Participation of Akt, Menin, and p21 in Pregnancy-Induced β-Cell Proliferation

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β-Cell mass increases during pregnancy to accommodate for insulin resistance. This increase is mainly due to β-cell proliferation, a process that requires intact prolactin receptor (Prlr) signaling. Signaling molecules that are known to regulate β-cell proliferation include Jak2, Akt, the tumor suppressor menin, and cell cycle proteins. Whether these pathways are involved in prolactin-mediated β-cell proliferation is unknown. Using the heterozygous prolactin receptor-null ($Prlr^{+/-}$) mice, we isolated pancreatic islets from both $Prlr^{+/+}$ and $Prlr^{+/-}$ mice on d 0 and 15 of pregnancy and examined the expression levels of these signaling molecules. In the wild-type mice ($Prlr^{+/+}$), both phospho-Jak2 and phospho-Akt expression in pancreatic islets increased during pregnancy, which were attenuated in the pregnant $Prlr^{+/-}$ mice. During pregnancy, menin expression was reduced by 50 and 20% in the $Prlr^{+/+}$ and the $Prlr^{+/-}$ mice, respectively, and the pregnant $Prlr^{+/-}$ mice had higher islet p18 levels than the $Prlr^{+/+}$ mice. Interestingly, between d 0 and 15 of pregnancy, expression of cyclin inhibitory protein $p21^{cip}$ was increased in the $Prlr^{+/+}$ mice, but this increase was blunted in the $Prlr^{+/-}$ mice. Lastly, we did not find any difference in the expression levels of cyclins D1, D2, and inhibitory kinases between the pregnant $Prlr^{+/+}$ and $Prlr^{+/-}$ mice. Therefore, we conclude that during pregnancy, placental hormones act through the prolactin receptor to increase β-cell mass by up regulating β-cell proliferation by engaging Jak2, Akt, menin/p18, and p21. Future studies will determine the relative contribution of these molecules in maintaining normal glucose homeostasis during pregnancy. **(*Endocrinology* 152: 847–855, 2011)**

Pancreatic β-cell mass and function exhibit significant plasticity so that in time of increased insulin requirement, such as obesity and pregnancy, β-cell number and insulin secretory capacity can increase significantly to meet this metabolic demand (1, 2). Several lines of evidence suggest that prolactin and/or placental lactogens are responsible for the pregnancy-associated changes in β-cell mass and function. First, during pregnancy, the increase in serum prolactin and placental lactogen levels parallels the increase in β-cell mass (3). Second, prolactin receptor, the receptor for both prolactin and placental lactogens, is present on pancreatic β-cells (4), and its expression increases during pregnancy (5). Furthermore, *in vitro* exposure of isolated islets to prolactin/placental lactogen showed that these hormones can increase insulin secretion and β-cell proliferation and lowers the threshold of glucose-stimulated insulin secretion, mimicking the effects of pregnancy on β-cells (6–8). Recently, we reported that intact prolactin receptors are required for maintenance of normal serum glucose during pregnancy (9). We found that transgenic mice with heterozygous deletion of the prolactin receptor ($Prlr^{+/-}$) have impaired glucose tolerance during pregnancy but normal glucose homeostasis during the nonpregnant state. In addition, the pregnant $Prlr^{+/-}$ mice have lower serum insulin levels in comparison with the $Prlr^{+/+}$ mice, which correlated with a reduced β-cell mass and a decreased β-cell proliferation rate in the pregnant $Prlr^{+/-}$ mice. These results suggest that during pregnancy, the action of pregnancy hormones is essential for maintaining adequate insulin responses by enhancing β-cell proliferation, thereby increasing β-cell mass.

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Abbreviations: CDK, Cyclin-dependent kinase; CIP, cyclin inhibitory protein; IR, insulin receptor; IRS, IR substrate; PI3K, phosphoinositol-3-kinase.

Significant efforts have been directed at deciphering the molecular mechanisms that regulate β-cell proliferation. One of the surprising and important earlier findings is that the insulin signaling pathway itself [insulin receptor (IR)/IR substrate (IRS)/phosphoinositol-3-kinase (PI3K)/Akt] is required for normal β-cell function (10). Studies in transgenic mice have found that ablation of the IR from pancreatic β-cells caused impaired glucose-stimulated insulin secretion, with a phenotype that closely resembles type 2 diabetes (11, 12). Deletion of IRS-2 has led to development of overt diabetes because these mice cannot increase β-cell mass to compensate for age-related insulin resistance (13). Downstream of the IR/IRS, Akt has been found to be important as well (14). Akt-null mice develop hyperglycemia and loss of β-cell mass with increased apoptosis (15), whereas overexpression of Akt in pancreatic β-cells increased cell size and total islet mass and prevented streptozotocin-induced diabetes (14, 16, 17). Taken together, these studies suggest that manipulation of the insulin signaling pathway within the pancreatic β-cells may lead to increased β-cell numbers.

The role of cell cycle proteins has also been examined. Both cyclins D1 and D2 are expressed in β-cells (18). Cyclin $D2^{-/-}$ mice have reduced β-cell mass and serum insulin levels as well as glucose intolerance (19). In contrast, cyclin $D1^{-/-}$ mice have a normal β-cell phenotype, but overexpression of cyclin D1 in human and rat islets enhances β-cell proliferation (20). Cyclin E is also expressed in the β-cells, and similar to the cyclin $D1^{-/-}$ mice, the cyclin $E^{-/-}$ mice had normal pancreatic β-cell growth and development (21). Nevertheless, it may potentially have a role in regulation of β-cell proliferation because one of its cofactors is the cyclin-dependent kinase (CDK)-2, another cell cycle protein that is abundantly expressed in the pancreatic β-cells. Recently, the tumor suppressor protein menin has been shown to directly regulate β-cell proliferation during pregnancy (22). Menin regulates gene transcription by promoting histone methylation. In islets, menin maintains expression of cyclin kinase inhibitors such as p27 and p18, which in turn block islet proliferation. Importantly, $menin^{+/-}$ mice developed insulinomas, whereas $p18^{-/-}$ mice had a 40% increase in β-cell mass (23). Furthermore, expression of menin, p27, and p18 are reduced during pregnancy, all at minimum levels on gestational d 13–18, coincide with the time of maximal β-cell proliferation. When menin is overexpressed in the islets, it results in reduced β-cell proliferation and β-cell mass during pregnancy, leading to gestational diabetes (22). Interestingly, prolactin infusion can also reduce menin, p27, and p18 expression while increasing β-cell proliferation (22), supporting menin as an important mediator of β-cell proliferation during pregnancy. Another cyclin kinase inhibitor, p21, may also be important during pregnancy because it is up-regulated in islets of mice that overexpress placental lactogen (24).

