent blood glucose levels are expressed relative to the basal glucose level. n = 5–7 for each genotype.

β-Cell proliferation rate was assessed by counting the percentage of β-cell that has incorporated BrdU. We found that during pregnancy, β-cell proliferation rate was blunted in the $Prlr^{+/-}$ mice in comparison with the $Prlr^{+/+}$ mice. Parallel to the β-cell mass results above, $Prlr^{+/+(+/+)}$ mice had the highest percentage of β-cells staining positive for BrdU (0.24 ± 0.03%), and $Prlr^{+/-(+/-)}$ had the lowest BrdU-positive β-cells (0.1 ± 0.04%), whereas $Prlr^{+/-(+/+)}$ and $Prlr^{+/+(+/-)}$ had intermediate BrdU incorporation rates (0.15 ± 0.03 and 0.18 ± 0.03%, respectively, n = 5–6) (Fig. 6, C and D).

Apoptosis was detected by TUNEL staining. As expected, β-cell apoptosis was a rare event in adult mice. By d 18 of pregnancy, the rate of apoptosis was approximately 0.1% in both wild-type and mutant mice ($Prlr^{+/+}$: 0.14 ± 0.05%; $Prlr^{+/-}$: 0.13 ± 0.08%). By P5, the rate of apoptosis was similarly increased in both wild-type and mutant mice ($Prlr^{+/+}$: 0.37 ± 0.19%; $Prlr^{+/-}$: 0.43 ± 0.23%) (Fig. 6, A and B). These results suggest that the heterozygous mutation of Prlr is not sufficient to affect β-cell apoptosis.

Discussion

Our studies have provided the first *in vivo* demonstration that PRL/PL signaling through the Prlr is required for maintaining normal glucose homeostasis and promoting an increase in β-cell mass during pregnancy. In studying $Prlr^{+/-}$ mice that have partial deficiency of Prlr expression, we found that during pregnancy, they showed defects in glucose tolerance and β-cell mass expansion, although their glucose homeostasis is

