

Ronadip R. Banerjee,^{1,2} Holly A. Cyphert,³ Emily M. Walker,³ Harini Chakravarthy,¹ Heshan Peiris,¹ Xueying Gu,¹ Yinghua Liu,¹ Elizabeth Conrad,³ Lisa Goodrich,⁴ Roland W. Stein,³ and Seung K. Kim^{1,5,6}



Gestational Diabetes Mellitus From Inactivation of Prolactin Receptor and MafB in Islet β -Cells

Diabetes 2016;65:2331–2341 | DOI: 10.2337/db15-1527

β -Cell proliferation and expansion during pregnancy are crucial for maintaining euglycemia in response to increased metabolic demands placed on the mother. Prolactin and placental lactogen signal through the prolactin receptor (PRLR) and contribute to adaptive β -cell responses in pregnancy; however, the in vivo requirement for PRLR signaling specifically in maternal β -cell adaptations remains unknown. We generated a floxed allele of *Prlr*, allowing conditional loss of PRLR in β -cells. In this study, we show that loss of PRLR signaling in β -cells results in gestational diabetes mellitus (GDM), reduced β -cell proliferation, and failure to expand β -cell mass during pregnancy. Targeted PRLR loss in maternal β -cells in vivo impaired expression of the transcription factor *Foxm1*, both G_1/S and G_2/M cyclins, tryptophan hydroxylase 1 (*Tph1*), and islet serotonin production, for which synthesis requires *Tph1*. This conditional system also revealed that PRLR signaling is required for the transient gestational expression of the transcription factor *MafB* within a subset of β -cells during pregnancy. *MafB* deletion in maternal β -cells also produced GDM, with inadequate β -cell expansion accompanied by failure to induce PRLR-dependent target genes regulating β -cell proliferation. These results unveil molecular roles for PRLR signaling in orchestrating the physiologic expansion of maternal β -cells during pregnancy.

Pregnancy is a unique acquired physiologic state of increased metabolic demand, requiring increased output of insulin by islet β -cells. This adaptive response is accomplished both

through enhanced insulin secretion and by β -cell proliferation and expansion in mice (1). Failure of adaptive β -cell expansion underlies dysregulated glucose homeostasis and progression to diabetes mellitus (2). Thus, the mechanisms underlying gestational β -cell proliferation are a focus of intensive ongoing investigation.

Pregnancy hormones are known regulators of β -cell growth and function (3). The lactogenic hormones prolactin and placental lactogen signal through the prolactin receptor (PRLR) and are crucial regulators of pregnancy adaptation in many maternal tissues (4). PRLR is expressed in both rodent and human pancreatic β -cells (5), and in vitro treatment of islets with prolactin has established it as a potent β -cell mitogen in both species (1). Although gene expression studies of islets during pregnancy identified strong induction of some prolactin signaling targets (6–8), the mechanisms underlying lactogen-stimulated changes in β -cells during pregnancy are incompletely understood. During transient β -cell proliferation and expansion during gestation, prior studies have reported increased expression of nuclear factors like FoxM1, the cyclin-dependent kinases cyclin A2 and cyclin B1, and *MafB* (6,9,10). However, the requirement for PRLR signaling to induce expression of these factors and the physiologic significance of the gestational *MafB*⁺ β -cell subpopulation are unknown.

Studies of β -cells during pregnancy in humans are confounded by practical and ethical challenges. Thus, animal studies remain critical for understanding β -cell biology during pregnancy (11). Mouse genetic studies of PRLR support a role in β -cell development and function; the global PRLR

¹Department of Developmental Biology, Stanford University School of Medicine, Stanford, CA

²Division of Endocrinology, Gerontology and Metabolism, Department of Medicine, Stanford University School of Medicine, Stanford, CA

³Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN

⁴Department of Neurobiology, Harvard Medical School, Boston, MA

⁵Division of Oncology, Department of Medicine, Stanford University School of Medicine, Stanford, CA

⁶Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, CA

Corresponding author: Seung K. Kim, seungkim@stanford.edu.

Received 4 November 2015 and accepted 11 May 2016.

This article contains Supplementary Data online at <http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db15-1527/-/DC1>.

© 2016 by the American Diabetes Association. Readers may use this article as long as the work is properly cited, the use is educational and not for profit, and the work is not altered.

knockout has glucose intolerance and reduced β -cell mass (12). Unfortunately, the global *Prlr* knockout mouse is sterile, precluding pregnancy studies (13). *Prlr*^{+/-} mice studied during pregnancy develop glucose intolerance and reduction in β -cell proliferation and mass expansion (14). Nevertheless, as loss of PRLR results in multiple abnormalities in other metabolic tissues that could indirectly influence β -cell function (15), it is essential to assess the consequences of targeted PRLR inactivation specifically in β -cells. In this study, we generated a conditional *Prlr* allele allowing Cre recombinase-mediated genetic ablation of PRLR signaling and identified a requirement for PRLR in molecular, hormonal, and proliferative adaptations by maternal β -cells in pregnancy. Collectively, our results suggest PRLR signaling is a master regulator of adaptive β -cell responses during pregnancy.

RESEARCH DESIGN AND METHODS

Creation of the Floxed *Prlr* Allele, β PRLRKO, and β MafBKO Mice

A targeting vector containing *Prlr* genomic DNA encompassing exons 4 through 9 were subcloned into plasmid PL253 containing a thymidine kinase cassette. Using recombinering, loxP and FRT-neo-FRT-loxP cassettes were placed flanking exon 5. The targeting vector was electroporated into C57BL/6J embryonic stem cells and clones selected using G418; validated clones were injected into 129 blastocysts generating chimeric males (Stanford Transgenic Core). Germ-line transmission of *Prlr*^f allele was identified by brown fur pups after crossing male chimeras with C57BL/6J females. Mouse tail genomic DNA was digested with NheI and Southern blotting performed with a 5' external probe to confirm a correctly targeted allele. The FRT-flanked neo cassette was removed by crosses with *FLPeR* mice (The Jackson Laboratory, Bar Harbor, ME), generating floxed PRLR mice (*Prlr*^{f/+}), which were backcrossed with C57BL/6J mice (The Jackson Laboratory) for more than eight generations. *Prlr*^{f/+} mice were crossed with mice with a transgene encoding Cre recombinase from rat insulin promoter elements (*RIP-Cre*) (16). Subsequently, *RIP-Cre;Prlr*^{f/+} males were crossed with *Prlr*^{f/+} females, generating β PRLRKO (*PRLR*^{f/f};*RIP-Cre*) and littermate *Prlr*^{f/+}, *Prlr*^{f/f}, *RIP-Cre;Prlr*^{f/+}, and *RIP-Cre;Prlr*^{f/+} controls. *MafB*^{f/f} mice (17) were crossed with *RIP-Cre*^M mice (18) and backcrossed to B6J mice at least six generations (see Supplementary Table 1: genotyping primers).

Mouse Husbandry, Breeding, and Experimentation

Mice were weaned 21–25 days after parturition. To avoid potential effects of altered maternal metabolism on offspring during in utero exposure, β PRLRKO females were solely used as experimental mice. Beginning at 8 weeks of age, β PRLRKO and control females were mated with wild-type FVB males. Vaginal plugs were scored at gestational day (GD) 0.5 and males removed. All experiments were repeated in at least two independent cohorts of mice. All procedures involving mice were approved and conducted in accordance with the Stanford Administrative Panel on

Laboratory Animal Care or Vanderbilt Animal Care and Use Program.

Tolerance Testing and Serum Metabolite Analysis

Intraperitoneal glucose and insulin tolerance testing were performed as previously described (19). An oral glucose tolerance test (GTT) was performed using a 22-gauge rigid gavage needle to deliver an intragastric glucose bolus of 2 g/kg body weight. Blood was collected by tail vein bleeding. Blood glucose levels were determined by glucometer (Bayer Contour; Bayer). Ad libitum fed or overnight (16 h)-fasted blood glucose levels were measured at 8:30 A.M. Serum insulin levels were performed by ELISA (Crystal Chem) following the manufacturer's directions.