It is unknown whether any of the above signaling pathways are activated in the pregnant islet in a prolactin-dependent manner. Therefore, the aim of this study is to determine which of the above signaling pathways are activated in the pregnant islets and whether dysregulation in any of these signaling pathways is detected in the $Prlr^{+/-}$ islets, providing a mechanism underlying the reduced β-cell proliferation in the $Prlr^{+/-}$ mice. Here, we report that dysregulation of the IRS-2, phospho-Jak2, phospho-Akt, and Akt as well as menin/p18 and p21 expression are observed in the pancreatic islets of pregnant heterozygous prolactin receptor-null mice ($Prlr^{+/-}$). Potentially, these signaling pathways converge on the cell cycle machinery leading to lower β-cell proliferation rate in the pregnant $Prlr^{+/-}$ mice.

Materials and Methods

Mice

Heterozygous prolactin receptor-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 $Prlr^{+/+}$ mice. The pups were genotyped as previously described (25). 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. Nonfasted blood glucose was determined using a glucometer (FastTake) by sampling from tail vein at 0800 h. 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.

Materials

Protease inhibitor Complete Mini Tablet is from Roche (Indianapolis, IN). Collagenase type V (C9263), dextran (D-4751), trypsin 1 mg tablets (T7168), and all chemicals are from Sigma Chemical Co. (St. Louis, MO). Sources of antibodies are specified below.

Immunohistochemistry

Pancreas was isolated from pregnant and nonpregnant mice, weighed, fixed in 4% paraformaldehyde/PBS solution at 4 C overnight, embedded in paraffin blocks, and then longitudinally serial sectioned to 7 μm. Every 40th tissue section was stained for insulin to identify β-cells. This provides at least 280 μm distance between sections stained, minimizing the possibility of sampling the same islet twice. Antigen retrieval was achieved by incubating tissue sections with 1 mg/ml trypsin (Sigma) at 37 C for 20 min. After 1 h of blocking with 1% goat serum/PBS at room temperature, tissues were incubated with primary antibody overnight at 4 C [guinea pig antiinsulin at 1:750 (Dako, Carpinteria, CA), diluted in 1% goat serum/PBS]. This was followed by 1 h incu-

bation with fluorophore-conjugated secondary antibodies [Cy3-anti-guinea pig (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted in 1% goat serum/PBS at 1:300, room temperature]. Bis-benzimide H 33342 trihydrochloride (0.1 μg/ml; Sigma) was added to the secondary antibody for nuclear staining. Sections were mounted using DakoCytomation fluorescent mounting medium and stored at 4 C.

Islet morphometry

For each pancreas section, consecutive images of adjacent nonoverlapping areas of the entire pancreas section were acquired using a Leica (Wetzlar, Germany) fluorescence microscope, and captured with a CoolSnap digital camera (26). Images were analyzed by ImageJ software to measure the insulin-positive area as well as the area of the entire pancreas section (identified by nuclear staining). β-Cell mass was calculated by multiplying the pancreas weight by the β-cell fraction (*i.e.* the ratio of insulin-positive cell area to total pancreatic tissue area on the entire section) (27). Results represent the average of four to five tissue sections per animal from three to five animals from each genotype.

Islet isolation

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

Protein expression analysis

Whole-cell protein extract were obtained by disrupting the isolated islets in lysis buffer [2% sodium dodecyl sulfate, 125 mM Tris (pH 7), 1 mM dithiothreitol in PBS, plus protease inhibitors: Complete Mini Tablets, 50 mM NaF, 10 nM okadaic acid, 1 mM Na_3VO_4, and 1 mM phenylmethylsulfonyl fluoride] (200 islets/100 μl), sonicated three times for 30 sec, followed by protein concentration determination using the Bradford methods. Protein was separated by SDS-PAGE (30 μg for menin and IRS-2 and 10 μg for all other molecules) and transferred onto polyvinylidene difluoride filters, blocked in 3% BSA at 4 C overnight, and then incubated with primary antibody [rabbit anti-Akt and anti-phospho-Akt antibody at 1:1000 (Cell Signaling Technology, Beverly, MA), rabbit anti-IRS-2 at 1:500 (Millipore, Bedford, MA), goat antimenin antibody at 1:1000 (Bethyl Laboratories, West Grove, PA), mouse anti-p27 Kip1 antibody at 1:1000 (BD Bioscience, Palo Alto, CA), rabbit anti-p18 antibody at 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-cyclin D1 at 1:1000 (Cell Signaling), rat anti-cyclin D2 at 1:1000 (Santa Cruz), rabbit anti-p21 at 1:500 (Santa Cruz), mouse anti-p16 at 1:1000 (Santa Cruz), goat anti-p15 at 1:500 (Santa Cruz), and rabbit anti-actin antibody at 1:1000 (Cell Signaling)] at room temperature for 2 h, followed by a 1-h incubation with horseradish peroxidase-conjugated secondary antibody [sheep anti-mouse antibody at 1:10,000 and donkey antirabbit at 1:10,000-15,000 (Amersham, Piscataway, NJ), donkey antigoat at 1:5,000–10,000 (Jackson), and donkey antirat at 1:5,000–10,000 (Jackson)]. Protein was visualized by the enhanced chemiluminescence method and scanned within the linear range using ImageJ software.

