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Pancreatic prolactin receptor signaling regulates maternal glucose homeostasis

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Abstract

Prolactin (PRL) signaling has been implicated in the regulation of glucose homeostatic adaptations to pregnancy. In this report, the PRL receptor (*Prlr*) gene was conditionally disrupted in the pancreas, creating an animal model which proved useful for investigating the biology and pathology of gestational diabetes including its impacts on fetal and placental development. In mice, pancreatic PRLR signaling was demonstrated to be required for pregnancy-associated changes in maternal β cell mass and function. Disruption of the *Prlr* gene in the pancreas resulted in fewer insulin-producing cells, which failed to expand appropriately during pregnancy resulting in reduced blood insulin levels and maternal glucose intolerance. This inability to sustain normal blood glucose balance during pregnancy worsened with age and a successive pregnancy. The etiology of the insulin insufficiency was attributed to deficits in regulatory pathways controlling β cell differentiation. Additionally, the disturbance in maternal blood glucose homeostasis was associated with fetal overgrowth and dysregulation of inflammation and PRL-associated transcripts in the placenta. Overall, these results indicate that the PRLR, acting within the pancreas, mediates maternal pancreatic adaptations to pregnancy. PRLR dysfunction is associated with glucose intolerance during pregnancy and pathological features consistent with gestational diabetes.

Key Words
▶ prolactin signaling
▶ gestational diabetes
▶ pregnancy
▶ pancreas

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Introduction

Glucose intolerance during pregnancy, known as gestational diabetes mellitus (GDM), is one of the most common pregnancy complications and affects nearly 17% of all pregnancies globally and ~7% in the United States (Newbern & Freemark 2011). Although diabetic symptoms associated with GDM disappear after delivery, its occurrence poses health risks for both the mother and fetus. GDM is associated with adverse pregnancy outcomes, including increased risk of miscarriage, hypertensive disorders, premature birth, cesarean delivery and development of metabolic disorders such as obesity and diabetes later in life (Vambergue & Fajardy 2011,

Zhu & Zhang 2016). GDM is a consequence of unsatisfactory pregnancy-dependent adaptations of pancreatic β cells (Rieck & Kaestner 2010).

Pregnancy is associated with significant metabolic demands that require adjustments in glucose homeostasis to meet the nutrient needs of the maternal–fetal unit (Huang *et al.* 2009, Rieck & Kaestner 2010). These metabolic adjustments are accompanied by a decline in maternal insulin sensitivity, which is often compensated by increasing maternal β cell mass and insulin secretion to maintain euglycemia (Parsons *et al.* 1992, Huang *et al.* 2009, Rieck & Kaestner 2010, Huang 2013). It is hypothesized that these maternal pancreatic adaptations are driven by the pregnancy hormones, prolactin (PRL) and placental lactogens (PLs), acting through the PRL receptor (PRLR) at the β cells within the islets of Langerhans (Freemark *et al.* 2002, Huang *et al.* 2009, Sorenson & Brelje 2009, Newbern & Freemark 2011).

PRL is a cytokine/hormone produced by the anterior pituitary and in some extra-pituitary sites (Bole-Feysot *et al.* 1998, Soares 2004, Horseman & Gregerson 2014). PRL is part of a larger protein family that includes hormones that are produced by the placenta with a similar spectrum of biological activities, referred to as PLs (Soares 2004). PRL and PLs have numerous biological functions, including regulation of lactation, morphogenesis, reproduction, metabolism and adaptations to physiological stressors (Bole-Feysot *et al.* 1998, Soares *et al.* 2007, Horseman & Gregerson 2014, Bernard *et al.* 2015). PRL and PLs act on their target cells through binding and activating the PRLR (Bole-Feysot *et al.* 1998, Soares 2004). The PRLR is a transmembrane receptor expressed in myriad of cell types throughout the body, including the pancreas (Bole-Feysot *et al.* 1998, Soares 2004, Horseman & Gregerson 2014, Bernard *et al.* 2015).

Previous *in vitro* studies using β cell cultures (Brelje *et al.* 1993, Vasvada *et al.* 2000) and *in vivo* experiments examining mice with global germline mutations at either *Prl* or *Prlr* loci (Vasvada *et al.* 2000, Freemark *et al.* 2002, Huang *et al.* 2009) have implicated a role for PRLR signaling in maternal islet adaptations. These studies have demonstrated that the PRLR is present on β cells and that its expression increases during pregnancy (Nagano & Kelly 1994). Overexpression of mouse PL-I (also known as PRL3D1) in β cells (Vasvada *et al.* 2000) or exposure of isolated islets to PRL or PLs (Brelje *et al.* 1993) coincides with pregnancy-associated increases in β cell proliferation, islet mass, insulin secretion, and a reduced threshold of glucose-stimulated insulin secretion. Conversely, homozygous nonpregnant or pregnant mice heterozygous

for the global *Prlr*-null allele have been reported to have decreased β cell mass, impaired glucose tolerance and a diminished insulin secretory response (Freemark *et al.* 2002, Huang *et al.* 2009). Collectively, these studies strongly support the idea that PRLR signaling is a key mediator of pregnancy-associated β cell proliferation, survival and insulin secretion, adaptations critical for maintaining maternal glycemic control.

Significant efforts have been directed at deciphering the *in vivo* role of PRLR signaling in regulating maternal islet cell adaptions and pregnancy-associated glucose homeostasis. Supporting data have been primarily accrued through experimentation with mice possessing global mutations at either *Prl* or *Prlr* loci (Vasvada *et al.* 2000, Freemark *et al.* 2002, Huang *et al.* 2009). Since *Prlr*-null mice are infertile, the experimental assessment of the *in vivo* role of PRLR signaling during pregnancy has been primarily restricted to assessment of maternal glucose homeostasis and pancreatic adaptations in mice heterozygous for the global *Prlr*-null allele (Huang *et al.* 2009). Although these investigations have suggested a role for PRLR signaling during maternal islet cell adaptations, the ubiquitous nature of PRLR expression does not permit a precise determination of the relative importance of PRLR signaling in the β cell versus some other targets in the body. To circumvent these impediments, Banerjee and colleagues have recently used the rat insulin promoter (RIP) *Cre* to conditionally delete *Prlr* from β cells (Banerjee *et al.* 2016). In their model, loss of PRLR signaling in β cells resulted in GDM, reduced β cell proliferation and impaired β cell mass expansion during pregnancy (Banerjee *et al.* 2016). The RIP-*Cre* has been shown to have significant *Cre* activity outside of the pancreas, including the hypothalamus (Wicksteed *et al.* 2010, Ladyman *et al.* 2017). Adding to the potential confusion, RIP-*Cre* has been demonstrated to develop glucose intolerance and impaired insulin secretion independent of a floxed target gene (Lee *et al.* 2006). Some of these actions may be the consequence of a human growth hormone minigene embedded in the RIP-Cre gene construct (Herrera 2000, Brouwers *et al.* 2014, De Faudeur *et al.* 2018).