Islet Isolation and Culture

Islets were isolated using retrograde perfusion of the pancreatic duct with collagenase, purified using density centrifugation, and cultured as previously described (20). Recombinant mouse prolactin (R&D Systems) was diluted in culture media to a final concentration of 500 ng/ml. Culture media was changed daily.

Imaging

Microscopy was performed on a Zeiss AxioM1 Fluorescence microscope with AxioVision software (Carl Zeiss). Confocal images were obtained using a Leica Sp2 microscope (Beckman Cell Sciences Imaging Facility; Leica Microsystems).

Quantifying β -Cell Mass and Proliferation

After weighing, the pancreas was fixed, embedded in optimal cutting temperature compound, and then frozen. Tissues were sectioned at 10- μ m intervals using a Leica 3050S Cryostat (Leica Microsystems). We immunostained tissue with anti-insulin antibody and DAPI and then analyzed sections separated by 200 μ m. β -Cell mass was measured using ImageJ (National Institutes of Health) to quantify insulin⁺ and total pancreas area in each section; β -cell area was the average of nine measured sections. The calculated β -cell mass = (β -cell area/total pancreas area) \times (total pancreas weight). To measure proliferation, we immunostained tissues using antibodies against insulin and Ki-67 and the proportion of Ki-67⁺ β -cells calculated as a percentage. At least 1,500 β -cells were counted for each animal; only clusters containing at least 20 β -cells were counted. The total number of islets per mouse was the sum of clusters counted in nine analyzed sections. Antibodies used are listed in Supplementary Table 2.

Quantitative PCR

Total RNA was isolated using TRIzol (Invitrogen) and reverse transcribed using the Ambion Retroscrip Kit (Ambion) according to the manufacturer's instructions. Quantitative RT-PCR (qPCRs) were performed with FastStart Universal Probe Master (Roche) and TaqMan assays (see Supplementary Table 3) on an ABI 7500 RealTime PCR System (Applied Biosystems). Assays were performed in technical replicates and normalized to mouse actin as a reference standard.

Western Blotting

Islets were lysed in 2× Bio-Rad loading buffer (Bio-Rad) containing 10% BME and then boiled for 5 min. Following electrophoresis on Bio-Rad TGX 4–20% gels (Bio-Rad), proteins were transferred onto nitrocellulose using the tank apparatus (Bio-Rad). Chemiluminescent detection was performed with SuperSignal West Pico (Thermo) and Carestream Kodak BioMax XAR film (Sigma-Aldrich).

Statistical Analysis

Data are presented as means ± SEM. Statistical significance was determined by unpaired two-tailed Student *t* test, repeated-measures ANOVA (RM-ANOVA), or χ^2 test as indicated using GraphPad Prism 6 (GraphPad Software, LaJolla, CA) or Microsoft Excel (Microsoft, Redmond, WA).

RESULTS

Conditional *Prlr* Inactivation in Islet β -Cells

To permit targeted PRLR signaling disruption, we generated mice harboring a Cre recombinase-sensitive *Prlr*^f allele (Fig. 1A and B) (see RESEARCH DESIGN AND METHODS). Intercrosses of *Prlr*^{f/+} mice generated *Prlr*^{f/f} mice and siblings with expected genotypes in Mendelian ratio (Fig. 1C and data not shown). To generate mice lacking PRLR in pancreatic β -cells (abbreviated β PRLRKO mice), we bred *RIP-Cre* mice to *Prlr*^f mice (see RESEARCH DESIGN AND METHODS). qPCR revealed that *Prlr* mRNA levels were reduced >95% in islets from β PRLRKO females as compared with littermate *Prlr*^{f/f} controls (Fig. 1D). Immunohistology and Western blotting demonstrated that β -cell PRLR protein was reduced or almost undetectable in virgin or pregnant female β PRLRKO mice (Fig. 1E and F). Conception, nursing, and rearing of pups by β PRLRKO mothers was similar to that of controls (see GESTATIONAL DIABETES MELLITUS IN β PRLRKO MICE). Thus, the *Prlr*^{f/f} mice permitted conditional genetic inactivation of *Prlr* in vivo.

Gestational Diabetes Mellitus in β PRLRKO Mice

We next investigated the consequences of disrupting β -cell PRLR signaling on glucose homeostasis. Adult virgin female and male β PRLRKO mice did not show alterations in glucose tolerance, weight, or β -cell mass, in contrast to defects observed in global *Prlr* knockout mice (Fig. 2A–C) (data not shown). Ad libitum fed glucose and insulin levels, insulin tolerance, and total pancreas mass also did not differ in 2- to 3-month-old virgin β PRLRKO females compared with age-matched controls (Supplementary Fig. 1A–D), the age when all subsequent pregnancy studies were performed.

By contrast, we observed pronounced defects of glucose regulation in pregnant β PRLRKO females. Maternal insulin demand peaks late in pregnancy, ~GD16.5 (10). On GD16.5, we observed marked glucose intolerance in β PRLRKO females (Fig. 2D) compared with control females (*Prlr*^{f/+} and *Prlr*^{f/f}). We also observed glucose intolerance in *RIP-Cre;Prlr*^{f/+} mice (Fig. 2D). Blood glucose levels during ad libitum feeding were also elevated in GD16.5 β PRLRKO mice (Fig. 2E). Expression of *Prlr* mRNA in *RIP-Cre;Prlr*^{f/+} mice was intermediate between β PRLRKO and control females (Supplementary Fig. 2A). However, weight gain in

pregnancy and litter sizes of β PRLRKO mothers were indistinguishable from that of controls (Supplementary Fig. 2B and C). Following parturition, β PRLRKO mothers lactated normally and nursed pups to weaning without impact on offspring survival rates (data not shown). Subsequent postpartum analyses showed that glucose intolerance and ad libitum glucose abnormalities in pregnant β PRLRKO mice resolved (Fig. 2F and G), a characteristic feature of gestational diabetes mellitus (GDM). Because some mouse strains harboring a *RIP-Cre* transgene develop glucose homeostasis abnormalities (21), we also evaluated *RIP-Cre* littermate controls but did not observe any differences in glucose tolerance in virgin, pregnant, or postpartum mice compared with controls (Fig. 2D, F, and H). Thus, β PRLRKO mice developed GDM.

Because GDM increases the risk of dysglycemia in future pregnancies, we investigated the effects of additional pregnancy on glucose regulation in β PRLRKO multigravida. During a second gestation in β PRLRKO females, glucose intolerance (Fig. 2H) and hyperglycemia in ad libitum feeding recurred (Fig. 2I). β PRLRKO females also developed evidence of worsened glucose control, including glycemia >200 mg/dL upon completion of the GTT (Fig. 2H) and fasting hyperglycemia (Fig. 2I), features not observed in the first pregnancy (Fig. 2D). Thus, we observed that glucose control worsened with additional pregnancies in β PRLRKO mice, similar to humans with multigravidity or GDM (22).

Failure to Proliferate and Expand β -Cells in Pregnant β PRLRKO Mice

To identify the basis for GDM in β PRLRKO mothers, we assessed insulin output and β -cell growth, features known to undergo adaptive enhancement in pregnancy (1). We found reduced serum insulin levels in β PRLRKO mice during GTT in late gestation (GD16.5), consistent with relative insulin deficiency (Fig. 3A and B). We sought to understand the basis for this relative insulin deficiency by examining insulin secretion in islets isolated from mice at GD16.5. Islets from β PRLRKO or control mice showed similar basal and glucose-stimulated insulin secretion (GSIS), normalized to insulin content (Fig. 3C), indicating the insulin deficiency observed in vivo was not a consequence of reduced GSIS. Insulin tolerance was also unchanged in β PRLRKO mothers at this gestational stage, excluding altered peripheral insulin sensitivity (Supplementary Fig. 3A). Next, we used the cellular marker Ki-67 to quantify β -cell proliferation and observed a threefold reduction of β -cell labeling in islets of GD16.5 β PRLRKO mice compared with controls (Fig. 3D and E). No change in total islet number was observed (Supplementary Fig. 3C).