Results

Pregnancy-induced increase in IRS-2 and Akt expression is blunted in the $Prlr^{+/-}$ mice

Recently, results from our *in vivo* data using a transgenic mouse with a heterozygous deletion of the prolactin receptor ($Prlr^{+/-}$) confirmed previous *in vitro* observations that placental hormones (prolactin and placental lactogen) up-regulate β-cell mass (2, 28) and that intact prolactin receptors are required for maintenance of normal glucose homeostasis during pregnancy (9). In comparison with the wild-type $Prlr^{+/+}$ mice, the mutant $Prlr^{+/-}$ mice express 50% less prolactin receptors in its islets (9), and the pregnant $Prlr^{+/-}$ mice had elevated nonfasted serum glucose ($Prlr^{+/-}$, 8.98 ± 0.22 mmol/liter; $Prlr^{+/+}$, 8.01 ± 0.25 mmol/liter; $P = 0.009$) (Fig. 1A) and reduced β-cell mass ($Prlr^{+/+}$, 3.06 ± 0.38 mg; $Prlr^{+/-}$, 2.01 ± 0.28 mg; $P = 0.04$) (Fig. 1B). It is important to note that the difference is specific to islets' response to pregnancy, because we detected no difference in any of the physiological parameters between the nonpregnant $Prlr^{+/+}$ and $Prlr^{+/-}$ mice. This difference in islet mass and serum

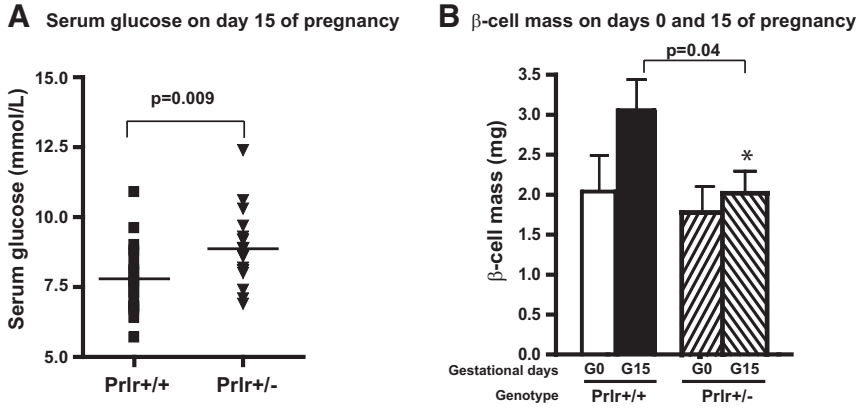

FIG. 1. Effects of heterozygous prolactin receptor deletion ($Prlr^{+/-}$) on serum glucose and β-cell mass during pregnancy. A, Serum glucose (millimoles/liter) were measured at 0800 h on nonfasted mice on d 15 of pregnancy (n = 15–20). B, β-cell mass (milligrams) on d 0 and 15 of pregnancy. β-cell mass was calculated as β-cell fraction (defined as the percent area of the pancreas section stained positive for insulin) multiplied by pancreas weight (n = 4–5). $Prlr^{+/+}$ are wild-type mice; $Prlr^{+/-}$ are heterozygous prolactin receptor-null mice. All experimental mice are 3–4 months old. *, $P < 0.05$ in comparison with the wild-type mice.

glucose was accompanied by a parallel difference in β-cell proliferation rate, such that β-cell proliferation rate in the $Prlr^{+/-}$ mice was approximately 40% of that of the $Prlr^{+/+}$ mice during pregnancy (9). To understand the mechanisms underlying the differential β-cell proliferation rate in the pregnant $Prlr^{+/+}$ *vs.* $Prlr^{+/-}$ mice, we isolated islets from both the pregnant and nonpregnant $Prlr^{+/+}$ and $Prlr^{+/-}$ mice and examined the expression and activity levels of the Jak2, IRS-2/Akt, menin/p18, and the cell cycle proteins pathways.

First, we examine the insulin signaling pathway, *i.e.* the expression of IRS-2 and Akt. IRS-2 is known to be essential for the regulation of β-cell mass (13, 29), and prolactin has been shown to activate IRS-2 (30). Here, we detected a moderate increase in IRS-2 expression during pregnancy (arbitrary units: nonpregnant in $Prlr^{+/+}$, 0.18 ± 0.03; pregnant $Prlr^{+/+}$, 0.31 ± 0.04), which was blunted in the $Prlr^{+/-}$ mice (arbitrary units: nonpregnant $Prlr^{+/-}$, 0.16 ± 0.01; pregnant $Prlr^{+/-}$, 0.23 ± 0.02; n = 10–20) (Fig. 2A). Downstream of IRS-2 in the insulin signaling pathway, Akt has also been shown to be important for regulation of β-cell proliferation (14). Here, we compare the expression levels of Akt in islets of nonpregnant *vs.* pregnant wild-type ($Prlr^{+/+}$) and mutant ($Prlr^{+/-}$) mice. In the $Prlr^{+/+}$ mice, expression of Akt in the islets was increased by 2.1-fold ($P = 0.01$) from d 0–15 of pregnancy (Fig. 2B). This increase was blunted in the $Prlr^{+/-}$ mice. Akt expression was significantly higher in the pregnant wild-type islets in comparison with the mutant islets (arbitrary units: $Prlr^{+/+}$, 2.83 ± 0.28; $Prlr^{+/-}$, 2.03 ± 0.29; $P = 0.05$; n = 9–21). We did not detect a significant difference in Akt expression levels between the nonpregnant $Prlr^{+/+}$ and $Prlr^{+/-}$ mice. Similarly, we found that Akt activation, as measured by Akt phosphorylation, was increased during pregnancy in the $Prlr^{+/+}$ mice, and this increased was blunted in the $Prlr^{+/-}$ mice (Fig. 2C).