In the current study, our goal was to investigate the role of PRLR signaling in maternal pancreatic adaptations to glucose homeostasis and islet function during pregnancy. To facilitate our investigation, we engineered a *Prlr* mutation in the mouse where exon 5, a region critical for PRLR activity (Brooks 2012), was conditionally deleted using pancreas-duodenum homeobox 1 (*Pdx1*) promoter *Cre* (Hingorani *et al.* 2003, Wicksteed *et al.* 2010, Arda *et al.* 2013, Snyder *et al.* 2013). The *Pdx1* promoter is active in

the pancreas during earlier phases of embryogenesis than the RIP promoter (Arda *et al.* 2013). Both *Pdx1-Cre* and RIP-*Cre* transgenic mice exhibit extra-pancreatic expression in the brain but most importantly expression is in spatially distinct regions of the brain (Honig *et al.* 2010, Wicksteed *et al.* 2010). Additionally, *Pdx1-Cre*, unlike RIP-*Cre*, does not contain the human growth hormone minigene (Herrera 2000, Hingorani *et al.* 2003) and its consequential artifacts. Similarities and differences achieved with *Pdx1-Cre* and RIP-*Cre* transgenic mice are informative and can provide insights into the importance of the pancreas in pregnancy-dependent glucose homeostasis. Using *Pdx1-Cre*, we show that pancreatic PRLR signaling controls pregnancy-dependent β cell expansion and prominently impacts maternal glucose homeostasis.

Materials and methods

Animals

All mice were maintained in accordance with institutional policies for the care and use of vertebrate animals in research using protocols approved by the University of Kansas Medical Center Animal Care and Use Committee. A targeting vector with *LoxP* sequences flanking Exon 5 of the mouse *Prlr* gene along with β-galactosidase (*LacZ*) and neomycin resistance (*Neo*) cassettes flanked by flippase (FLP) recognition target (*FRT*) sites within Intron 4 of the *Prlr* gene was designed and generated by the European Conditional Mouse Mutagenesis Program (EUCOMM) (Bradley *et al.* 2012). The targeting vector was transfected into E14Tg2a (129P2/OlaHsd) embryonic stem cells and G418-resistant clones were isolated and validated by PCR genotyping, DNA sequencing and karyotyping. Correctly targeted ES cells were used to generate chimeric mice via injection into C57BL/6 blastocysts by the Transgenic and Gene Targeting Facility of the University of Kansas Medical Center. Chimeras with an extensive agouti contribution were backcrossed with C57BL/6 mice, and germline transmission of the targeted allele was confirmed by PCR and genomic sequencing. The *FRT-LacZ-neo* cassette was removed by crossing with a FLP recombinase mouse (Jackson Laboratory; Stock No. 009086) (Farley *et al.* 2000) to generate *Prlr*-floxed mice (*Prlr*f/f, control). *Prlr*-floxed mice were crossed with mice expressing *Cre* recombinase under mouse *Pdx1* promoter (*Pdx1-Cre*, Jackson Laboratory; Stock No. 014647) (Hingorani *et al.* 2003), generating pancreas-specific *Prlr* conditional knockout mice (*Prlr*$^{Pdx1-d/d}$).

Pdx1-Cre transgenic mice and *Gt(ROSA)26Sor*$^{tm4(ACTB-tdTomato,-EGFP)Luo}$/J also known as ROSA$^{mT/mG}$ reporter mice were obtained from the Jackson Laboratory (Stock No. 007576). Both lines were initially generated on a mixed C57BL/6 × 129 (129X1/SvJ × 129S1/Sv) F1 background and backcrossed several generations onto C57BL/6. The ROSA$^{mT/mG}$ reporter transgene is driven by a chicken β actin promoter from the *Gt(ROSA)26Sor* genomic locus (Muzumdar *et al.* 2007). In the absence of *Cre* recombinase, ROSA$^{mT/mG}$ mice constitutively express membrane-targeted tdTomato (mT), a red fluorescent protein. When bred to mice expressing *Cre* recombinase, the mT cassette is excised in the *Cre*-expressing tissue(s), allowing expression of membrane-targeted enhanced green fluorescent protein (EGFP, mG) (Muzumdar *et al.* 2007, Snyder *et al.* 2013).

Genotyping was performed on DNA isolated from tail biopsies obtained just prior to weaning of litters (Ain *et al.* 2004, Alam *et al.* 2007, Bu *et al.* 2016, 2017) using primers provided in Table 1.

Validation of *Pdx1-Cre* specificity

To validate the specificity of *Pdx1-Cre* recombinase, we crossed *Pdx1-Cre* transgenic mice (*Pdx-Cre*+) with ROSA$^{mT/mG}$ heterozygotes. For imaging studies, 12-week-old littermates were killed and imaged simultaneously to establish optimal exposure times and to control for auto-fluorescence, particularly of the gastrointestinal tract. Fluorescence imaging of the abdominal cavity was performed using an IVIS small animal imaging system (Perkin-Elmer) with tdTomato or EGFP fluorescence illumination settings (tdTomato: 545/30 excitation and 598/55 emission; EGFP 475/40 excitation and emission 530/50).

Tissue collection

*Prlr*f/f mice were crossed with transgenic *Pdx1-Cre* mice to produce *Prlr*$^{Pdx1-d/d}$ and *Prlr*f/f offspring. Males and females of appropriate age were caged together overnight. The presence of a seminal plug in the vagina was designated gestation day (gd) 0.5. Pancreas and placental tissues were collected on gd 15.5 and weighed. Placental tissues were frozen in dry-ice-cooled heptane for immunohistochemical staining or snap-frozen in liquid nitrogen and stored at −80°C until processing. Pancreas tissues were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for histological analysis or stored in

Table 1 Primers for genotyping and qRT-PCR analyses.