Consistent with the observed reduction of Ki-67⁺ β -cells in pregnant β PRLRKO mice, β -cell area and mass were reduced approximately twofold compared with pregnant *Prlr*^{f/f} and *RIP-Cre* controls (Fig. 3F and G and Supplementary Fig. 3C and D). Compared with nonpregnant β PRLRKO females (Fig. 2C) (0.89 ± 0.06 mg), pregnant β PRLRKO females (Fig. 3G) (1.91 ± 0.16 mg) exhibited a significant

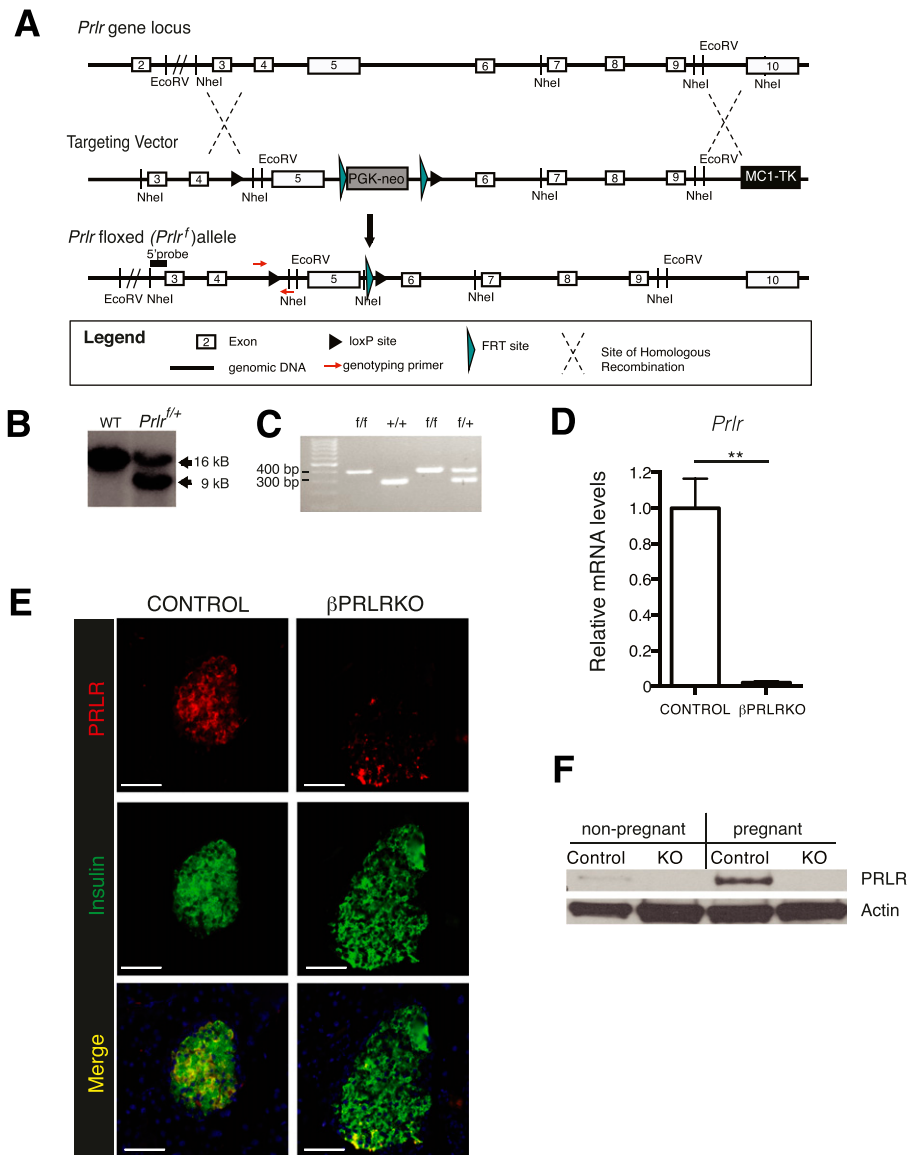


Figure 1—Conditional inactivation of PRLR signaling in pancreatic β -cells (β PRLRKO). **A**: Schematic diagram (not to scale) depicting the *Prlr* gene locus, targeting vector sequence, and *Prlr^f* allele structure (see RESEARCH DESIGN AND METHODS). **B**: Southern blot of genomic DNA from mice demonstrating germline transmission of *Prlr^f*. Introduction of a *NheI* site downstream of the loxP cassette resulted in a 9-kb (targeted allele) rather than a 16-kb fragment (wild-type [WT] allele). **C**: Genotyping PCR of a litter depicting representative progeny of all potential *Prlr* genotypes following a cross of *Prlr^{f/+}* mice. **D**: mRNA levels of *Prlr* in β PRLRKO females (*RIP-Cre;Prlr^{f/f}*) and control littermates (*Prlr^{f/f}*). $n = 6$ mice/group. **E**: Immunofluorescence images of islets from β PRLRKO female and control mice stained for PRLR and insulin (scale bar, 100 μ m). **F**: Western blot of PRLR expression from islets isolated from virgin β PRLRKO female and controls. Actin was used as a loading control. $**P \leq 0.01$ by *t* test.

increase of β -cell mass during pregnancy ($P = 0.004$), suggesting PRLR-independent mechanisms of gestational mass expansion are active yet insufficient to compensate for the loss of PRLR signaling. Thus, loss of PRLR led to reduced β -cell proliferation and mass expansion in pregnant β PRLRKO.

Disrupted β -Cell Cycle Gene Expression and Serotonin Signaling in β PRLRKO Mice

To investigate the molecular basis of reduced gestational β -cell proliferation in β PRLRKO mice, we measured gene

expression. Gestational induction of known PRLR signaling targets like *Prlr* itself and cytokine-inducible Src homology 2-containing protein (*Cish*), a negative regulator of PRLR signaling (8), was markedly reduced as expected (Fig. 4A). Tryptophan hydroxylase 1 (*Tph1*) is highly induced by pregnancy in β -cells (6,23) and catalyzes the rate-limiting step of islet serotonin synthesis. In turn, serotonin contributes to β -cell proliferation through autocrine and paracrine effects (6). In β PRLRKO pregnant mice, islet *Tph1* induction was eliminated (Fig. 4B), and immunoreactive serotonin in islets was absent during