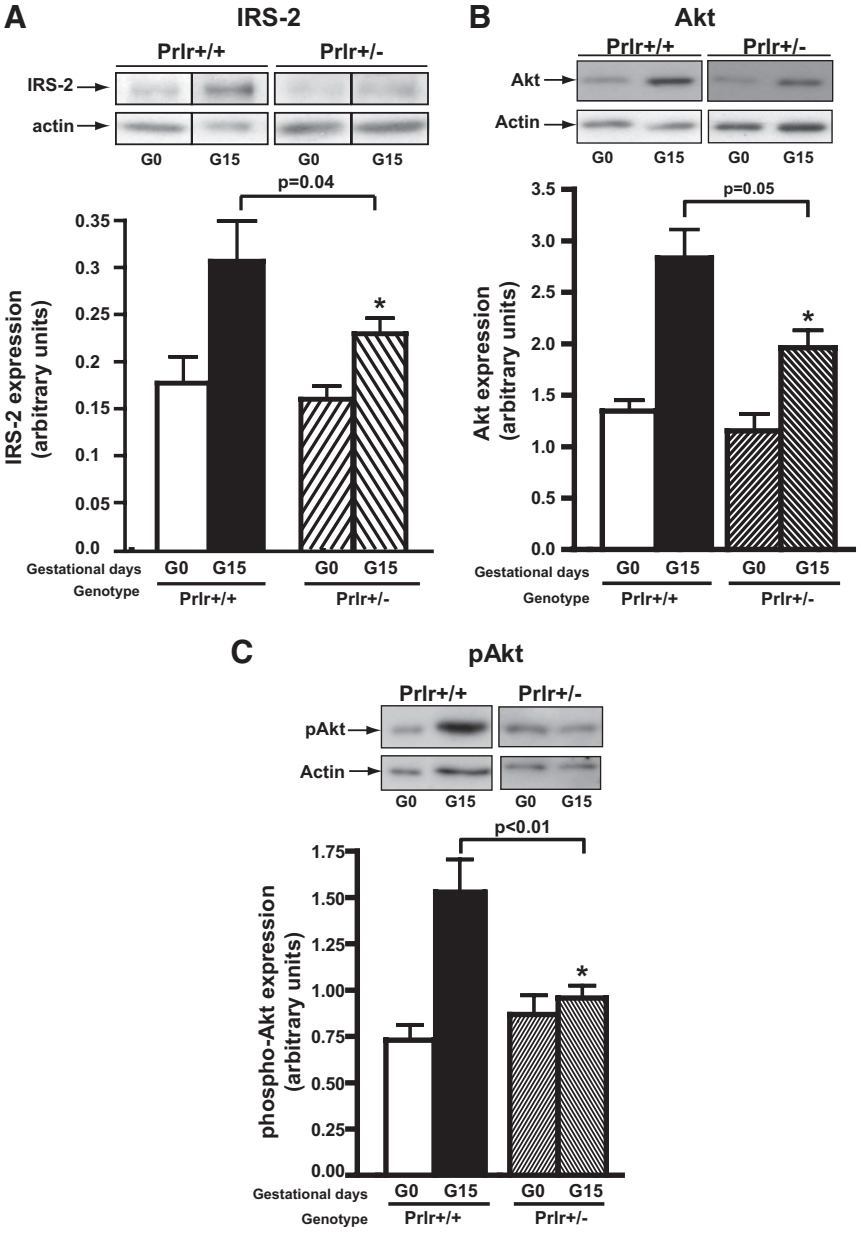

FIG. 2. Pregnancy-associated increases in IRS-2, Akt, and phospho-Akt expression were reduced in the $Prlr^{+/-}$ mice. Whole-cell lysates were prepared from isolated islets, and 10–30 μg protein from each mouse was immunoblotted for IRS-2 (A), Akt (B), phospho-Akt (C), and actin. Each lane represents one individual mouse. The OD of the IRS-2/Akt/pAkt bands were normalized to that of actin, which was used as the loading control. A representative blot is shown. Results represent mean ± SEM of nine to 21 mice from at least three separate experiments. G0, Gestational d 0 (*i.e.* nonpregnant); G15, gestational d 15. $Prlr^{+/+}$ are wild-type mice; $Prlr^{+/-}$ are heterozygous prolactin receptor-null mice. All experimental mice are 3–4 months old. *, $P < 0.05$ in comparison with the wild-type pregnant mice.

Pregnancy-induced reduction in menin expression is blunted in the $Prlr^{+/-}$ mice

Next, we examine whether the tumor suppressor menin is involved in Prlr-mediated increase in β-cell proliferation of pregnancy. In $Prlr^{+/+}$ mice, menin protein levels decreased by 50% between d 0 and 15 of pregnancy (Fig. 3A). In contrast, we did not detect a significant reduction in menin expression in the $Prlr^{+/-}$ mice during pregnancy. Downstream of menin, the expression of the inhibitory kinase p18 decreased

during pregnancy in the Prlr$^{+/+}$ mice (Fig. 3B). However, p18 expression was higher in the islets of pregnant Prlr$^{+/-}$ mice in comparison with the Prlr$^{+/+}$ mice, correlating with the decreased β-cell proliferation rate of the pregnant Prlr$^{+/-}$ mice. Interestingly, similar to findings by Zhang *et al.* (31), we did not observe a significant reduction in p27 protein expression during pregnancy (Fig. 3C).