Target	Forward	Reverse
Genotyping primers		
Pdx1-Cre	CTGGACTACATCTTGAGTTGCAGG	ACGGTGTACGGTCAGTAAATTTG
Prlr14732	ATGCCACTTTCCAAGGTCTG	GCCATTGCAGCTGTAGTCAA
qRT-PCR primers		
Prl5a1	ATGCGGCTGTCTAAGATTCAAC	CTTCCATGATACATCTGGGCAC
Prl8a8	ACCCACGGATGGAAACATTTG	TGCAGCTCTGAAAACAATCTCAT
Prl7a2	GCCTCTGTACCTTTGAGTAGCA	CGCAGTTCCATGTTGAGGTTTTT
Tnfa	CCAGTGTGGGAAGCTGTCTT	AAGCAAAAGAGGAGGCAACA
Cxcl2	AACATCCAGAGCTTGAGTGTGA	TTCAGGGTCAAGGCAAACTT
Socs1	CTGCGGCTTCTATTGGGGAC	AAAAGGCAGTCGAAGGTCTCG
Il10	TGGCCTTGTAGACACCTTGG	AGCTGAAGACCCTCAGGATG
Prlr	AAAACATGTCATCTGCACTT	TGGTAGGTGGCAACCATTTT
Foxm1	CTGATTCTCAAAAGACGGAGGC	TTGATAATCTTGATTCCGGCTGG
Mafa	AGGAGGAGGTCATCCGACTG	CTTCTCGCTCTCCAGAATGTG
Ins2	GCTTCTTCTACACACCCATGTC	AGCACTGATCTACAATGCCAC
Ngn3	CCGGATGACGCCAAACTTA	CATAGAAGCTGTGGTCCGCTATG
Amy2a	TTGCCAAGGAATGTGAGCGAT	CCAAGGTCTTGATGGGTTATGAA
Pdx1	CCCCAGTTTACAAGCTCGCT	CTCGGTTCCATTCGGGAAAGG
Sox17	GATGCGGGATACGCCAGTG	CCACCACCTCGCCTTTCAC
Hnf4a	CACGCGGAGGTCAAGCTAC	CCCAGAGATGGGAGAGGTGAT
Tph1	ACTGGAGAATAGAACACCAGAGC	TGTAACAGGCTCACATGATTCTC

RNAlater stabilization solution (Thermo Fisher Scientific, catalog No. AM7020) at −80°C until processed.

RNA isolation, cDNA synthesis and transcript measurements

Total RNA was isolated from tissues from second pregnancies using TRIzol reagent (Thermo Fisher Scientific, catalog No. 15596018). cDNA was synthesized from 1 µg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, catalog No. 4368813). Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed on a reaction mixture (20 µL) containing cDNA diluted five times with water and PowerSYBR Green PCR Master Mix (Applied Biosystems, catalog No. 4367659) using specific primer (250 nM) sequences (Table 1). Amplification and florescence detection were carried out using an ABI 7500 Real Time PCR system (Applied Biosystems) for 40 cycles (95°C for 10 min; 92°C for 15 s; 60°C for 1 min; 95°C for 15 s; 60°C for 15 s; and 95°C for 15 s). Relative transcript expression was calculated by ΔΔCt method and normalized to 18S rRNA.

Western blotting

Maternal pancreas tissues obtained from second pregnancies were homogenized in RIPA lysis buffer (Santa Cruz Biotechnology; catalog No. sc-24948A) supplemented with Halt Protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, catalog No. 78443). Protein concentrations were determined by the DC protein assay (Bio Rad). A total of 50 µg of protein per reaction sample were separated on 4–20% ExpressPlus PAGE Gels (GenScript, Piscataway, NJ, USA; catalog Nos. M42012 and M42015) and transferred to PVDF Blotting Membrane (GE Healthcare; catalog No. 10600023). Following transfer, membranes were blocked in 5% non-fat milk in Tris-buffered saline with 0.1% Tween 20, for non-specific binding and subsequently probed with specific primary antibodies to SRY-box 17 (SOX17; 1:500, Santa Cruz Biotechnology, catalog No. sc-130295), phosphorylated signal transducer and activator of transcription 5 (STAT5^{pTyr694}, DH47E7, 1:500, Cell Signaling Technology, catalog No. 4322), PDX1 (D59H3; 1:1000, Cell Signaling Technology, catalog No. 5679) and glyeraldehyde-3-phosphate dehydrogenase (GAPDH, 1:300, Abcam, catalog No. ab9485). Immunoreactive proteins were visualized by Luminata Crescendo Western HRP Substrate (Millipore, catalog No. WBLUR0500) according to the manufacturer's protocol.

Histology, immunofluorescence and immunohistochemistry

Nonpregnant and gd15.5, *Prlr$^{f/f}$* and *Prlr$^{Pdx1-d/d}$* mice were killed, and the entire pancreas was removed, weighed and fixed in 4% paraformaldehyde-PBS solution at 4°C overnight. Tissues were dehydrated, embedded in paraffin and sectioned at 7 µm. Every 40th section was stained for

insulin (1:250, Cell Signaling Technology, catalog No. C27C9 or 1:100, GeneTex, Irvine, CA, USA, catalog No. GTX27842) to identify β cells and β cell mass. Briefly, for each pancreas section, adjacent nonoverlapping areas of the entire pancreas section were imaged using fluorescence microscopy. NIH ImageJ software was used to measure β cell (insulin-positive) area and total pancreas area. The number of islets (defined as insulin-positive cell clusters at least 25 μm in diameter) were counted and mean islet size was calculated as the ratio of total insulin-positive cell area to the total islet number. β cell fraction was measured as the ratio of the insulin-positive cell area to the total tissue area represented on the section. Finally, β cell mass was determined by multiplying the β cell fraction by the weight of the pancreas.

Intraperitoneal glucose tolerance test (IGTT) and insulin measurements

IGTTs were performed on non-pregnant, gd 15.5 pregnant and postpartum day 4 females. Mice were fasted for 6 h with free access to water and then injected intraperitoneally (i.p.) with a D-glucose solution (2 g/kg body weight). Blood glucose levels were measured from the tail vein using the OneTouch Ultra Smart blood glucose monitoring system (Lifescan, Milpitas, CA, USA) just before the i.p. injection (time-0) and at 15, 30, 60, 90 and 120 min post injection. Glucose excursions were measured using standard methods. Blood samples (30 μL) were collected at time-0 and 30-min post glucose injection for measurement of insulin using a mouse insulin enzyme-linked immunosorbent assay kit (Crystal Chem, Elk Grove Village, IL, USA, catalog No. 90080).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6 software with either two-way ANOVA followed by Bonferroni multiple comparisons or unpaired Student's t tests applied when appropriate. Data are represented as mean±S.D. with the statistical significance level set at $P < 0.05$.

Results

Generation and validation of pancreatic disruption of the mouse *Prlr* gene

A germ line allele possessing *LoxP* sequences flanking Exon 5 of the *Prlr* gene was successfully generated (Fig. 1A).