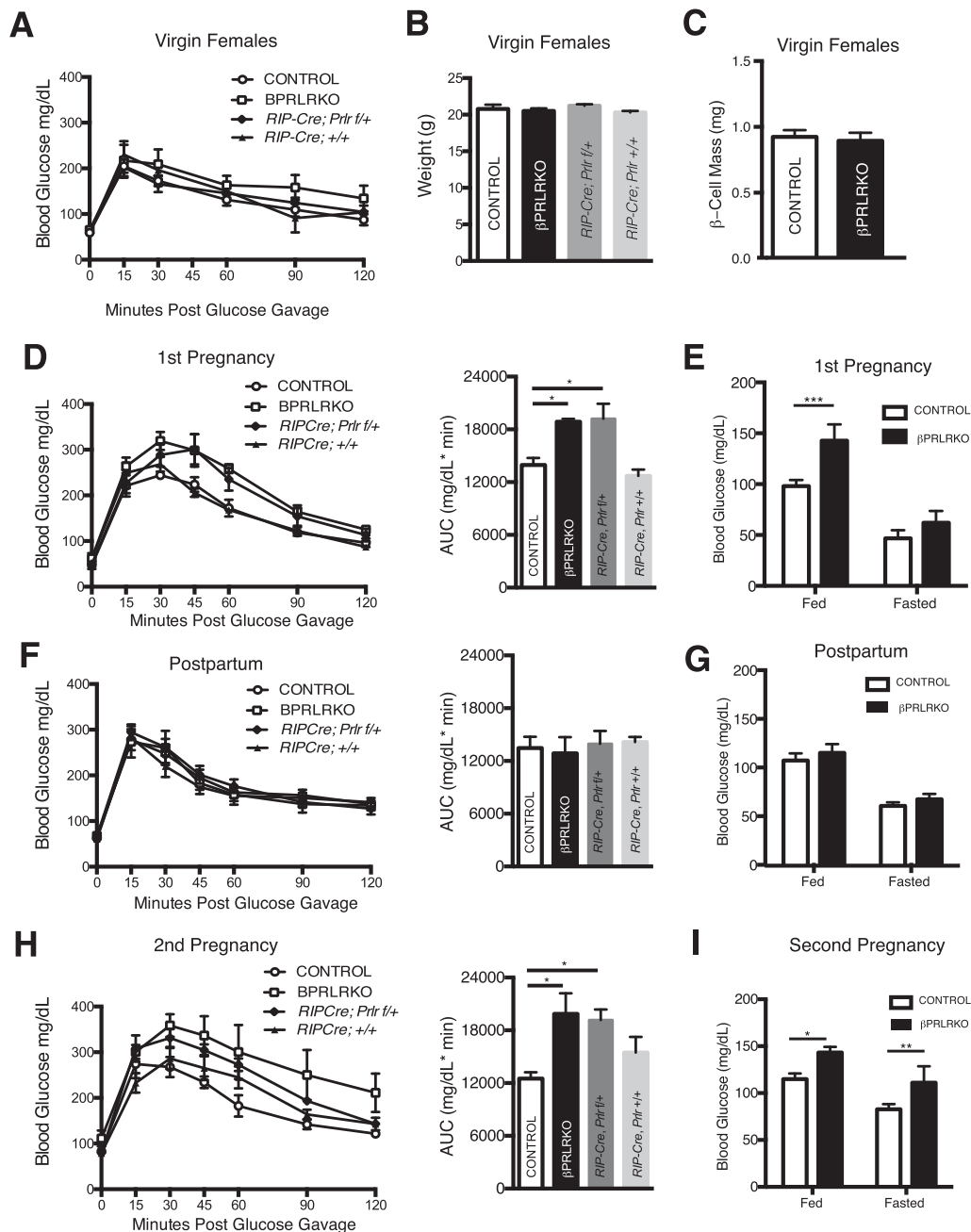


Figure 2— β PRLRKO females have GDM. Virgin β PRLRKO females and control littermates were followed through pregnancy, postpartum, and a second pregnancy. *A*: GTT. *B*: Body weights. *C*: β -Cell mass in virgin females. *D–I*: Glucose tolerance with corresponding area under the curve (AUC) for each genotype and ad libitum fed and overnight fasted glucose for β PRLRKO females and controls. *D* and *E*: First pregnancy. *F* and *G*: Postpartum. *H* and *I*: Second pregnancy. $n = 5–8$ mice/group. * $P < 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ for RM-ANOVA (GTT) or *t* test (blood glucose).

gestation (Fig. 4C). Thus, β -cell PRLR is required for islet serotonin production during pregnancy. Prior work suggests that lactogens alter expression of genes encoding β -cell cyclins, cyclin-dependent kinase inhibitors, and transcription factors *Foxm1* and *Foxd3* (4,10,24–26). In β PRLRKO pregnant females, qPCR with isolated islets at GD16.5 revealed reductions in *Ccna2*, *Ccnb1*, *Ccnb2*, and *Ccnd1* (which, respectively, encode cyclins A2, B1, B2, and D1), and *Foxm1*, all previously implicated as regulators of

gestational β -cell proliferation (6,8,10) (Fig. 4D). By contrast, we found that expression of *Ccnd2* (Fig. 4D), *Men1*, *Cdkn2c* (p18), *Cdkn1b* (p27), and *Foxd3* (Supplementary Fig. 4A), which prior studies indicate are regulated by lactogen signaling in pregnancy (24–27), were unaffected in β PRLRKO islets at GD16.5. Moreover, reduced *Foxd3* expression during gestation (24) still occurred in β PRLRKO females, indicating PRLR signaling is not required for *Foxd3* downregulation in pregnancy (Supplementary Fig. 4A).

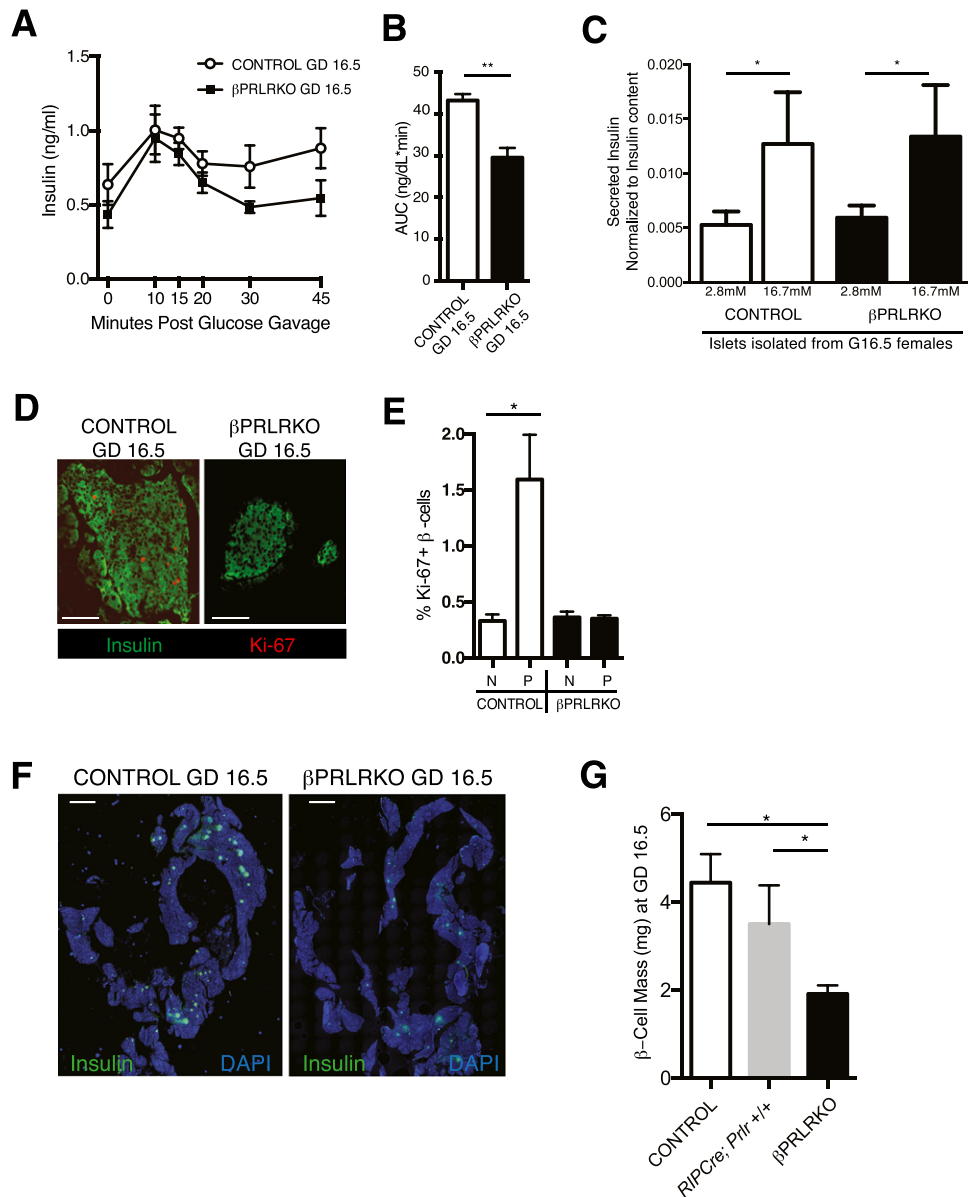


Figure 3— β PRLRKO females have reduced β -cell proliferation and mass during pregnancy. Serum insulin levels during GTT (A) and corresponding area under the curve (AUC) measurements (B) in GD16.5 pregnant β PRLRKO females and controls (*Prlr^{f/f}*). $n = 4$ mice/group. $**P \leq 0.01$ by *t* test. C: GSIS on islets isolated from GD16.5 β PRLRKO females ($n = 4$) and controls ($n = 6$). D: Representative islet images of GD16.5 pancreas sections from β PRLRKO females and controls with Ki-67 immunofluorescence, insulin, and DAPI (scale bar, 100 μ m). E: The percentage of β -cells with Ki-67⁺ nuclei staining in nonpregnant (N) and pregnant (P) (GD16.5) β PRLRKO females and controls. F: Representative images of pancreas sections from GD16.5 β PRLRKO females and controls used for β -cell area. Insulin immunofluorescence (green) and DAPI (blue) (scale bar, 2 mm). G: Calculated β -cell mass in GD16.5 β PRLRKO, *RIP-Cre; Prlr^{+/+}*, and control (*Prlr^{f/f}*) females. $n = 3$ or 4 mice/group. $*P < 0.05$ by *t* test.