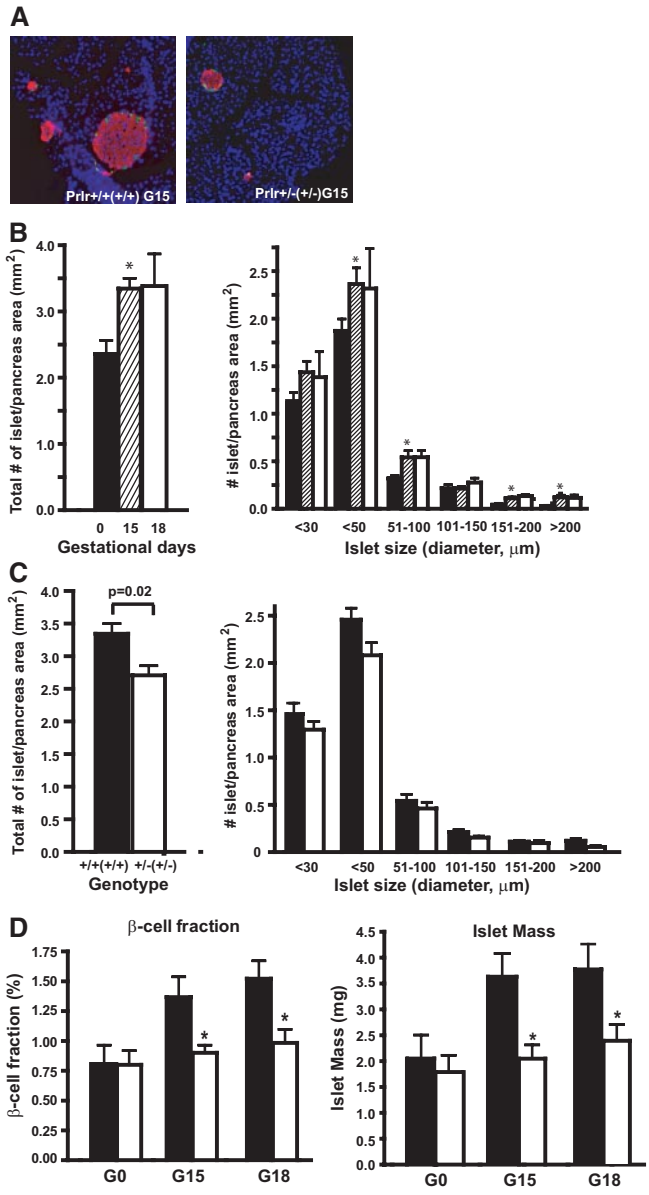

FIG. 4. Effects of heterozygous Prlr deletion on islet number and mass during pregnancy. A, Pancreas sections from pregnant mice (G15 and G18) or nonpregnant mice (G0) were immunostained for insulin (*red*) and glucagon (*green*) to identify islets. Representative images from a $Prlr^{+/+(+/+)}$ and a $Prlr^{+/-(+/-)}$ pregnant pancreas are shown. B, The number of islets (defined as insulin-positive cells or cell clusters) in $Prlr^{+/+}$ mice are expressed per pancreas area (square millimeters). Islets of various sizes are grouped by diameters. *Black bar*, G0; *striped bar*, G15; *white bar*, G18. *, $P < 0.05$ in comparison with the number of islets in the nonpregnant mice for the same size category. C, During pregnancy (G15), the total number of islets (per square millimeter of pancreas area) was reduced in the $Prlr^{+/-(+/-)}$ mice in comparison with the $Prlr^{+/+(+/+)}$ mice (*left*), most notably with fewer very small and small islets (diameter < 30 and 50 μm) (*right*). D and E, β-Cell fraction (D), defined as the percent area of the pancreas section stained positive for insulin, and β-cell mass (E), derived from β-cell fraction multiplied by the pancreas weight, were compared between $Prlr^{+/+(+/+)}$ and $Prlr^{+/-(+/-)}$ mice. Results represent mean ± SE; n = 4–5 mice of each genotype, and at least five sections from each mouse were analyzed. For C–E, *black bar*, $Prlr^{+/+(+/+)}$; *white bar*, $Prlr^{+/-(+/-)}$. *, $P < 0.05$ in comparison with the wild-type mice at the same gestational stage.