To validate *Pdx1-Cre* recombinase activity within the pancreas, we used the ROSA^mT/mG indicator mouse, which constitutively expresses a conditional tdTomato transgene that converts to GFP expression following exposure to *Cre* recombinase. Bioluminescent images acquired using an IVIS small animal imaging system showed that tdTomato was ubiquitously expressed with brighter expression within the pancreas of the ROSA^mT/mG littermate lacking *Pdx1-Cre*, whereas GFP expression was evident within the pancreas and duodenum of *Pdx1-Cre*/ROSA^mT/mG littermates (Supplementary Fig. 1, see section on supplementary data given at the end of this article) confirming the activity of *Pdx1-Cre* recombinase (Snyder *et al.* 2013). To demonstrate the tissue specificity of the *Prlr* gene disruption, PCR was performed on genomic DNA samples from various tissues of *Prlr^Pdx1-d/d* mice. The deleted *Prlr* allele was only present in pancreatic DNA from mice of this genotype (Fig. 1B). Using specific primers, qRT-PCR verified the deletion of Exon 5 of the *Prlr* gene in RNA from pancreatic tissue of *Prlr^Pdx1-d/d* mice (Fig. 1C).

We next investigated PRLR signaling in the pancreas of control (*Prlr^f/f*) and pancreatic PRLR-deficient (*Prlr^Pdx1-d/d*) mice. It has been previously reported that PRLR signaling, in β cells, is mediated at least in part by activation of STAT5 (Brelje *et al.* 2002, Friedrichsen *et al.* 2003, Huang *et al.* 2009). Consequently, we determined the activation state of STAT5 by assessing its phosphorylation on Tyr-694. We also examined the expression of known transcriptional targets of PRLR signaling in pancreatic tissue (tryptophan hydroxylase-1, *Tph1*; Huang *et al.* 2009, Rieck & Kaestner 2010; forkhead box M1, *Foxm1*; Zhang *et al.* 2005, 2010; MAF BZIP transcription factor A, *Mafa*; Zhang *et al.* 2005) of pregnant *Prlr^f/f* and *Prlr^Pdx1-d/d* mice. STAT5p^Tyr694 protein was diminished in tissue sections and lysates prepared from pancreatic tissue of gd 15.5 *Prlr^Pdx1-d/d* vs *Prlr^f/f* mice (Fig. 2A and B). qRT-PCR results further demonstrated that *Tph1*, *Foxm1* and *Mafa* were expressed at lower levels in gd 15.5 pancreatic tissue from *Prlr^Pdx1-d/d* compared to *Prlr^f/f* mice (Fig. 2C).

In summary, loss of the pancreatic *Prlr* in *Prlr^Pdx1-d/d* mice resulted in decreased pancreatic phosphorylation of STAT5 concomitant with diminished abundance of pancreatic *Tph1*, *Foxm1* and *Mafa* transcripts, confirming the efficacy of interfering with β cell PRLR signaling.

Pancreatic deficient *Prlr* mice have impaired pregnancy-dependent glucose homeostasis

PRLR signaling has been implicated in the regulation of pancreatic adaptations to pregnancy (Vasvada *et al.* 2000,
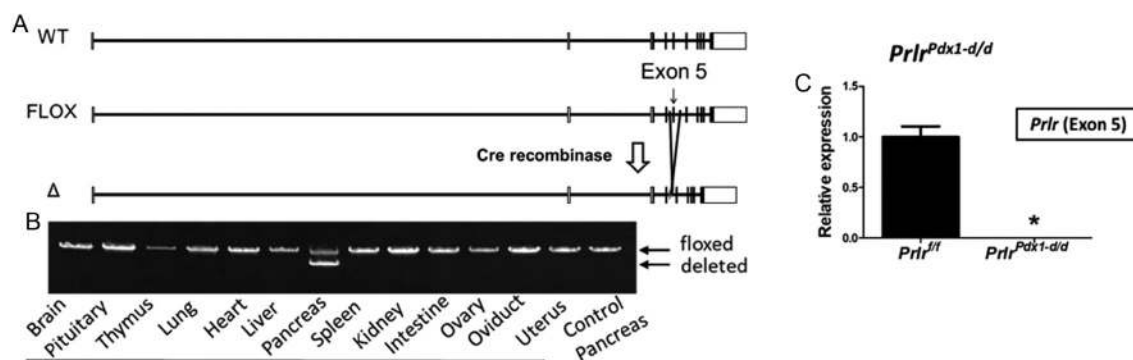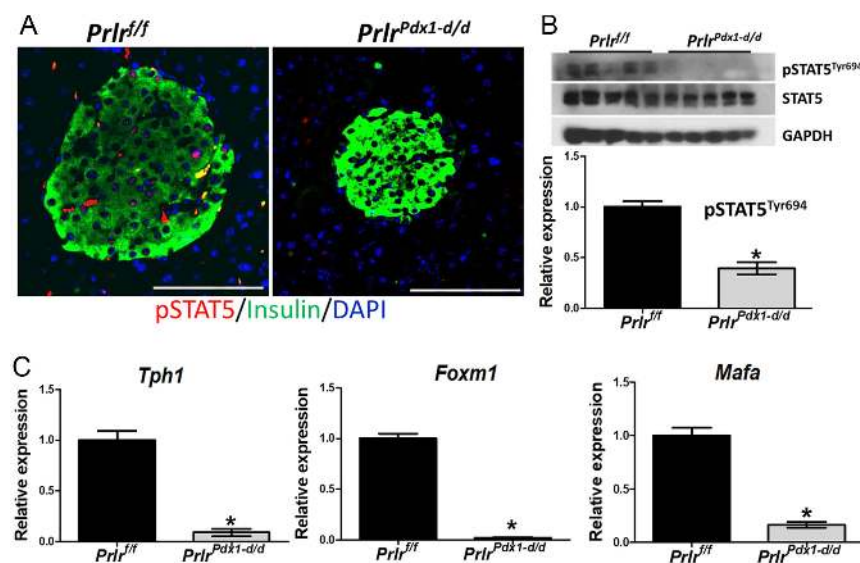
Figure 1

Generation and characterization of *Pdx1-Cre*-driven disruption of the floxed *Prlr* allele. (A) Schematic representations of the mouse *Prlr* gene, the floxed *Prlr* allele and the null *Prlr* allele following deletion of floxed Exon 5 with *Pdx1-Cre* recombinase. (B) PCR of DNA from various tissues of *Prlr^Pdx1-d/d* mice and control pancreas. (C) qRT-PCR verification of the absence of the contributions of Exon 5 to the *Prlr* transcript in pancreas tissue of *Prlr^Pdx1-d/d* mice (mean ± S.D., Student's *t* test, *$P < 0.05$).