Collectively, our in vivo targeted genetic studies demonstrate that PRLR signaling is required for serotonin signaling and gene expression changes controlling adaptive maternal β -cell expansion.

To assess the formal possibility that GDM might indirectly alter gene expression in pregnant β PRLRKO mice, we measured the response of isolated β PRLRKO islets to lactogen treatment in vitro. PRLR activates several intracellular pathways, including Jak2/Stat5, a major mediator of its signal transduction (28). As expected,

purified mouse prolactin robustly induced phosphorylation of Stat5 in control islets (Supplementary Fig. 4B). By contrast, phospho-Stat5 was reduced in islets from *RIP-Cre; Prlr^{f/f}* mice and absent in β PRLRKO islets treated with prolactin (Supplementary Fig. 4B). We next examined gene expression in cultured islets treated with prolactin for 24 h in concentrations comparable to peak lactogen levels in pregnancy (29). In controls, PRLR target genes, including *Prlr*, *Cish*, and *Tph1*, were induced to levels similar to those at GD16.5; this induction was

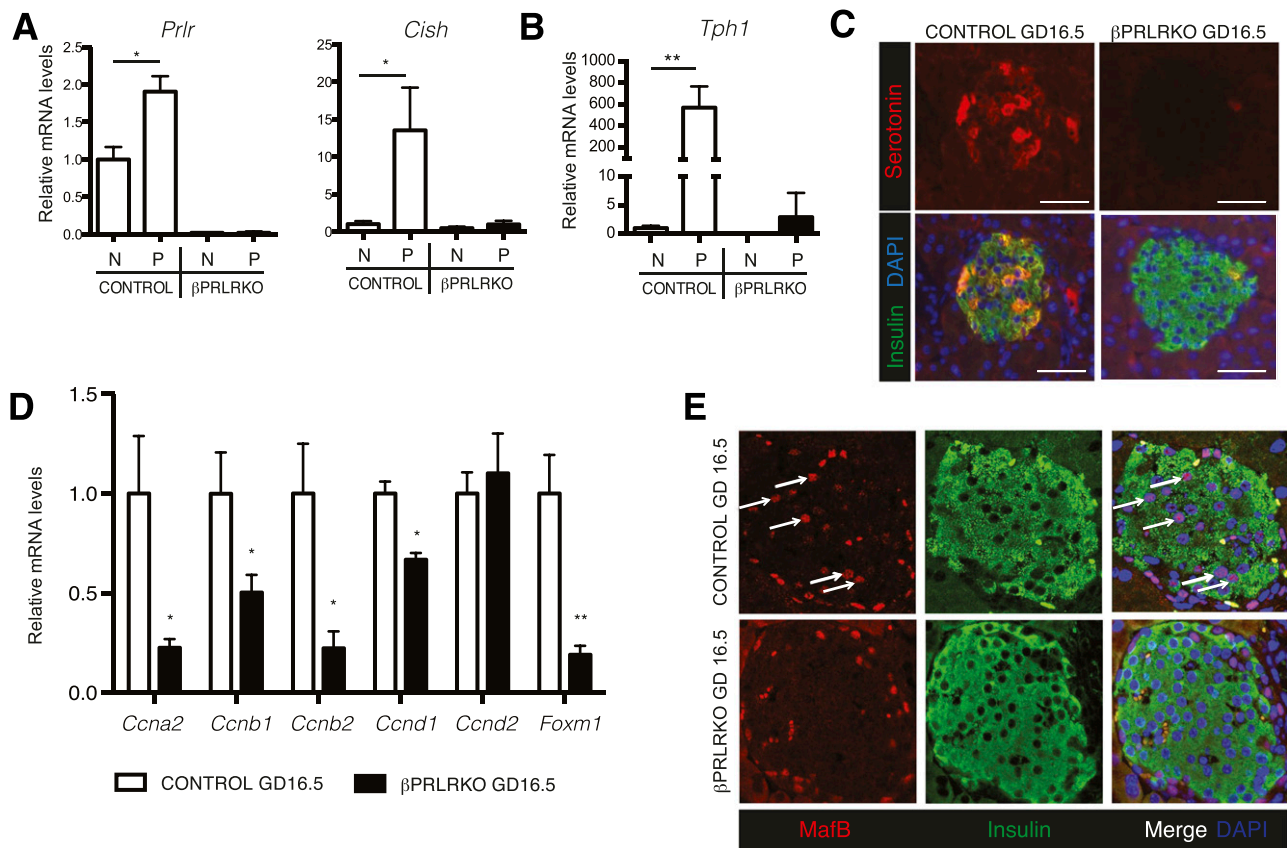


Figure 4—Altered cell cycle regulators and serotonin signaling in β PRLRKO islets during pregnancy. mRNA levels of *Prlr* and *Cish* (A) and *Tph1* (B) in nonpregnant (N) or pregnant (P) β PRLRKO females (black bars) and controls (open bars). C: Immunofluorescence of islet serotonin, insulin, and DAPI (scale bar, 50 μ m). D: Relative mRNA levels of *Ccna2*, *Ccnb1*, *Ccnb2*, *Ccnd1*, *Ccnd2*, and *Foxm1* in β PRLRKO females and controls at GD16.5. E: Confocal immunofluorescence images of islets from GD16.5 β PRLRKO females and controls (original magnification $\times 40$). MafB (red), insulin (green), and DAPI (blue) with MafB⁺Ins⁺ cells highlighted (white arrows). For mRNA studies, pregnant samples were from GD16.5 and expression levels normalized to nonpregnant controls = 1. $n = 3$ –8 mice/group. * $P < 0.05$, ** $P \leq 0.01$ by t test.

eliminated in β PRLRKO islets (Supplementary Fig. 4C). FoxM1 expression was reported to increase in whole islets exposed in vitro to placental lactogen (10), but we did not detect consistent FoxM1 induction after treatment of islets with lactogens up to 4 days in culture (data not shown). These in vitro data are consistent with gene expression changes of β PRLRKO islets observed in vivo and suggest that altered expression of PRLR target genes reflects disrupted intrinsic islet signaling, not secondary effects of altered metabolism in GDM.

MafB Expression in β -Cells During Pregnancy Requires PRLR Signaling

In pancreatic islets from adult mice, the transcription factor MafB is exclusively expressed in α -cells (30), except during pregnancy when we detected transient expression in a subset of β -cells (9). This contrasts with MafB expression within both α -cells and β -cells during development (30,31). The physiologic significance of MafB expression in β -cells during pregnancy was unknown. We observed that up to 25% of maternal insulin⁺ (abbreviated Ins⁺) cells produced MafB by GD16.5 (Supplementary Fig. 5A). MafB⁺ β -cells diminished by postpartum day

7 and were undetectable by postpartum day 28 (Supplementary Fig. 5B). The maternal MafB⁺Ins⁺ cells at GD16.5 also produced Pdx1, a key transcription factor and regulator of β -cells (Supplementary Fig. 5C). MafB expression in adult α -cells promotes glucagon production, but we did not observe bihormonal Ins⁺Glucagon⁺ cells in maternal islets (data not shown). Strikingly, we found that MafB⁺ β -cells were absent from maternal β PRLRKO islets at GD16.5 or other gestational stages (Fig. 4E and data not shown). Thus, β -cell MafB expression in pregnancy requires PRLR signaling.

MafB Loss Impairs Gestational β -Cell Expansion and Promotes GDM

To identify physiologic roles for MafB in maternal β -cells, we generated a β -cell-specific deletion of MafB (β MafBKO) (see RESEARCH DESIGN AND METHODS). Nonpregnant β MafBKO mice had normal glucose tolerance (Fig. 5A), but showed glucose intolerance during pregnancy compared with control MafB^{f/f} mice (Fig. 5B). This glucose intolerance was similar to that in β PRLRKO mice (Fig. 2). Additionally, both β -cell proliferation (Fig. 5C) and mass (Fig. 5D) were reduced in β MafBKO mice during pregnancy but not in virgin females.