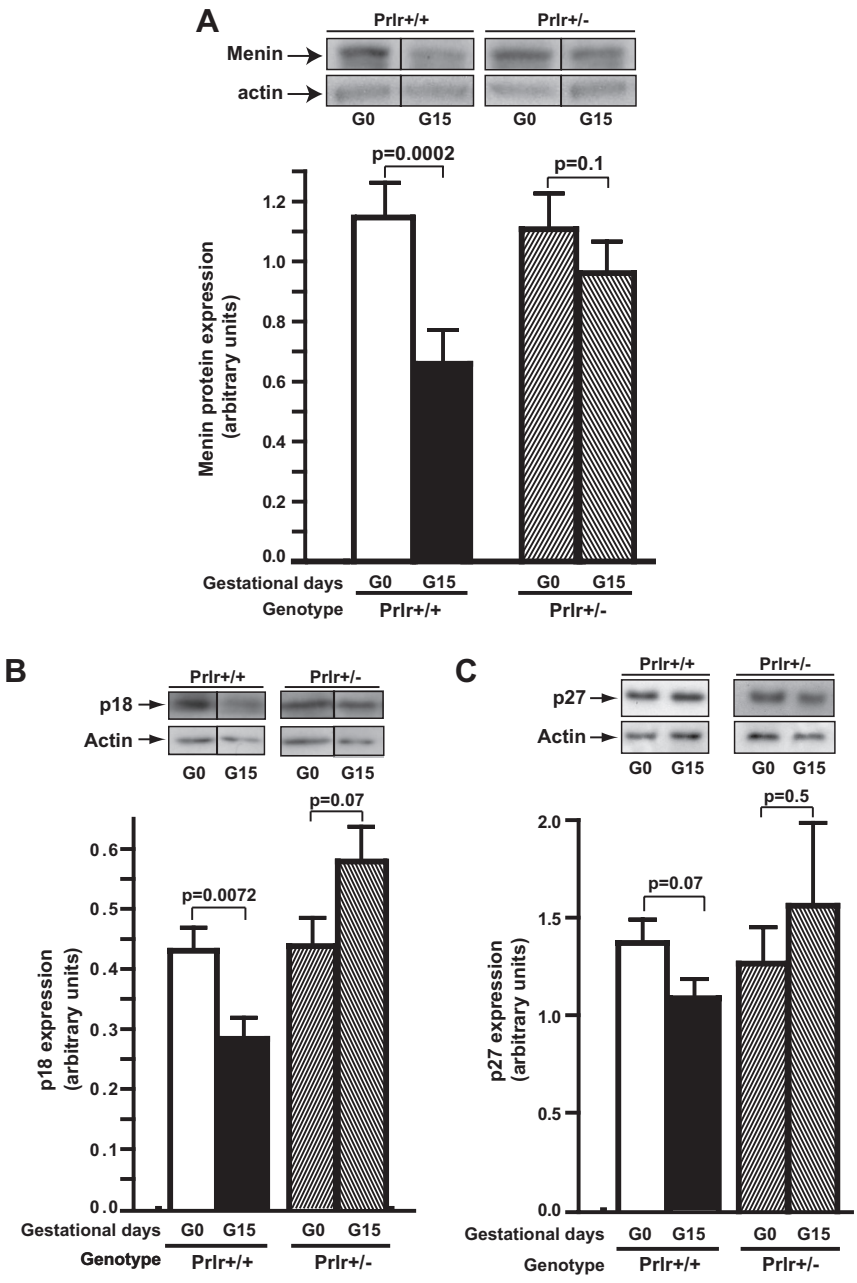

FIG. 3. Pregnancy-induced reduction in menin expression was blunted in the Prlr$^{+/-}$ mice. Whole-cell lysates were prepared from isolated islets, and 30 μg of protein from each mouse was immunoblotted for menin (A), p18 (B), p27 (C), and actin. The OD of the menin, p18, and p27 bands were normalized to that of actin, which was used as the loading control. Each lane represents one individual mouse. A representative blot is shown. Results represent mean ± SEM of 15–25 mice from at least three separate experiments. G0, Gestational d 0 (*i.e.* nonpregnant); G15, gestational d 15. Prlr$^{+/+}$ are wild-type mice; Prlr$^{+/-}$ are heterozygous prolactin receptor-null mice. All experimental mice are 3–4 months old.

The increase in p21 expression during pregnancy is diminished in Prlr$^{+/-}$ mice

Previous work from Cozar-Castellano *et al.* (18) found that in islets of transgenic mice overexpressing placental lactogen, expression of the cyclin inhibitory protein (CIP) p21 was specifically and significantly increased, whereas the expression of other CIPs (*i.e.* p27 and p57) were not affected. Here, we found that during pregnancy, p21 expression was increased by 2.00 ± 0.24-fold in the wild-type mice (Fig. 4). However, this increase was not observed in the pregnant Prlr$^{+/-}$ mice.

Next, we proceeded to examine the expression levels of cyclins and cyclin inhibitory kinases in islets during pregnancy. To our surprise, during pregnancy, we observed only a modest increase in cyclin D1 and D2 protein expression that was not statistically significant (Fig. 5, A and B). Similarly, cyclin E expression was unchanged during pregnancy (data not shown). When we examined the expression levels of inhibitory kinases, we found that expression of p16 and p15 were comparable between the islets of pregnant and nonpregnant mice (Fig. 5, C and D).

Pregnancy-induced increase in Jak2 activation is blunted in the Prlr$^{+/-}$ mutant mice

It is well established that prolactin activates the Jak2/Stat5 pathway (32, 33) and that prolactin receptors directly engages Jak2 in response to ligand binding. Here, we found that during pregnancy, there is a 2-fold increase in Jak2 phosphorylation in the Prlr$^{+/+}$ mice, whereas in the Prlr$^{+/-}$ mice, the increase is significantly blunted (Fig. 6). This change in Jak2 activation, however, was not accompanied by a change in Jak2 expression, because we cannot detect significant change in Jak2 expression during pregnancy in either the wild-type Prlr$^{+/+}$ or the Prlr$^{+/-}$ mutant mice (Fig. 6).