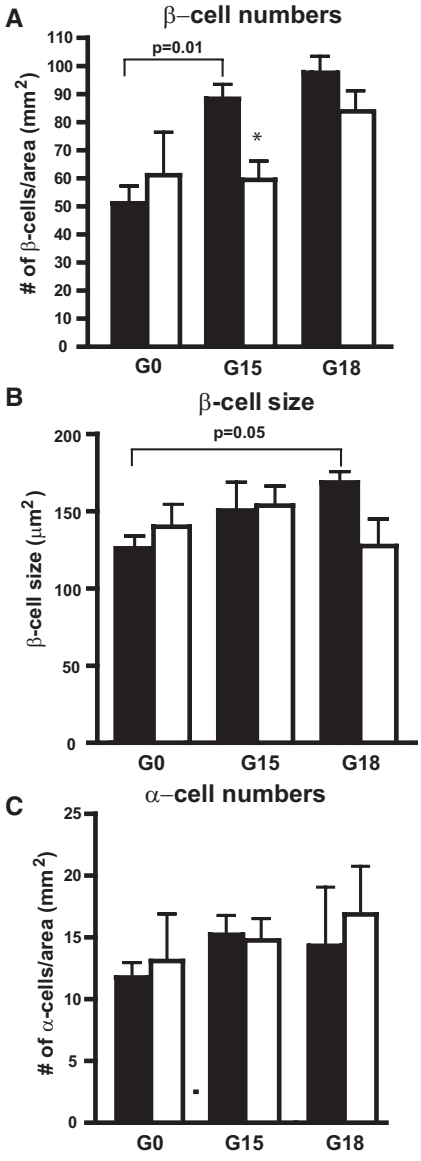

FIG. 5. Effects of heterozygous Prlr deletion on cell numbers and size during pregnancy. A and C, Pancreas sections from pregnant mice (G15 and G18) or nonpregnant mice (G0) were immunostained with antiglucagon and antiinsulin antibodies to identify α- and β-cells, respectively. Number of cells per pancreas area (square millimeter) was counted for each pancreas section, and results are expressed as mean ± SE. B, β-Cell cross-sectional area or size was calculated by dividing the total insulin-positive areas by the number of insulin-positive cells on each pancreas section. n = 4–5 mice of each genotype, and four to five sections from each mouse were analyzed. *Black bar*, $Prlr^{+/+(+/+)}$; *white bar*, $Prlr^{+/-(+/-)}$. *, $P < 0.05$ in comparison with the $Prlr^{+/+(+/+)}$ mice at the same gestational stage.

indistinguishable from that of the wild-type mice before pregnancy. At both d 15 and 18 of pregnancy, $Prlr^{+/-}$ mice had higher nonfasted blood glucose and greater glucose excursion during an IPGTT. We chose to study these time points because previous studies found the highest β-cell proliferation rate on G14-G15, with a leveling off of β-cell proliferation on G18-G20 (5, 9). It is important to point out that the difference in glucose homeostasis is not a result of a difference in insulin sensitivity, because the wild-type and the mutant mice had comparable insulin sensitivity. Some studies have demonstrated that PRL decreases insulin sensitivity (21). Therefore, if anything, insulin sensitivity would have been expected to increase in Prlr mutants. Perhaps, therefore, the degree of glucose intolerance might be underestimated in our $Prlr^{+/-(+/-)}$ mutant mice.