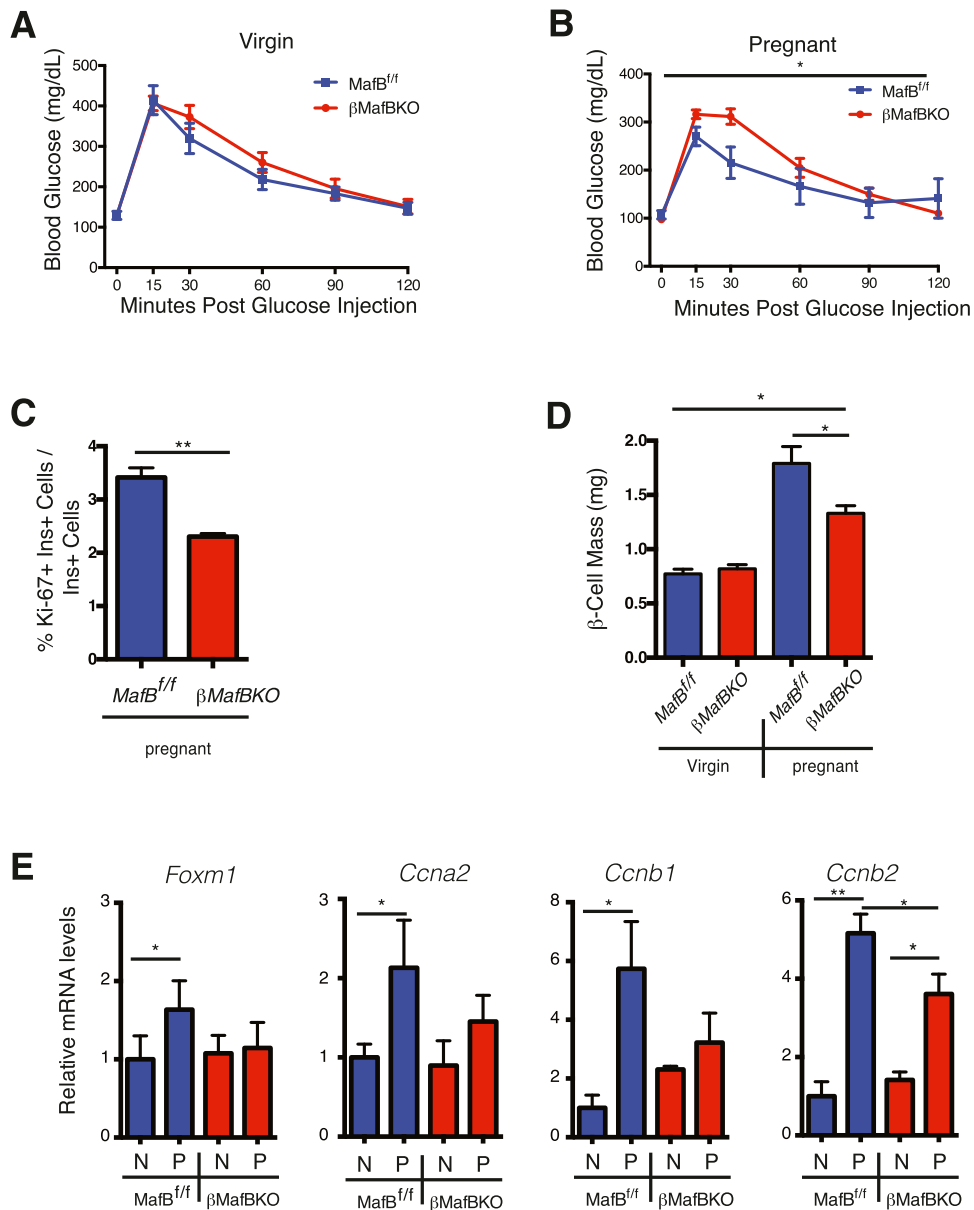


Figure 5— β MafBKO mice have GDM, reduced β -cell proliferation, and gene expression changes that overlap with β PRLRKO mice. GTT in β MafBKO females (*MafB^{fl/fl};RIP-Cre^M*) and controls (*MafB^{fl/fl}*) as virgins (A) and during pregnancy (B) at GD15.5. The percentage of Ki-67⁺ nuclei within β -cells (Ki-67⁺Ins⁺/total Ins⁺ cells) (C) and β -cell mass (mg) in virgin and GD15.5 β MafBKO and controls (D). E: Relative mRNA levels of *Foxm1*, *Ccna2*, *Ccnb1*, and *Ccnb2* in nonpregnant (N) or GD15.5 (P) β MafBKO females and controls. Expression levels were normalized to N controls = 1. $n = 3$ –8 mice/group. * $P < 0.05$, ** $P \leq 0.01$ for RM-ANOVA (GTT) or t test.

Like β PRLRKO mice, pregnant β MafBKO mice failed to induce *Foxm1*, *Ccna2*, or *Ccnb1* in pregnancy (Fig. 5E). Although levels of *Ccnb2* mRNA increased with pregnancy in β MafBKO females, this induction was blunted compared with pregnant controls (Fig. 5E). The *RIP-Cre^M* allele used to generate β MafBKO mice also encodes human growth hormone that can activate PRLR signaling (32). Consistent with these prior findings, we observed significant elevations of PRLR signaling targets *Cish* and *Tph1* in nonpregnant female *RIP-Cre^M* mice (Supplementary Fig. 5D). By contrast, *Prlr* expression in nonpregnant β MafBKO was similar to controls and was significantly induced during

pregnancy. However, the induction was blunted compared with pregnant controls, including *RIP-Cre^M* pregnant females (Supplementary Fig. 5D). Despite this apparent partial activation of PRLR signaling, female β MafBKO mice did not show elevated *Foxm1* or *Ccna2* levels at baseline, and induction of both genes during pregnancy was blunted compared with pregnant *RIP-Cre^M* controls (Fig. 5E and Supplementary Fig. 5E). Moreover, despite elevated *Tph1* expression, β MafBKO islets showed clear reduction of serotonin compared with control mice at GD16.5 (Supplementary Fig. 5F). Thus, maternal islet serotonin production requires MafB expression in

gestation. Together with our finding that MafB induction fails in β PRLRKO mothers, these data indicate that MafB is regulated by PRLR signaling and required for β -cell adaptations during pregnancy.

DISCUSSION

Genetic and cell biology studies have suggested that PRLR signaling is a crucial regulator of β -cell expansion (12,14,29,33), but prior loss-of-function studies were performed in global PRLR knockouts rather than examining PRLR loss specifically in β -cells. Our studies reveal a clear requirement for PRLR in β -cell function in vivo. A prior study of conventional PRLR knockout mice identified glucose intolerance, reduced body weight, and reduced β -cell mass in adult mice of both sexes (12), but it was unclear if these reflected islet or nonislet effects. By contrast, we observed normal glucose homeostasis and β -cell mass in adult prepartum β PRLRKO mice and no alteration in adult body weight. Our results suggest that defective PRLR signaling in nonislet tissues likely contributes to body weight and glucose homeostasis abnormalities previously observed in nonpregnant mice with global PRLR knockout. Some of these differences may also be related to genetic background differences between 129Sv and B6 mouse strains, known to affect PRLR-dependent phenotypes (34). Additional studies have indicated a role for PRLR signaling in fetal and early postnatal islet development (35). *RIP-Cre* expression commences in the fetal pancreas and has been used by us and others to inactivate β -cell genes in the fetal and neonatal period (20). Thus, β PRLRKO mice might be predicted to display defective developmental expansion of β -cells; however, virgin adult β PRLRKO mice displayed no alteration in β -cell mass. Further studies are required to assess the role of PRLR in embryonic or perinatal β -cell development.

