Maternal insulin resistance and transient hyperglycemia impact the metabolic and endocrine phenotypes of offspring

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Kahraman S, Dirice E, De Jesus DF, Hu J, Kulkarni RN. Maternal insulin resistance and transient hyperglycemia impact the metabolic and endocrine phenotypes of offspring. Am J Physiol Endocrinol Metab 307: E906–E918, 2014. First published September 23, 2014; doi:10.1152/ajpendo.00210.2014.—Studies in both humans and rodents suggest that maternal diabetes leads to a higher risk of the fetus developing impaired glucose tolerance and obesity during adulthood. However, the impact of hyperinsulinemia in the mother on glucose homeostasis in the offspring has not been fully explored. We aimed to determine the consequences of maternal insulin resistance on offspring metabolism and endocrine pancreas development using the LIRKO mouse model, which exhibits sustained hyperinsulinemia and transient increase in blood glucose concentrations during pregnancy. We examined control offspring born to either LIRKO or control mothers on embryonic days 13.5, 15.5, and 17.5 and postpartum days 0, 4, and 10. Control offspring born to LIRKO mothers displayed low birth weights and subsequently rapidly gained weight, and their blood glucose and plasma insulin concentrations were higher than offspring born to control mothers in early postnatal life. In addition, concentrations of plasma leptin, glucagon, and active GLP-1 were higher in control pups from LIRKO mothers. Analyses of the endocrine pancreas revealed significantly reduced β-cell area in control offspring of LIRKO mothers shortly after birth. β-Cell proliferation and total islet number were also lower in control offspring of LIRKO mothers during early postnatal days. Together, these data indicate that maternal hyperinsulinemia and the transient hyperglycemia impair endocrine pancreas development in the control offspring and induce multiple metabolic alterations in early postnatal life. The relatively smaller β-cell mass/area and β-cell proliferation in these control offspring suggest cell-autonomous epigenetic mechanisms in the regulation of islet growth and development.

Maternal insulin resistance; intrauterine environment; offspring metabolism; endocrine pancreas development; hyperinsulinemia

MATERNAL METABOLIC STATUS DURING PREGNANCY is important for fetal growth and development, since the fetus is completely dependent upon its mother for nutrition. Studies have reported that adverse experiences during fetal life can impair fetal development and cause permanent metabolic adaptations in the offspring that would influence their long-term health by increasing the risk for developing insulin resistance, type 2 diabetes, obesity, and/or cardiovascular disease in adulthood (8). To understand the role of intrauterine environment on fetal growth and development, investigators have used various animal models of maternal overnutrition [obesity (29), high-fat diet (15)] and maternal malnutrition (low-protein diet, low-energy diet) (11) and have also investigated other conditions affecting mothers during pregnancy [e.g., gestational diabetes (34), hyperglycemia (16), hypoxia (46), anemia (24), and glucocorticoid exposure (23)] (2). However, the effects of insulin resistance on a background of transient hyperglycemia in the mother on alterations in metabolism and pancreas development in the offspring remain unclear.

Insulin resistance contributes to a range of serious health problems ranging from diabetes to heart disease and cancer (4), and its increasing prevalence in adults, including women of childbearing age, makes this syndrome a growing concern worldwide. Since insulin resistance alters the metabolic status in the affected individuals, its presence in women during pregnancy has the potential to be detrimental to growth and metabolism in the offspring. Thus, insulin resistance directly impacts pregnant women and also their offspring.

In this study, we used a mouse model of insulin resistance to determine how maternal insulin resistance affects metabolism and endocrine pancreas development in the offspring. To this end, we investigated the liver-specific insulin receptor knock-out (LIRKO) mouse, in which the insulin receptor gene is deleted specifically in the liver by Cre-loxP-mediated recombination (27). LIRKO females were hyperinsulinemic with normal random blood glucose levels before the onset of pregnancy and displayed a transient increase in blood glucose levels and glucose intolerance. They became more insulin resistant compared with the pregestational state and developed pronounced diabetic phenotypes as a result of pregnancy. These results indicate that LIRKO females exhibit significant metabolic alterations during pregnancy and can be used as a potential model to study the effects of hyperinsulinemia and transient hyperglycemia on fetal growth and development. Our data using this unique model indicated that offspring born to LIRKO mothers have multiple metabolic alterations and are characterized by a reduction in β-cell reserves during early postnatal life.