Freemark *et al.* 2002, Huang *et al.* 2009, Huang 2013). To examine the impact of pancreatic *Prlr*-null mutation on glucose homeostasis, we performed IGTT on virgin and pregnant *Prlr^f/f* and *Prlr^Pdx1-d/d* mice at various ages. Inactivation of PRLR signaling in the pancreas did not affect body weight of either virgin (Fig. 3A) or pregnant mice (Fig. 3D). Loss of PRLR signaling in the pancreas did not significantly affect blood glucose levels in nonpregnant females (Fig. 3B and C). However, pregnant (gd 15.5) mice possessing a pancreatic PRLR deficiency had significantly elevated fasting blood glucose and impaired glucose tolerance in comparison to control pregnant mice. Furthermore, the inability to sustain normal blood glucose balance during pregnancy persisted with increased maternal age and a second pregnancy (Fig. 3E, F, G, H and I). *Prlr^Pdx1-d/d* dams returned to normal glycemic control 4 days postpartum (Fig. 3I). Insulin responses to bolus

glucose injection were significantly blunted in *Prlr^Pdx1-d/d* dams versus age-matched *Prlr^f/f* dams (Fig. 3H). Thus, pregnant mice possessing a pancreatic deficit in PRLR signaling exhibit poor glucose homeostasis, including impairments in glucose-dependent insulin responses.

Loss of pancreatic PRLR signaling results in diminished β cell mass

During pregnancy, maternal islets go through structural and functional changes to maintain glycemic control. In rodents, the number and size of maternal β cells increase during mid-gestation resulting in an increased capacity for insulin production (Freemark *et al.* 2002, Huang *et al.* 2009, Rieck & Kaestner 2010, Huang & Chang 2014). Here, we show that relative to control mice, both virgins and pregnant mice possessing a pancreatic PRLR

Figure 2

Characterization of PRL-responsive genes in the pancreas from pregnant *Prlr^f/f* and *Prlr^Pdx1-d/d* dams on gd 15.5. (A) Immunofluorescence staining for phosphorylated STAT5^pTyr694 protein (pSTAT5; red), insulin (green), and 4′,6-diamidino-2-phenylindole (DAPI) binding (blue), scale bar: 100 µm. (B) Detection of pSTAT5 protein abundance by western blotting and quantification by densitometry. (C) qRT-PCR measurements of transcripts for known targets of PRLR signaling within the pancreas: *Tph1*, *Foxm1* and *Mafa*. Statistical analyses: $n = 5$, mean ± S.D., Student's *t* test, *$P < 0.05$.
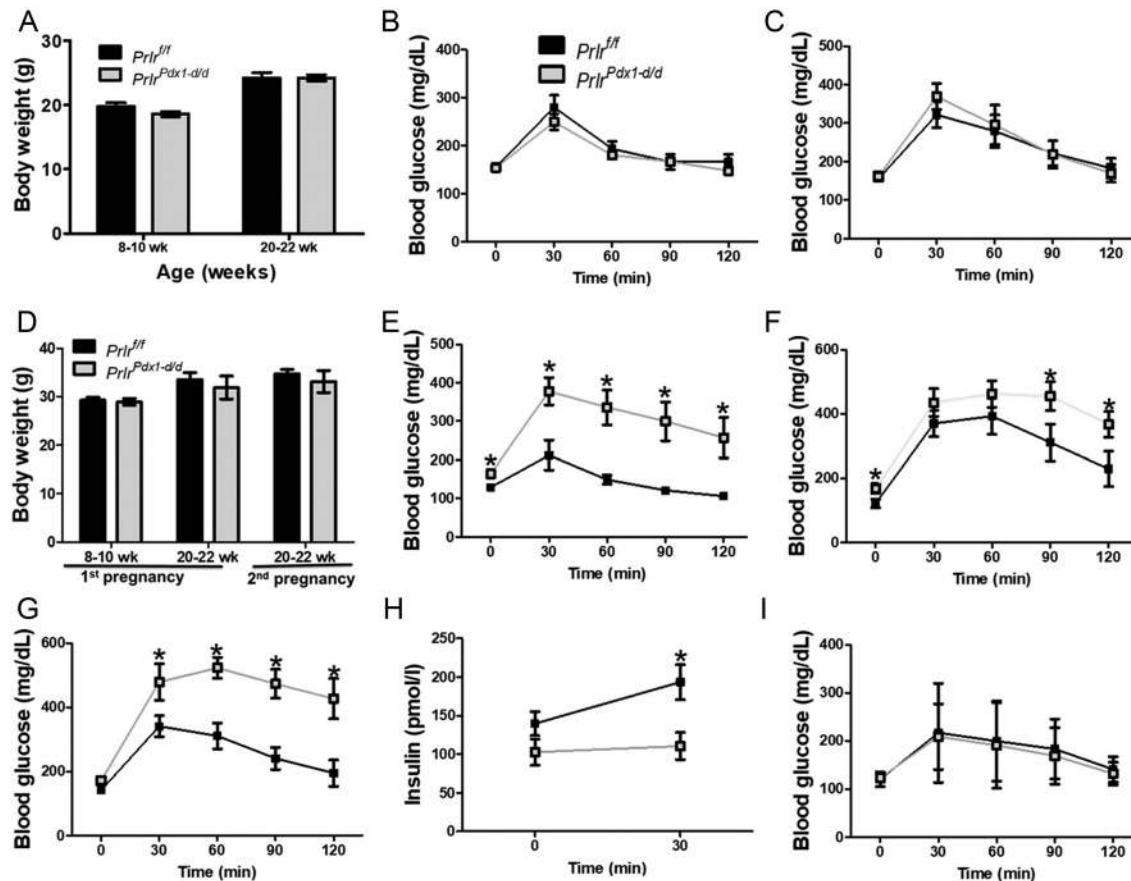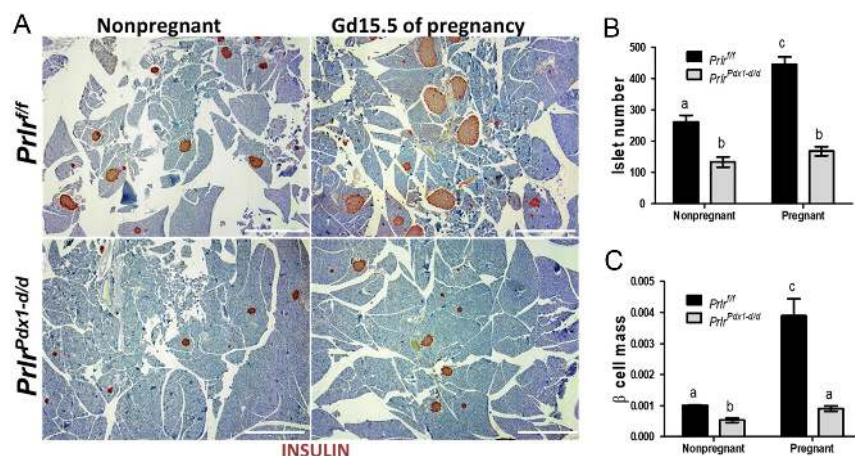
Figure 3