Discussion

Although several signaling pathways such as IRS-2, Akt, Jak2/Stat5b, and MAPK have been shown to be activated

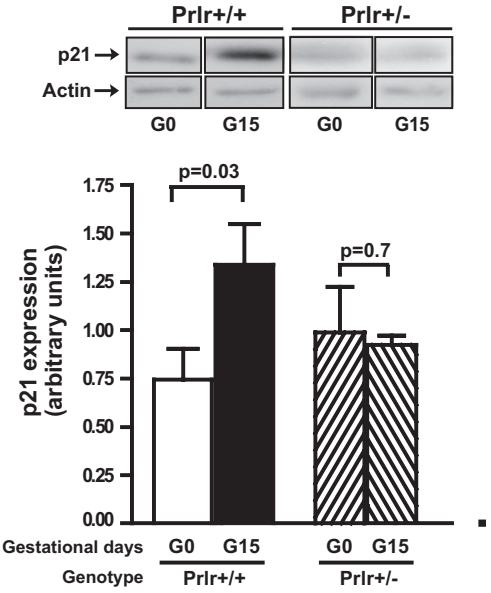

FIG. 4. p21 expression was up-regulated during pregnancy, and this response was blunted in the $Prlr^{+/-}$ mice. Whole-cell lysates were prepared from isolated islets, and 10 μg protein from each mouse was immunoblotted for p21 and actin. The OD of the p21 band was normalized to that of actin, which was used as the loading control. Each lane represents one individual mouse. A representative blot is shown. Results represent mean ± SEM of eight to 14 mice from at least three separate experiments. G0, Gestational d 0 (*i.e.* nonpregnant); G15, gestational d 15. $Prlr^{+/+}$ are wild-type mice; $Prlr^{+/-}$ are heterozygous prolactin receptor-null mice. All experimental mice are 3–4 months old.

by prolactin *in vitro*, whether these signaling pathways are activated by prolactin *in vivo* is unknown (34). This study aimed to determine the signaling pathways that are involved in regulating prolactin-mediated β-cell proliferation during pregnancy. To achieve this aim, we chose to compare the expression levels of signaling molecules in isolated islets of the pregnant $Prlr^{+/+}$ *vs.* the $Prlr^{+/-}$ mice, because we have previously shown that the $Prlr^{+/-}$ mice had reduced β-cell mass and β-cell proliferation rate in comparison with the $Prlr^{+/+}$ mice during pregnancy. We have made several novel observations here. First, the expression of IRS-2 in the pancreatic islets was up-regulated during pregnancy, and this increase was dependent on intact prolactin receptor signaling. Second, there was a significant increase in the protein expression and activation of Akt in the pregnant islet, and this increase was blunted when prolactin receptor expression was reduced in the β-cells, as was in the $Prlr^{+/-}$ mice. Third, expression of the tumor suppressor menin and its downstream effector p18 were down-regulated during pregnancy, and intact Prlr was required to maintain this response. Fourth, expression of the CIP p21 was increased during pregnancy, and this increase was blunted in the pregnant $Prlr^{+/-}$ mice. Lastly, the pregnancy-associated increase in Jak2 activation was blunted in the $Prlr^{+/-}$ mice. Together, these results represent the first *in vivo* demonstration that molecules from several distinct signaling pathways are implicated in prolactin-mediated regulation of β-cell proliferation during pregnancy.

Multiple signaling pathways participate in regulating β-cell proliferation. The insulin signaling pathway itself (IR/IRS/PI3K/Akt) is required for normal β-cell function (10). IRS-2 has an important role in β-cell proliferation (13) and we observed a moderate increase in IRS-2 expression in islets from pregnant $Prlr^{+/+}$ mice, which was blunted in the $Prlr^{+/-}$ mice (Fig. 2). Downstream of the IRS proteins, Akt also plays a role in regulating islet mass. The Akt2-null mice developed hyperglycemia and loss of β-cell mass with increased apoptosis (15), whereas overexpression of a constitutively active Akt1 in β-cells resulted in an increase in islet mass and β-cell proliferation (16), accompanied by an increase in pancreatic insulin content and mild hypoglycemia (17). In the context of pregnancy, Amaral *et al.* (30) reported that in the pregnant rat islets, expression of Akt in islets is up-regulated in comparison with the nonpregnant animal. Here, we found that during pregnancy, Akt expression in the islet increased by 2.1-fold in the $Prlr^{+/+}$ mice but only 1.7-fold in the $Prlr^{+/-}$ mice, and the expression level was significantly higher in the pregnant $Prlr^{+/+}$ mice in comparison with the $Prlr^{+/-}$ mice (Fig. 2B). More importantly, we found a significant increase in Akt activation during pregnancy, as measured by expression of phospho-Akt, and this increase was blunted in the $Prlr^{+/-}$ mice (Fig. 2C). Taken together, these results suggest that the lower β-cell proliferation rate we observed in the pregnant $Prlr^{+/-}$ mice may be in part due to the lower Akt activity.

Downstream of Akt, several signaling pathways may participate in the regulation of β-cell proliferation. In transgenic mice overexpressing a constitutively active Akt, Fatrai *et al.* (35) reported a 4.1-fold increase in cyclin D1, a 1.9-fold increase in cyclin D2, but no change in the expression levels of cyclin D3 or any of the inhibitory kinases, *i.e.* p15, p16, and p18. The observation that cyclin D2-null mice have decreased β-cell mass, reduced insulin levels, and glucose intolerance (19, 36) supports its potential role as an effector of Akt-mediated β-cell proliferation. Here, we found that during pregnancy, with a 2.1-fold increase in phospho-Akt in the $Prlr^{+/+}$ mice, there is a modest but not statistically significant 1.4-fold increase in cyclin D2 with no change in cyclin D1 expression levels. We also did not observe any changes in expression levels of p16 or p15 (Fig. 5). This result needs to be interpreted with some caution because we observed wide variations in cyclin D2 expression level between each mouse, despite sampling over 20 mice in both the pregnant and nonpregnant conditions.