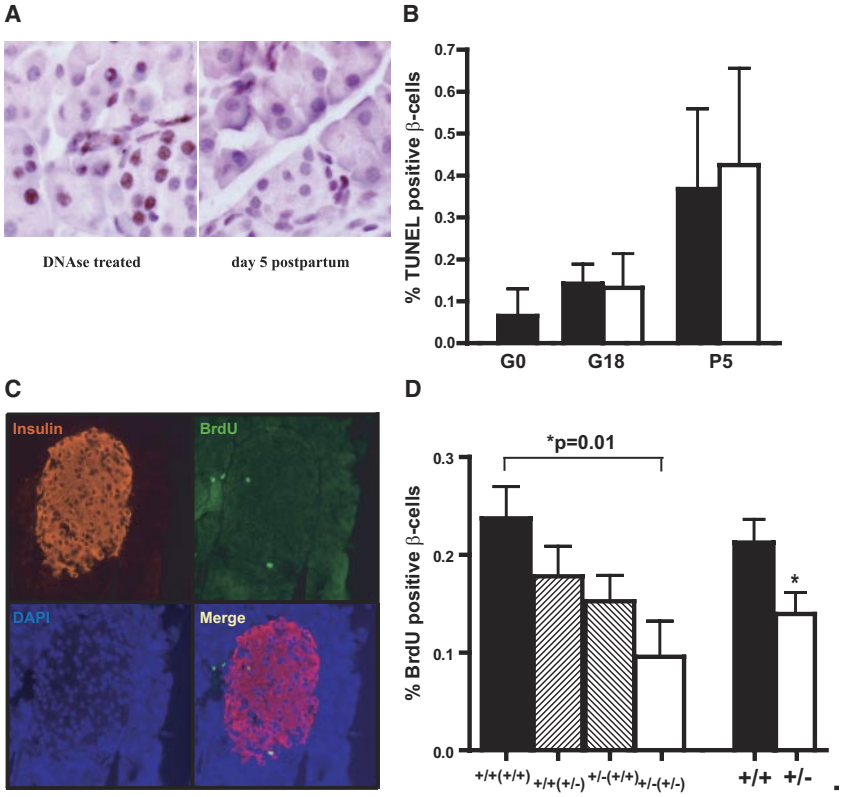

FIG. 6. Effects of heterozygous Prlr deletion on β-cell apoptosis and proliferation. A and B, Pancreas sections from nonpregnant (G0) and pregnant (G18) mice and mice on d 5 after delivery (P5) were processed to detect apoptotic cells by TUNEL staining. A, Representative image of a pancreas section treated with deoxyribonuclease (as positive control for DNA breakage) and a pancreas section from a mouse on P5 are presented. B, The percentage of β-cells stained positive for TUNEL were counted for each pancreas section, and results are expressed as mean ± SE; n = 3–4 mice of each genotype, and three to four sections from each mouse were analyzed. *Black bar*, $Prlr^{+/+(+/+)}$; *white bar*, $Prlr^{+/-(+/-)}$. C and D, Pancreas sections from pregnant (G15) mice were processed to detect BrdU incorporation by immunostaining with an anti-BrdU antibody. C, Representative image of a pancreas section immunostained for BrdU and insulin. D, The percentage of β-cells stained positive for BrdU were counted for each pancreas section, and results are expressed as mean ± SE; n = 3–4 mice of each genotype, and four to five sections from each mouse were analyzed. Genotypes of the mice are as indicated on the x-axis. *, $P < 0.05$ in comparison with the wild-type mice.

Our results showed that the impaired glucose tolerance in the pregnant mutant mice is accompanied by a failure in β-cell mass expansion. In comparison with wild-type mice, the pregnant $Prlr^{+/-}$ mice had significantly lower β-cell proliferation rate, lower β-cell fraction, and lower islet mass. Interestingly, in wild-type mice, we observed a significant increase in β-cell number from d 0–15 of pregnancy, followed by a more modest increase between d 15 and d 18. β-cell size, however, increased only slightly between d 0 and 15 of pregnancy and more dramatically between d 0 and 18. Therefore, consistent with previous studies, our results suggest that Prlr is important for both β-cell hyperplasia (22, 23) and hypertrophy during pregnancy. It is important to point out that all of the differences in $Prlr^{+/-}$ mice were observed in pregnant mice only, and none of the above parameters differed during the nonpregnant state. Freemark *et al.* reported previously that Prlr homozygous mutants had a reduction in β-cell mass and impaired glucose tolerance in nonpregnant mice, but they did not report the glucose homeostasis of heterozygous pregnant mice (16). Therefore, our results are consistent and expand their findings, showing that under physiological stress (*i.e.* pregnancy) heterozygous deletion of Prlr is sufficient to elicit abnormality in glucose homeostasis and reduced ability to increase β-cell mass.

β-Cell mass is not the sole determinant of insulin levels and glucose homeostasis during pregnancy. PRL increases the insulin mRNA and protein levels (24) and up-regulates the expression and activity of glucokinase, an enzyme that metabolizes glucose in β-cells and regulates insulin secretion (10). Therefore, a decrease in PRL-regulated insulin synthesis and glucokinase activity may also contribute to the decrease in steady-state insulin and the increase in glucose levels we observed in nonfasted pregnant $Prlr^{+/-}$ mice. In addition, the $Prlr^{+/-}$ mice also secrete less insulin during the first 30 min of the IPGTT. This can simply be an extension of the above point, *i.e.* $Prlr^{+/-}$ mice synthesize less insulin and therefore secrete less insulin upon stimulation. The decrease is quite profound though, reflecting the possibility that the heterozygous pregnant mutant mice may have an insulin secretory defect independent of its low insulin content, such that the peak in insulin secretion might be achieved later than 30 min after an ip glucose injection. Indeed, PRL up-regulates GLUT2 (8), which is required for glucose-mediated insulin secretion, and it is possible that $Prlr^{+/-}$ mice have less GLUT2 on their β-cells and therefore reduced glucose-stimulated insulin secretion. Whether some or all of these potential intracellular metabolic and signaling changes occurred as a consequence of Prlr deletion will be subject to future studies.