MATERIALS AND METHODS

Animals

Control (IRlox/lox:alb-Cre−/+ ) and LIRKO (IRlox/lox:alb-Cre−/− ) mice were maintained on the C57BL/6 background after back-crossing to 12 generations and bred at the Joslin Animal Facility on a 12:12-h light-dark cycle with ad libitum water and food. Female mice were caged with males, and mating was confirmed by the presence of vaginal plaque checked in the morning. The presence of vaginal plaques was considered to represent pregnancy day 0.5. Fetuses or neonates were euthanized together with their mothers at key stages during normal mouse pancreatic development [embryonic day E13.5, E15.5, and E17.5, newborn [postnatal day 0 (P0)], P4, and P10]. Fetuses/neonates were counted, weighed, and euthanized by decapitation, and blood was collected from cervical vessels. Pancreata were rapidly digested, weighed, and fixed in Z-fix or 4% parafor-
maldehyde solution. Perigonadal white adipose tissues from 10-day-old offspring were dissected, divided into two parts, and either rapidly frozen in liquid nitrogen or fixed in Z-fix overnight at 4°C. Sexes and genotypes were determined by PCR analysis of genomic DNA obtained from tail snap (22). All procedures were approved by the Joslin Diabetes Center Institutional Animal Care and Use Committee and performed in accordance with National Institutes of Health (NIH) guidelines.

**Oral Glucose Tolerance Tests**

All mice were subjected to oral glucose tolerance tests (OGTT) on day 15.5 of pregnancy. Mice were fasted overnight for 14 h, followed by glucose administration (2.5 g/kg body wt) using oral gavage. Blood glucose was measured using an automatic glucometer (Glucometer Elite; Bayer) immediately before (time 0) and 15, 30, 60, and 120 min after the injection.

**Insulin Tolerance Test**

An insulin tolerance test was performed on control and LIRKO females before pregnancy, on day 15.5 of pregnancy, and after delivery (L0 and L4). The mice were fasted for 3 h (between 7 and 10 AM) and were injected intraperitoneally with 1 U/kg body wt insulin (Humulin R, U-100; Eli Lilly). Blood glucose concentrations were measured from the tail vein using an automatic glucometer before (time 0) and 15, 30, 45, and 60 min after insulin injection.

**Pregnancy Hormone Concentrations**

The levels of progesterone (Cusabio Biotech), prolactin (Calbiochem), and estradiol (Calbiochem) were measured by ELISA in the Joslin Specialized Assay Core.

**Blood Glucose, Plasma Insulin, Leptin, Glucagon, Glucagon-Like Peptide-1, and C-peptide Concentrations**

Ad libitum glucose levels were measured by a glucometer using tail vein blood. Plasma insulin, leptin, glucagon, glucagon-like peptide-1 (GLP-1), and C-peptide concentrations were measured in the Joslin Specialized Assay Core.

**Measurement of Adipocyte Size**

Five-micrometer sections of paraffin-embedded perigonadal white adipose tissues from 10-day-old pups were stained with hematoxylin and eosin. Five digital images from nonoverlapping fields were obtained at ×20 magnification from each section from four different animals per group. Pictures were analyzed using Cell Profiler image analysis software, and adipocyte pixel area was converted to adipocyte diameter (3, 6). A total of 2,000 cells (CC group) or 1,250 cells (CL group) were analyzed per section.

**RNA Extraction and Quantitative RT-PCR**

Perigonadal white adipose tissue from 10-day-old pups was homogenized in Trizol (Life Technologies) using Bullet Blender (Next Advance, Averill Park, NY) at speed 9 for 5 min at 4°C. Total RNA was extracted using the Direct-zol RNA MiniPrep Kit (Zymo Research, Irvine, CA) according to the manufacturer’s instructions. Complementary DNA (cDNA) was generated from total RNA with the High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA) and used for quantitative RT-PCR with iTag Universal SYBR Green (Bio-Rad Laboratories). Expression was normalized to TATA box-binding protein. Primer sequences are listed in Table 6.