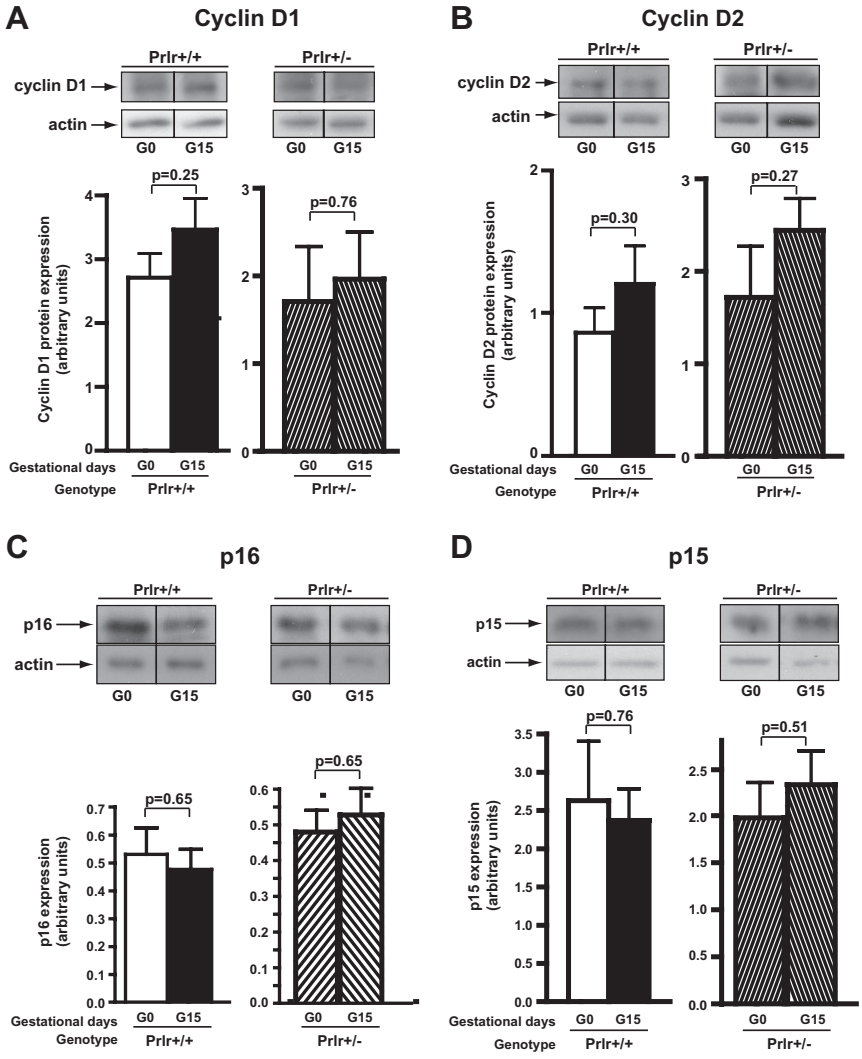

FIG. 5. Expression levels of the cyclin Ds and inhibitory kinases were unchanged during pregnancy. Whole-cell lysates were prepared from isolated islets, and 10 μg protein from each mouse was immunoblotted for cyclin D1(A), cyclin D2 (B), p15 (C), p16 (D), and actin. The OD of the band was normalized to that of actin. A representative blot is shown. Each lane represents an individual mouse. Results represent mean ± SEM of eight to 26 mice from three separate experiments. G0, Gestational d 0 (*i.e.* nonpregnant); G15, gestational d 15. $Prlr^{+/+}$ are wild-type mice; $Prlr^{+/-}$ are heterozygous prolactin receptor-null mice. All experimental mice are 3–4 months old.

In contrast to the lack of Akt-stimulated increase in cyclin and inhibitory kinase expression, the expression of the CIP $p21^{cip}$ was significantly up-regulated in the constitutively active Akt mice (35). Moreover, transgenic mice with islet-specific overexpression of placental lactogen has a significantly increased $p21^{cip}$ level with no effects on expression levels of cyclins, other CIPs, or inhibitory kinases (18). In the islets of pregnant $Prlr^{+/+}$ mice, $p21^{cip}$ expression was up-regulated, but this increase was diminished in the pregnant $Prlr^{+/-}$ mice (Fig. 4). This parallel in expression pattern of phospho-Akt and $p21^{cip}$ in the islets of pregnant mice suggest that in pregnancy, prolactin-mediated up-regulation of Akt activity leads to an increase in $p21^{cip}$ expression and that in the pregnant $Prlr^{+/-}$ mice, the absence of pregnancy-associated increase in $p21^{cip}$ expression may be a consequence of the lower Akt activity in the $Prlr^{+/-}$ mice. $p21^{cip}$ is an interesting molecule. Although it is generally considered an inhibitor of cell cycle progression, because it is a cofactor for the cyclin D-CDK4 complex, it can also accelerate cell cycle progression (37). Here, the increase in $p21^{cip}$ expression in pregnancy suggests that in the presence of a mitogen (*i.e.* prolactin), CIP such as $p21^{cip}$ attempt to limit the extent of cell proliferation so that tumorigenesis does not occur. It follows that in the pregnant $Prlr^{+/-}$ mice, the lower $p21^{cip}$ expression should lead to increased β-cell proliferation instead of the blunted β-cell proliferation as we observed in our previous study (9). A potential explanation for these seemly inconsistent observations may lie in the role of $p21^{cip}$ as a stabilizer of the cyclin D-CDK4 complex (37). It appears that $p21^{cip}$ and $p27^{cip}$ are necessary for the assembly, stability, and nuclear localization of the cyclin D-CDK4 complex, which is required for cell cycle progression (37). In the $Prlr^{+/-}$ mice, the lower $p21^{cip}$ level may contribute to less efficient assembly of the cyclin D-CDK4 complex, leading to lower cell proliferation rate. Interestingly, $p21^{cip}$-overexpressing mice recover better from streptozotocin-induced diabetes with emergence of a population of Pdx-1-positive proliferating, nonendocrine cells, suggesting that if the preexisting β-cells cannot proliferate due to high $p21^{cip}$ expression, progenitors are reactivated to form β-cells in the context of β-cell injury (38).