Glucose homeostasis in control (*Prlr^f/f*) and pancreatic *Prlr* null (*Prlr^Pdx1-d/d*) mice. (A) Body weights for virgin *Prlr^f/f* (8–10 week: *n* = 16; 20–22 week: *n* = 9) and *Prlr^Pdx1-d/d* (8–10 week: *n* = 24; 20–22 week: *n* = 10). (B) Intraperitoneal glucose tolerance tests (IGTT) for virgin *Prlr^f/f* (8–10 week: *n* = 16) and *Prlr^Pdx1-d/d* (8–10 week: *n* = 24). (C) IGTT for virgin *Prlr^f/f* (20–22 week: *n* = 9) and *Prlr^Pdx1-d/d* (20–22 week: *n* = 10) females following 6 h of fasting. (D) Body weights for pregnant *Prlr^f/f* (8–10 week-first pregnancy: *n* = 9; 20–22 week-first pregnancy: *n* = 9; 20–22 week-second pregnancy: *n* = 6) and *Prlr^Pdx1-d/d* (8–10 week-first pregnancy: *n* = 9; 20–22 week-first pregnancy: *n* = 6; 20–22 week-second pregnancy: *n* = 6) females on gd 15.5. (E, F, G, H and I) IGTT were performed on gd 15.5 female mice after 6 h of fasting: (E) 8–10 week old-first pregnancy *Prlr^f/f* (*n* = 9) and *Prlr^Pdx1-d/d* (*n* = 9) females; (F) 20–22 week old-first pregnancy *Prlr^f/f* (*n* = 9) and *Prlr^Pdx1-d/d* (*n* = 6) females; (G) 20–22 week old-second pregnancy *Prlr^f/f* (*n* = 6) and *Prlr^Pdx1-d/d* (*n* = 6) females. (H) Serum insulin levels at baseline (0) and 30 min following an intraperitoneal injection of glucose in 20–22 week-old-second pregnancy *Prlr^f/f* (*n* = 6) and *Prlr^Pdx1-d/d* (*n* = 6) female mice. (I) IGTT were performed on 20–22 week-old postpartum day 4 *Prlr^f/f* and *Prlr^Pdx1-d/d* female mice following their second pregnancy. Statistical analyses: mean ± s.d., Student's *t* test, *$P < 0.05$.

deficit possessed significantly fewer β cells (Fig. 4A and B). β cells in *Prlr^Pdx1-d/d* mice failed to optimally expand during gestation leading to a diminished fractional area of insulin-positive cells (Fig. 4A) and a reduced β cell mass (Fig. 4C). Differences were not noted in the sizes of the pancreas between *Prlr^f/f* and *Prlr^Pdx1-d/d* mice. The deficit in insulin-producing cells is consistent with sub-optimal glucose-stimulated insulin secretion and poor maternal glucose control observed in *Prlr^Pdx1-d/d* dams.

PRLR signaling and pancreas adaptations to pregnancy

To elucidate molecular mechanisms intrinsic to the abnormal β cell adaptations in *Prlr^Pdx1-d/d* animals, we next analyzed the expression of genes associated with cell differentiation in pancreatic tissue from pregnant mice. qRT-PCR analyses showed that loss of pancreatic *Prlr* was associated with a significant decrease in the expression of transcripts linked to β cell differentiation and function, including *Pdx1*, *Sox17*, *Hnf4a*, *Ngn3* and *Ins2* (Fig. 5A), and an increase in the expression of an acinar cell biomarker, *Amy2a* (Fig. 5B). Consistent with the transcript analysis, immunoblotting revealed a significant reduction in SOX17 protein expression in pancreas lysates from pregnant *Prlr^Pdx1-d/d* compared to *Prlr^f/f* dams (Fig. 5C). Differences were not noted for pancreatic transcript expression in virgin *Prlr^f/f* and *Prlr^Pdx1-d/d* mice (Supplementary Fig. 2). Our data show that loss of pancreatic PRLR signaling negatively affects islet growth-dependent adaptations to pregnancy.

Figure 4
Insulin-positive β cell distributions in pancreatic tissues from nonpregnant and gd 15.5 pregnant *Prlr$^{f/f}$* and *Prlr$^{Pdx1-d/d}$* female mice. (A) Immunohistochemical staining for insulin (brown), scale bar: 100 μm. (B) Quantification of insulin-positive islets/pancreas. (C) Quantification of β cell mass (g). Statistical analyses: nonpregnant ($n = 5$) and pregnant ($n = 5$), mean ± s.d., analysis of variance followed by Bonferroni *post hoc* test. Group means identified by different letters are significantly different, ($P < 0.05$). A full colour version of this figure is available at https://doi.org/10.1530/JOE-18-0518.

Loss of pancreatic PRLR signaling affects fetal growth and placental gene expression

GDM is associated with abnormalities in placental function and fetal growth (Vambergue & Fajardy 2011, Jarmuzek *et al.* 2015). Given the impaired glycemic control during pregnancy, we compared placental and fetal weights and placental gene expression in control and pancreatic PRLR-deficient dams. Although placental weights did not exhibit significant differences (Fig. 6A), gd 15.5 fetal and postnatal day 1 pup weights were significantly larger in second pregnancies from dams with a pancreatic deficiency in PRLR signaling (Fig. 6C and D). The disruption in pancreatic PRLR signaling was also associated with a significant dysregulation of placental PRL family and inflammation-related transcript expression (Fig. 6E and F).

Similar to GDM, mice with impaired pancreatic PRLR signaling exhibit maternal glucose intolerance, placental dysfunction and fetal overgrowth.