A potentially interesting observation is an increase in the number of very small (diameter <30 μm or fewer than five cells) and small (diameter 30–50 μm or fewer than 15 cells) islets between d 0 and 15 of pregnancy (Fig. 4B). Although these small islets are unlikely to make significant contributions to overall β-cell mass (7), this observation suggests that during early pregnancy, not only is there an increase in islet size by β-cell proliferation but also new, small islets are being formed. It is tempting to speculate that these small islets are formed from progenitor cells present in adult pancreas (25). An alternative interpretation is that if all islets, large or small, increase in size at a fixed percentage, this will increase the probability of detecting the edge of an islet, which would appear as a small islet. And indeed, we found that during pregnancy, the number of islets increased in almost all size categories (Fig. 4B) and that the number of small islets when expressed as a percentage of total number of islets did not increase during pregnancy, consistent with the concept that β-cell proliferation occurs in both large and small islets. However, these observations do not exclude the possibility of new islet formation during pregnancy, which remains an intriguing question requiring further exploration.

Another novel finding is that a reduction in Prlr expression did not affect β-cell apoptosis. Apoptosis is a rare event in adult

pancreas (3, 19). In both $Prlr^{+/+}$ and $Prlr^{+/-}$ mice, we observed a slight increase in apoptosis toward the end of pregnancy, and as expected, significant apoptosis occurred after delivery of the pups, returning the β-cell mass to that of the prepregnant state (3). Although we did not observe any difference in apoptosis rate between the wild-type and mutant mice, it was possible that Prlr status could affect apoptosis rate, because PRL is known to reduce apoptosis in other cell types (26). However, the fact that we observed only about one apoptotic cell for every 1000 β-cells on d 18 of pregnancy indicates that differential rates of apoptosis likely has a minimal impact on β-cell mass during pregnancy.

Upon examination of pedigrees, it became clear that maternal genotype has a significant effect on the phenotype of the daughters during pregnancy. $Prlr^{+/-}$ mice derived from $Prlr^{+/-}$ mothers had the highest nonfasted serum glucose levels, whereas $Prlr^{+/+}$ mice derived from $Prlr^{+/+}$ mothers had the lowest and $Prlr^{+/-}$ mice derived from $Prlr^{+/+}$ mothers and $Prlr^{+/+}$ mice derived from $Prlr^{+/-}$ mothers had intermediate serum glucose levels. The difference in serum glucose correlated inversely with serum insulin level. It is well described that maternal genotype and environment influences the phenotype of the offspring. One target organ of the fetal programming process is the pancreas. Animal studies have demonstrated that *in utero* exposure to malnutrition, hyperglycemia, and/or hyperinsulinemia is associated with impaired glucose tolerance and reduced glucose-stimulated insulin secretion in the offspring (27–29). Furthermore, these pups are born with altered pancreatic islet structure, pancreatic hypertrophy, and an increased number of small islets (30). Most importantly, these already hypertrophied pancreata do not increase in size and function to accommodate for pregnancy-induced insulin resistance when these mice themselves become pregnant; they develop gestational diabetes, leading to a vicious cycle of diabetes that begets more diabetes. Our results are consistent with the above hypothesis.

In conclusion, we have found that Prlr is required to maintain normal glucose homeostasis during pregnancy. Heterozygous deletion of Prlr results in impaired glucose tolerance and reduced serum insulin as well as reduced β-cell mass and number during pregnancy. The effect on β-cell number is likely through reduced proliferation of either existing β-cells and/or precursors but not through increased apoptosis. Further study is required to understand the mechanisms underlying Prlr-dependent β-cell proliferation.

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