The relationship between the menin/p18/p27 pathway, Akt, and $p21^{cip}$/cyclins is also an unsolved question. Prolactin stimulates the phosphorylation and nuclear translocation of signal transducer and activator of transcription 5 (STAT5), inducing expression of *Bcl6*, which directly associates with and represses transcription of *Men1*, reducing menin expression (22). This reduction in menin expression leads to reduced p18 and $p27^{cip}$ expression, which allows for increased β-cell proliferation. When menin is overexpressed in the β-cells, there is a corresponding increase in p18 and $p27^{cip}$ expression with

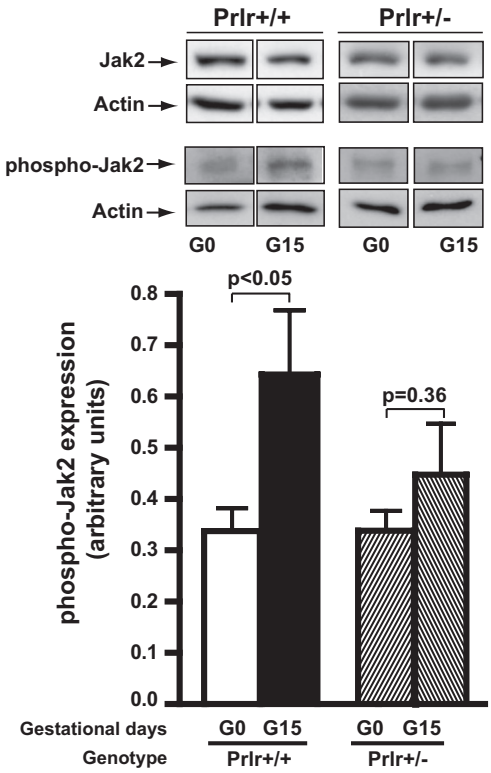

FIG. 6. Pregnancy-associated increase in Jak2 phosphorylation was diminished in the $Prlr^{+/-}$ mice. Whole-cell lysates were prepared from isolated islets, and 40 μg protein from each mouse was immunoblotted for phospho-Jak2, Jak2, and actin. A representative blot is shown. Each lane represents an individual mouse. Results represent mean ± SEM of eight to 18 mice from three separate experiments. G0, Gestational d 0 (*i.e.* nonpregnant); G15, gestational d 15. $Prlr^{+/+}$ are wild-type mice; $Prlr^{+/-}$ are heterozygous prolactin receptor-null mice. All experimental mice are 3–4 months old.

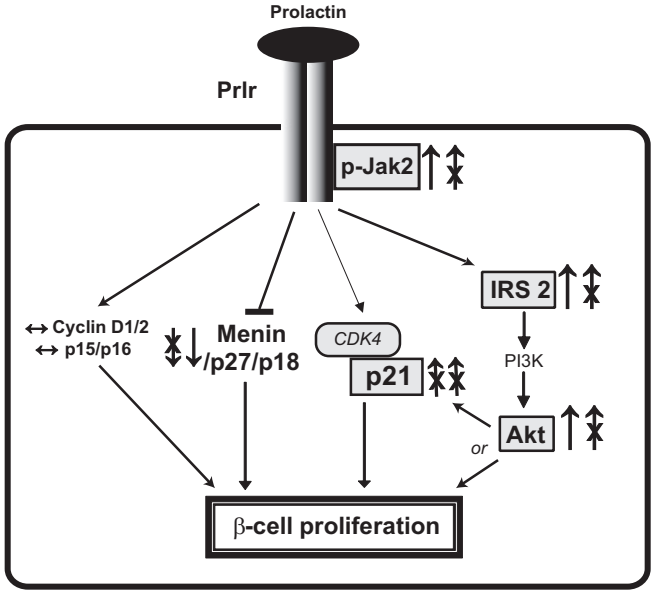

FIG. 7. The proposed model of prolactin-mediated regulation of β-cell proliferation during pregnancy. In response to prolactin, there is an increase in phospho- Jak-2, IRS-2, phospho-Akt (and Akt), and p21 expression, whereas menin and p18 expression are suppressed. Both IRS-2 and Akt are known to participate in up-regulation of β-cell proliferation. p21 may be regulated via an Akt-dependent pathway, and its up-regulation may increase β-cell proliferation via stabilizing the formation of the cyclin D-CDK4 complex and regulated progression through the cell cycle. In mice heterozygous for a deletion in the prolactin receptor, all of these responses were blunted. ↑, Increased; ↓, decreased; ↔, no change; x, blunted; Prlr, prolactin receptor.

reduction in β-cell proliferation and more importantly, these transgenic mice have elevated blood glucose during pregnancy (22). Here, we found that the pregnancy-induced reduction in menin and p18 expression observed in the $Prlr^{+/+}$ mice was blunted in the $Prlr^{+/-}$ mice. Currently, there is no evidence of cross talk between the Akt and the menin/p18/$p27^{cip}$ pathway in the β-cell, although in pituitary adenoma, activation of the PI3K/Akt pathway can lead to increased expression of mixed lineage leukemia-1 (MLL1), which functions in a complex with menin (39).

In summary, we found that during pregnancy, expression of phospho-Jak2, IRS-2, phospho-Akt, Akt, and $p21^{cip}$ are increased, whereas menin and p18 expression are suppressed in isolated islets. These results are consistent with the model that prolactin increases the expression of Akt, which in turn increases $p21^{cip}$ expression. This slightly elevated level of $p21^{cip}$ serves two functions: it prevents uncontrolled cell proliferation but at the same time stabilizes the formation of the cyclin D-CDK4 complex and regulated progression through the cell cycle. Independent of the Akt/$p21^{cip}$/cyclin D/CDK4 interactions, pregnancy-mediated reduction in menin/p18 and up-regulation of Jak2 activation allows for enhanced β-cell proliferation. In the pregnant $Prlr^{+/-}$ mice, all these responses are blunted, contributing to the decreased β-cell proliferation rate observed in these mice (Fig. 7). Future studies will need to address the potential cross talk between these pathways (34) and, more importantly, how they can be manipulated to yield controlled β-cell proliferation and regulated expansion of β-cell mass.

Acknowledgments

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