Discussion

Pregnancy is associated with significant metabolic demands that require adjustments in glucose homeostasis to meet the needs of the maternal–fetal unit. These adaptations involve a decline in maternal insulin sensitivity, which leads to a compensatory increase in maternal β cell mass and insulin secretion to maintain glucose homeostasis (Rieck & Kaestner 2010, Newbern & Freemark 2011). Failure to achieve optimal maternal β cell mass and function during pregnancy can result in insulin insufficiency and GDM, leading to immediate and/or long-term health complications for the mother and fetus (Vambergue & Fajardy 2011, Zhu & Zhang 2016). Although pancreatic β cell dysfunction is a primary cause of diabetes, we unfortunately do not understand all the molecular targets required for establishing and maintaining proper functional maternal pancreatic β cells during pregnancy. In this study, we established a mouse
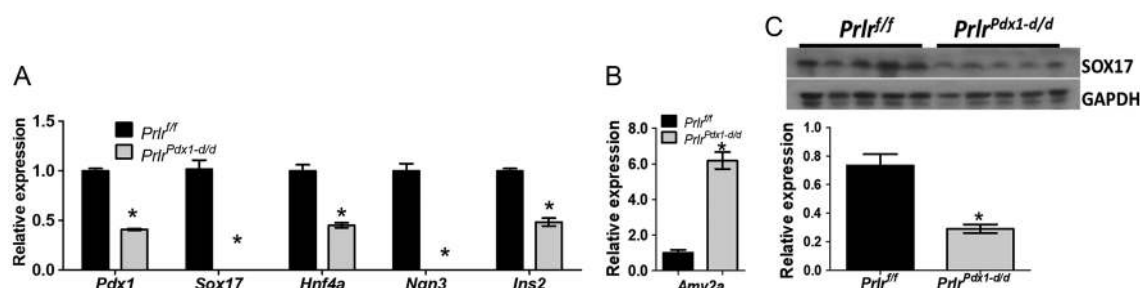
Figure 5
PRLR signaling and maternal pancreas gene expression. qRT-PCR measurements in maternal pancreatic tissue of selected transcripts associated with β cell (A) and acinar cell differentiation (B) from the second pregnancy of gd 15.5 *Prlr$^{f/f}$* and *Prlr$^{Pdx1-d/d}$* mice. Cycle threshold (Ct) values for pancreatic transcript expression at gd 15.5 *Prlr$^{f/f}$* and *Prlr$^{Pdx1-d/d}$* mice (mean Ct values ± s.d.): *Pdx1* (*Prlr$^{f/f}$*: 20.68 ± 0.46 vs *Prlr$^{Pdx1-d/d}$*: 22.88 ± 0.58) *Sox17* (*Prlr$^{f/f}$*: 20.45 ± 0.78 vs *Prlr$^{Pdx1-d/d}$*: 26.75 ± 0.67), *Hnf4a* (*Prlr$^{f/f}$*: 23.93 ± 0.97 vs *Prlr$^{Pdx1-d/d}$*: 27.40 ± 0.65), *Ngn3* (*Prlr$^{f/f}$*: 20.20 ± 0.35 vs *Prlr$^{Pdx1-d/d}$*: 30.02 ± 0.30), *Ins2* (*Prlr$^{f/f}$*: 15.39 ± 0.47 vs *Prlr$^{Pdx1-d/d}$*: 18.19 ± 0.65) and *Amy2a* (*Prlr$^{f/f}$*: 18.36 ± 0.87 vs *Prlr$^{Pdx1-d/d}$*: 14.19 ± 0.75). Relative expression refers to the ratio of *Prlr$^{Pdx1-d/d}$* transcript levels to *Prlr$^{f/f}$* transcript levels. (C) Detection of SOX17 protein abundance by western blotting and quantification by densitometry. Relative expression refers to the ratio of *Prlr$^{Pdx1-d/d}$* protein levels to *Prlr$^{f/f}$* protein levels. Statistical analyses: $n = 5$, mean ± s.d., Student's *t* test, *$P < 0.05$.
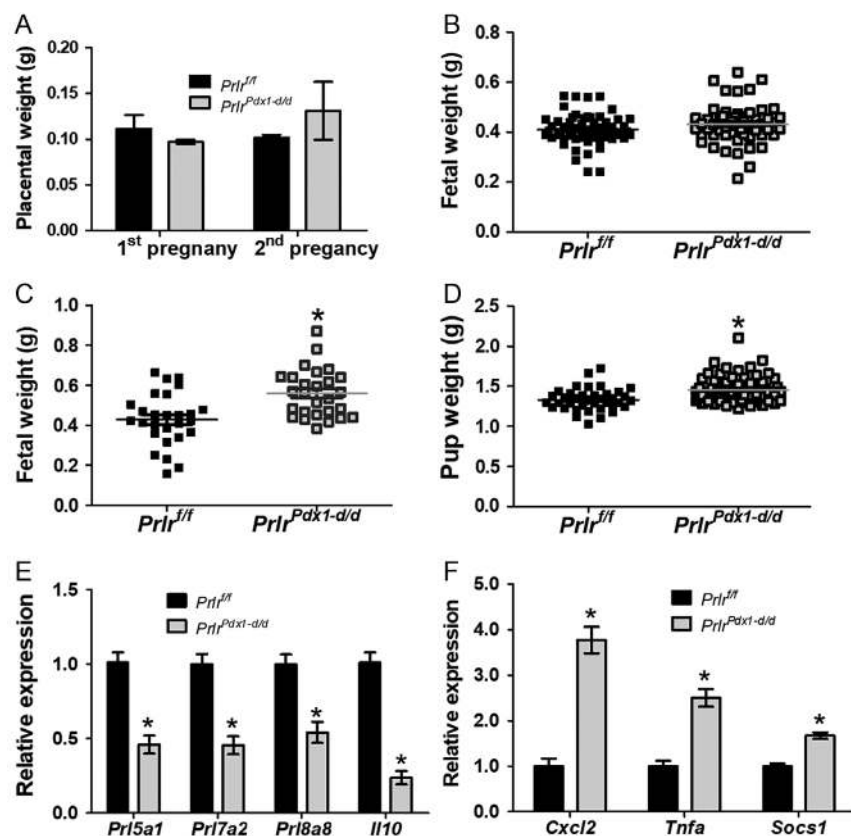
Figure 6

Loss of pancreatic PRLR signaling affects fetal growth and placental gene expression. Gestation day 15.5 placental weights from first and second pregnancies (A) and fetal weights from first (B) and second (C) pregnancies of *Prlr^f/f* (first pregnancy: *n* = 57; second pregnancy: *n* = 27) and *Prl^Pdx1-d/d* (first pregnancy: *n* = 54; second pregnancy: *n* = 27). (D) Postnatal day 1 pup weights were also measured (*Prlr^f/f*, *n* = 40; *Prl^Pdx1-d/d*, *n* = 69). qRT-PCR measurements in gd 15.5 placental tissue of selected downregulated (E) and upregulated (F) transcripts from *Prlr^f/f* (*n* = 6) and *Prlr^Pdx1-d/d* (*n* = 6) genotypes. Relative expression refers to the ratio of *Prl^Pdx1-d/d* transcript levels to *Prlr^f/f* transcript levels. Statistical analyses: mean ± s.d., Student's *t* test, *$P < 0.05$.

model with a *Prlr* gene disruption in the pancreas using *Pdx1-Cre* to test the hypothesis that PRLR signaling is required for pregnancy-dependent adaptations in maternal β cell mass and function. We report that inactivation of PRLR signaling using the *Pdx1-Cre* undermines β cell mass expansion and function, leading to impaired maternal glucose homeostasis, placental gene dysregulation and fetal overgrowth. We tracked the etiology of the insufficiency in β cells to deficits in regulatory pathways controlling their differentiation. The findings support a role for pancreatic PRLR signaling in mediating maternal islet adaptations to pregnancy.