PRLR signaling is thought to involve transduction through multiple effectors, including signal transducer and activator of transcription 5 (*Stat5*). However, mice carrying a β -cell-specific deletion of *Stat5* did not develop GDM (36). Thus, although *Stat5* is considered a principal mediator of PRLR signaling, our results suggest that other signal transduction pathways such as mitogen-activated protein kinase/extracellular signal-regulated kinase or phosphatidylinositol 3-kinase/Akt (4) are critical for gestational β -cell adaptation. Supporting this model, recent studies have demonstrated that prolactin induction of *Tph1* is coordinated by activation of all three pathways (37). β -Cell-specific deletion of *Tph1* has been reported, but gestational studies were not performed (38). Mice with pancreas-specific deletion of *Foxm1* display profound GDM, but also exhibit prepregnancy defects in β -cell mass that likely reflect *Foxm1* roles in physiological β -cell proliferation (10,39). Collectively, these prior studies support the view that targets of PRLR signaling regulate gestational β -cell expansion.

The significance of MafB⁺ β -cells during pregnancy was previously unknown. Our studies demonstrate intact PRLR

signaling is required for the induction of MafB in a subset of β -cells during pregnancy. In this study, we report that MafB expression is necessary for proliferation of β -cells in pregnancy. Moreover, MafB was required for pregnancy-induced islet serotonin production and *Foxm1* upregulation. Prior studies identified roles for MafB in islet development and postnatal expansion, in which it regulates terminal differentiation and maturation (17,31); a role for MafB in proliferation has also been established in pathological settings (40). Our studies reveal a role in physiologic proliferation of gestation, in which MafB⁺ β -cells maintain their hallmark features, including production of insulin and Pdx1 and suppression of glucagon. This contrasts with re-expression of MafB in α -like β -cells observed after *Pdx1* or *Foxo1* loss, in which β -cell dedifferentiation contributes to diabetes pathogenesis (41). Thus, in normal pregnancy, transient expression of MafB by Ins⁺ cells does not appear to reflect dedifferentiation. Future studies will be necessary to identify the specific mechanisms by which *MafB*, *Foxm1*, and *Tph1* coordinate and control proliferation and mass expansion during gestation.

We and others have previously described additional putative PRLR signaling targets, including *Men1*, *p18*, *p27*, and *Foxd3* (14,25,26,42). For example, we found prolactin was sufficient to induce *Men1* gene expression changes in islets and that *Men1* induction was sufficient to impair maternal β -cell expansion (25). However, in β PRLRKO pregnant mice, we did not detect altered expression of *Men1*, *p18*, or *p27*, suggesting PRLR signaling is not necessary for regulating these factors. These findings may reflect differences intrinsic to gain- and loss-of-function models, in vitro and in vivo experimentation, mouse strain differences, or yet-unidentified modifying factors that contribute to the regulation of these genes during pregnancy. Pregnancy is a complex, transient, polyhormonal state, and our findings highlight the importance of in vivo loss-of-function models for genetic analysis of putative target genes.

Brouwers et al. (32) report of human growth hormone minigene effects on islets in mice harboring transgenes like *RIP-Cre^M* highlights the importance of using *RIP-Cre^M* control littermates in physiologic studies. The β PRLRKO mice lack the very receptor believed to mediate the augmentation of β -cell mass by human growth hormone (32). Thus, the β PRLRKO model should be resistant to possible confounding changes caused by the *RIP-Cre* transgene.

Prior studies suggest that human β -cells expand in pregnancy, but the molecular and cellular basis of this is not clear (43,44). For example, Butler et al. (43) have suggested that this expansion may reflect β -cell neogenesis rather than proliferation, a possibility consistent with the recent finding of maternal β -cell neogenesis in rodents (10,45). A recent study identified single nucleotide polymorphisms in the 5' untranslated region of *PRLR* associated with GDM risk in humans (46). Intriguingly, induction of *TPH1* and islet serotonin also occurs in human pregnancy (6), but it is not known whether this

reflects increased β -cell PRLR signaling. However, PRLR expression is much lower in human than rodent β -cells (47), and MafB expression, although restricted to α -cells in adult rodents, is also present in β -cells from a nonpregnant human (48). Other correlative studies have implicated a more general role for PRLR signaling in human glucose control and metabolism. Higher prolactin levels are associated with a reduced risk of glucose intolerance and diabetes in both men and women (49). A human PRLR disease-associated mutation causing familial hyperprolactinemia (50) was recently described, although information on diabetes or glucose homeostasis in this family was not reported. Thus, further studies are needed to assess the role of PRLR signaling in human β -cell biology in both gestational and nongestational settings.

In summary, we produced and characterized mice lacking PRLR in β -cells during gestation and found these mice developed hallmark features of GDM. These studies establish the *in vivo* requirement of PRLR in β -cells for serotonin production and for modulating expression of genes critical for β -cell proliferation during pregnancy and identify a novel role for MafB in gestational proliferation. PRLR signaling is thought to modulate the development and function of many tissues (5), including bone, immunity, adipose metabolism, and breast and prostate cancer pathogenesis (15). Thus, the conditional *Prlr^f* allele developed in this study will be useful to examine PRLR signaling in other settings relevant to human health.

Acknowledgments. The authors thank members of the Kim laboratory and Drs. Hail Kim (Korea Advanced Institute of Science and Technology), Justin Annes (Stanford University School of Medicine), and Mitchell Lazar (Perelman School of Medicine, University of Pennsylvania) for helpful discussions and advice. Kartik Viswanathan helped with initial stages of the project, and Dr. Pei Wang (School of Medicine, UT Health Sciences Center, San Antonio, TX) provided help with design and construction of the *Prlr^f* allele.

Funding. R.R.B. is supported by a National Institute of Diabetes and Digestive and Kidney Diseases Mentored Clinical Scientist Research Career Development Award (K-08 DK091359). H.A.C. is supported by National Institutes of Health (NIH) National Research Service Award 1F-32 DK-102283. H.C. is supported by a JDRF postdoctoral award. H.P. is supported by American Diabetes Association grant 1-16-PDF-086. Work in the laboratory of R.W.S. is supported by National Institutes of Health/National Institute of Diabetes and Digestive and Kidney Diseases grants DK-090570, DK-089572, and DK-050203. The laboratory of S.K.K. is supported by the Snyder Foundation and grants from the NIH, JDRF, and Helmsley Trust. S.K.K. is a Howard Hughes Medical Institute Investigator.

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. R.R.B., R.W.S., and S.K.K. conceived and designed the experiments and wrote the manuscript. R.R.B., H.A.C., E.M.W., H.C., H.P., X.G., Y.L., and E.C. performed experiments and analyzed data. L.G. provided MafB floxed mice. All authors reviewed and edited the manuscript. S.K.K. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

References

1. Sorenson RL, Brelje TC. Adaptation of islets of Langerhans to pregnancy: beta-cell growth, enhanced insulin secretion and the role of lactogenic hormones. *Horm Metab Res* 1997;29:301–307

2. Halban PA, Polonsky KS, Bowden DW, et al. β -cell failure in type 2 diabetes: postulated mechanisms and prospects for prevention and treatment. *J Clin Endocrinol Metab* 2014;99:1983–1992

3. Ernst S, Demirci C, Valle S, et al. Mechanisms in the adaptation of maternal beta-cells during pregnancy. *Diabetes Manag (Lond)* 2011;1:239–248

4. Goffin V, Binart N, Touraine P, Kelly PA. Prolactin: the new biology of an old hormone. *Annu Rev Physiol* 2002;64:47–67

5. Freemark M, Driscoll P, Maaskant R, Petryk A, Kelly PA. Ontogenesis of prolactin receptors in the human fetus in early gestation. Implications for tissue differentiation and development. *J Clin Invest* 1997;99:1107–1117

6. Kim H, Toyofuku Y, Lynn FC, et al. Serotonin regulates pancreatic beta cell mass during pregnancy. *Nat Med* 2010;16:804–808

7. Layden BT, Durai V, Newman MV, et al. Regulation of pancreatic islet gene expression in mouse islets by pregnancy. *J Endocrinol* 2010;207:265–279

8. Rieck S, White P, Schug J, et al. The transcriptional response of the islet to pregnancy in mice. *Mol Endocrinol* 2009;23:1702–1712