Disrupting pancreatic PRLR signaling did not affect blood glucose levels in nonpregnant females; however, on gd 15.5, *Prlr^Pdx1-d/d* dams had elevated fasting blood glucose and impaired glucose tolerance which returned to normal glycemic control 4 days postpartum. These results are consistent with previous studies using mice heterozygous for the global *Prlr*-null allele (Huang *et al.* 2009) or RIP-*Cre* mediated inactivation of *Prlr* gene (Banerjee *et al.* 2016). In these studies, disruption of *Prlr* resulted in impaired maternal glucose homeostasis. Our data together with previous work support an *in vivo* role for pancreatic PRLR signaling as a key regulator of

maternal glucose homeostasis during murine pregnancy. Some evidence is consistent with a role for PRLR signaling in human pregnancy. For example, SNPs of the *PRLR* gene can increase the risk of GDM by more than two-fold (Le *et al.* 2013) and serum PRL levels in human pregnancies predict postpartum β cell function and the risk of diabetes with lower levels being associated with poor β cell function and higher risk of diabetes (Retnakaran *et al.* 2016). Collectively, these results indicate that pancreatic PRLR signaling is involved in regulating maternal blood glucose homeostasis during pregnancy *in vivo* and that its inactivation can predispose the mother to poor glycemic control during pregnancy.

We observed that female mice lacking the *Prlr* gene in the pancreas have fewer insulin-producing cells, which fail to expand appropriately during pregnancy. The reduced number of β cells together with compromised pregnancy-induced β cell mass expansion in *Prlr^Pdx1-d/d* dams is a potential contributing factor to the observed glucose intolerance and insulin secretion dysregulation. Indeed, others have reported decreased β cell mass, impaired glucose tolerance and a diminished insulin secretory response following PRLR inactivation (Freemark *et al.* 2002, Huang *et al.* 2009, Arumugam *et al.* 2014,

Banerjee *et al.* 2016). Conversely, overexpression of PL in β cells (Vasvada *et al.* 2000) or exposure of isolated islets to PRL or PLs (Brelje *et al.* 1993), coincide with pregnancy-associated increases in β cell proliferation, islet mass, insulin secretion and a reduced threshold of glucose-stimulated insulin secretion. Taken together, these results strongly support the idea that PRLR signaling is a mediator of β cell mass, survival and insulin secretion; adaptations critical during pregnancy to prevent pathological maternal glucose intolerance and its consequences.

Prlr^*Pdx1-d/d* female mice have fewer insulin-producing cells, which fail to expand appropriately during pregnancy, and diminished pancreatic expression of genes pivotal to the regulation of islet differentiation, including *Pdx1*, *Sox17*, *Hnf4a* and *Ngn3*. PDX-1 is a master regulator of islet development and function and controls insulin expression and other hormones produced by islet cells of the adult pancreas (Ohlsson *et al.* 1993, Miller *et al.* 1994, Gu *et al.* 2002, 2003). SOX17 is a transcription factor critical for pancreatic development (Spence *et al.* 2009) and regulates several factors involved in insulin trafficking and secretion in β cells (Jonatan *et al.* 2014). Loss of SOX17 results in improper secretion of insulin, β cell dysfunction and GLUT2 expression leading to a prediabetic state in mice (Jonatan *et al.* 2014). HNF4A directly regulates genes involved in glucose transport and glycolysis (Stoffel & Duncan 1997, Gupta *et al.* 2007). Mice carrying a null mutation in *Hnf4a* have impaired glucose-stimulated insulin secretion (Gupta *et al.* 2005, Miura *et al.* 2006). In humans, mutations in *HNF4A* are strongly associated with adult-onset diabetes (Yamagata *et al.* 1996, Stoffel & Duncan 1997, Harries *et al.* 2008) and recently haploinsufficiency *HNF4A* mutations have been associated with increased birthweight and macrosomia (Pearson *et al.* 2007). NGN3 is a transcription factor required for the development of pancreas and its expression defines progenitors that develop into endocrine cells of the pancreas (Gradwohl *et al.* 2000, Herrera *et al.* 2002, Gu *et al.* 2003, Rukstalis & Habner 2009, Wang *et al.* 2009, Gomez *et al.* 2015, Sheets *et al.* 2018). During midgestation, *Ngn3* expression has been reported to increase in maternal endocrine and exocrine compartments of the pancreas where it is thought to play a role in β cell neogenesis and proliferation (Zhang *et al.* 2010, Søstrup *et al.* 2014). The identification of a role for PRLR signaling in the β cell differentiation emerged in this investigation as a consequence of utilization of *Pdx1-Cre* to disrupt the *Prlr* gene. *Pdx1-Cre* is activated during early stages of pancreas development, in contrast to the activation of RIP-*Cre*, which is activated in terminally differentiated β cells (Herrera *et al.* 2002, Gu *et al.* 2003, Lee *et al.* 2006, Wicksteed *et al.* 2010, Arda *et al.* 2013, Banerjee *et al.* 2016).

In summary, our data using a *Pdx1-Cre* conditional *Prlr* mutant mouse model support a role for pancreatic PRLR signaling in the regulation of pregnancy-dependent glucose homeostasis and strengthen earlier observations using the RIP-*Cre* conditional *Prlr*-mutant mouse (Banerjee *et al.* 2016). The similarities of the phenotypes in these two conditional mouse models are important. Although, *Pdx1-Cre* and RIP-*Cre* are islet-targeting *Cre* recombinases, they exhibit extra-pancreatic activities and in the case of RIP-*Cre* other potential artifacts. New insights into mechanisms underlying PRLR-dependent islet expansion were linked to the regulation of pathways controlling β cell differentiation. We also found that impairments in maternal glucose homeostasis led to placental gene dysregulation and fetal overgrowth. The findings reinforce the experimental value of implementation of multiple *Cre* recombinases in the dissection of critical events regulating physiological processes.

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