9. Pechhold S, Stouffer M, Walker G, et al. Transcriptional analysis of intracytoplasmically stained, FACS-purified cells by high-throughput, quantitative nuclease protection. *Nat Biotechnol* 2009;27:1038–1042

10. Zhang H, Zhang J, Pope CF, et al. Gestational diabetes mellitus resulting from impaired beta-cell compensation in the absence of FoxM1, a novel downstream effector of placental lactogen. *Diabetes* 2010;59:143–152

11. Pasek RC, Gannon M. Advancements and challenges in generating accurate animal models of gestational diabetes mellitus. *Am J Physiol Endocrinol Metab* 2013;305:E1327–E1338

12. Freemark M, Avril I, Fleenor D, et al. Targeted deletion of the PRL receptor: effects on islet development, insulin production, and glucose tolerance. *Endocrinology* 2002;143:1378–1385

13. Ormandy CJ, Camus A, Barra J, et al. Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. *Genes Dev* 1997;11:167–178

14. Huang C, Snider F, Cross JC. Prolactin receptor is required for normal glucose homeostasis and modulation of beta-cell mass during pregnancy. *Endocrinology* 2009;150:1618–1626

15. Ben-Jonathan N, Hugo ER, Brandebourg TD, LaPensee CR. Focus on prolactin as a metabolic hormone. *Trends Endocrinol Metab* 2006;17:110–116

16. Herrera PL. Adult insulin- and glucagon-producing cells differentiate from two independent cell lineages. *Development* 2000;127:2317–2322

17. Yu WM, Appler JM, Kim YH, Nishitani AM, Holt JR, Goodrich LV. A Gata3-Mafb transcriptional network directs post-synaptic differentiation in synapses specialized for hearing. *eLife* 2013;2:e01341

18. Postic C, Shiota M, Niswender KD, et al. Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic beta cell-specific gene knock-outs using Cre recombinase. *J Biol Chem* 1999;274:305–315

19. Banerjee RR, Rangwala SM, Shapiro JS, et al. Regulation of fasted blood glucose by resistin. *Science* 2004;303:1195–1198

20. Goodyer WR, Gu X, Liu Y, Bottino R, Crabtree GR, Kim SK. Neonatal β cell development in mice and humans is regulated by calcineurin/NFAT. *Dev Cell* 2012;23:21–34

21. Lee JY, Ristow M, Lin X, White MF, Magnuson MA, Hennighausen L. RIP-Cre revisited, evidence for impairments of pancreatic beta-cell function. *J Biol Chem* 2006;281:2649–2653

22. Solomon CG, Willett WC, Carey VJ, et al. A prospective study of pregravid determinants of gestational diabetes mellitus. *JAMA* 1997;278:1078–1083

23. Schraenen A, Lemaire K, de Faudeur G, et al. Placental lactogens induce serotonin biosynthesis in a subset of mouse beta cells during pregnancy. *Diabetologia* 2010;53:2589–2599

24. Plank JL, Frist AY, LeGrone AW, Magnuson MA, Labosky PA. Loss of Foxd3 results in decreased β -cell proliferation and glucose intolerance during pregnancy. *Endocrinology* 2011;152:4589–4600

25. Karnik SK, Chen H, McLean GW, et al. Menin controls growth of pancreatic beta-cells in pregnant mice and promotes gestational diabetes mellitus. *Science* 2007;318:806–809
26. Arumugam R, Fleenor D, Lu D, Freemark M. Differential and complementary effects of glucose and prolactin on islet DNA synthesis and gene expression. *Endocrinology* 2011;152:856–868
27. Huang C. Wild-type offspring of heterozygous prolactin receptor-null female mice have maladaptive β -cell responses during pregnancy. *J Physiol* 2013;591:1325–1338
28. Rieck S, Kaestner KH. Expansion of beta-cell mass in response to pregnancy. *Trends Endocrinol Metab* 2010;21:151–158
29. Brelje TC, Allaire P, Hegre O, Sorenson RL. Effect of prolactin versus growth hormone on islet function and the importance of using homologous mammosomatotropic hormones. *Endocrinology* 1989;125:2392–2399
30. Artner I, Le Lay J, Hang Y, et al. MafB: an activator of the glucagon gene expressed in developing islet alpha- and beta-cells. *Diabetes* 2006;55:297–304
31. Artner I, Bianchi B, Raum JC, et al. MafB is required for islet beta cell maturation. *Proc Natl Acad Sci U S A* 2007;104:3853–3858
32. Brouwers B, de Faudeur G, Osipovich AB, et al. Impaired islet function in commonly used transgenic mouse lines due to human growth hormone minigene expression. *Cell Metab* 2014;20:979–990
33. Vasavada RC, Garcia-Ocaña A, Zawalich WS, et al. Targeted expression of placental lactogen in the beta cells of transgenic mice results in beta cell proliferation, islet mass augmentation, and hypoglycemia. *J Biol Chem* 2000;275:15399–15406
34. Binart N, Melaine N, Pineau C, et al. Male reproductive function is not affected in prolactin receptor-deficient mice. *Endocrinology* 2003;144:3779–3782
35. Auffret J, Freemark M, Carré N, et al. Defective prolactin signaling impairs pancreatic β -cell development during the perinatal period. *Am J Physiol Endocrinol Metab* 2013;305:E1309–E1318
36. Lee JY, Gavrilova O, Davani B, et al. The transcription factors Stat5a/b are not required for islet development but modulate pancreatic beta-cell physiology upon aging. *Biochim Biophys Acta* 2007;1773:1455–1461
37. Iida H, Ogiwara T, Min MK, et al. Expression mechanism of tryptophan hydroxylase 1 in mouse islets during pregnancy. *J Mol Endocrinol* 2015;55:41–53
38. Kim K, Oh CM, Ohara-Imaizumi M, et al. Functional role of serotonin in insulin secretion in a diet-induced insulin-resistant state. *Endocrinology* 2015;156:444–452
39. Zhang H, Ackermann AM, Gusarova GA, et al. The FoxM1 transcription factor is required to maintain pancreatic beta-cell mass. *Mol Endocrinol* 2006;20:1853–1866
40. Lu J, Hamze Z, Bonnafant R, et al. Reexpression of oncoprotein MafB in proliferative β -cells and Men1 insulinomas in mouse. *Oncogene* 2012;31:3647–3654
41. Talchai C, Xuan S, Lin HV, Sussel L, Accili D. Pancreatic β cell de-differentiation as a mechanism of diabetic β cell failure. *Cell* 2012;150:1223–1234
42. Hughes E, Huang C. Participation of Akt, menin, and p21 in pregnancy-induced beta-cell proliferation. *Endocrinology* 2011;152:847–855
43. Butler AE, Cao-Minh L, Galasso R, et al. Adaptive changes in pancreatic beta cell fractional area and beta cell turnover in human pregnancy. *Diabetologia* 2010;53:2167–2176
44. Van Assche FA, Aerts L, De Prins F. A morphological study of the endocrine pancreas in human pregnancy. *Br J Obstet Gynaecol* 1978;85:818–820
45. Hakonen E, Ustinov J, Palgi J, Miettinen PJ, Otonkoski T. EGFR signaling promotes β -cell proliferation and survivin expression during pregnancy. *PLoS One* 2014;9:e93651
46. Le TN, Elsea SH, Romero R, Chaiworapongsa T, Francis GL. Prolactin receptor gene polymorphisms are associated with gestational diabetes. *Genet Test Mol Biomarkers* 2013;17:567–571
47. Chen H, Kleinberger JW, Takane KK, et al. Augmented Stat5 signaling bypasses multiple impediments to lactogen-mediated proliferation in human β -cells. *Diabetes* 2015;64:3784–3797
48. Dai C, Brissova M, Hang Y, et al. Islet-enriched gene expression and glucose-induced insulin secretion in human and mouse islets. *Diabetologia* 2012;55:707–718
49. Wang T, Lu J, Xu Y, et al. Circulating prolactin associates with diabetes and impaired glucose regulation: a population-based study. *Diabetes Care* 2013;36:1974–1980
50. Newey PJ, Gorvin CM, Cleland SJ, et al. Mutant prolactin receptor and familial hyperprolactinemia. *N Engl J Med* 2013;369:2012–2020