**Table 1. Measurements of body weight, blood glucose, and serum insulin in control and LIRKO dams**

<table>
<thead>
<tr>
<th>Body Weight, g</th>
<th>Weight Gain/Pup, g</th>
<th>Blood Glucose, mg/dl</th>
<th>Serum Insulin, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 7–10)</td>
<td>LIRKO (n = 6–9)</td>
<td>Control (n = 7)</td>
<td>LIRKO (n = 7)</td>
</tr>
</tbody>
</table>
| G0 | 20.4 ± 0.7 | 21.4 ± 1.1 | 0.88 ± 0.06 | 0.91 ± 0.07 | 123.8 ± 3.7 | 137.0 ± 16.5 | 0.38 ± 0.12 | 4.54 ± 1.12
| G13.5 | 27.0 ± 1.0*** | 27.1 ± 1.1** | 1.25 ± 0.07† | 1.32 ± 0.11§ | 125.8 ± 9.0 | 131.3 ± 23.1 | ND | ND
| G15.5 | 30.5 ± 1.0*** | 30.0 ± 1.0*** | 1.77 ± 0.08§ | 1.70 ± 0.06§ | 109.4 ± 9.0 | 196.3 ± 55.3‡ | 1.43 ± 0.12* | 8.08 ± 1.05‡
| G17.5 | 34.8 ± 1.2*** | 33.2 ± 1.4*** | 97.3 ± 8.3* | 121.6 ± 9.3‡ | 123.1 ± 9.0 | 121.6 ± 9.3‡ | 1.21 ± 0.80 | 6.30 ± 1.74‡
| L0 | 26.4 ± 0.8*** | 27.6 ± 1.6** | 109.7 ± 6.2* | 147.5 ± 17.2 | 113.0 ± 12.3 | 127.0 ± 1.7 | 1.79 ± 0.50* | 3.21 ± 0.35‡
| L4 | 25.3 ± 0.7*** | 26.9 ± 0.7*** | 121.7 ± 4.1 | 148.0 ± 25.5 | 121.7 ± 4.1 | 148.0 ± 25.5 | 2.20 ± 0.68** | 4.07 ± 0.82
| L10 | 25.7 ± 1.7*** | 26.9 ± 0.6** | ND | ND | ND | ND | ND | ND

Values are means ± SE. LIRKO, liver-specific insulin receptor knockout; G, gestational days; L, lactation days (L0 is first day of lactation); ND, not determined. ***P < 0.001 vs. G0; **P < 0.01 vs. G0; †P < 0.001 vs. G13.5; §P < 0.05 vs. G15.3; *P < 0.05 vs. G15.5; ‡P < 0.05 vs. control.

*Fig. 1. Breeding scheme. Female control mice were crossed to male control mice to produce control offspring exposed to a normal intrauterine environment (CC, control offspring from control mother). Female liver-specific insulin receptor knockout (LIRKO) mice were crossed to male control mice to produce control offspring exposed to an insulin-resistant intrauterine environment (CL, control offspring from LIRKO mother; LL, LIRKO offspring from LIRKO mother). All studies were focused on comparing control pups (CC vs. CL). IR, insulin receptor; Alb, albumin.*
MATERIAL INSULIN RESISTANCE IMPACTS OFFSPRING METABOLISM

Table 2. Measurements of OGTT in control and LIRKO dams on day 15.5 of pregnancy

<table>
<thead>
<tr>
<th>Blood Glucose, mg/dl</th>
<th>Control (n = 6)</th>
<th>LIRKO (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time, min</td>
<td>Control (n = 6)</td>
<td>LIRKO (n = 6)</td>
</tr>
<tr>
<td>0</td>
<td>84.8 ± 4.8</td>
<td>80.1 ± 5.3</td>
</tr>
<tr>
<td>15</td>
<td>269.3 ± 11.0</td>
<td>319.9 ± 17.4*</td>
</tr>
<tr>
<td>30</td>
<td>326.0 ± 9.4</td>
<td>394.0 ± 12.9**</td>
</tr>
<tr>
<td>60</td>
<td>255.2 ± 32.9</td>
<td>362.3 ± 36.5**</td>
</tr>
<tr>
<td>120</td>
<td>111.8 ± 23.6</td>
<td>272.9 ± 24.1***</td>
</tr>
</tbody>
</table>

Glucose AUC mg·min·dl⁻¹ 26,848.8 ± 1,722.0 38,752.5 ± 1,902.6***

Values are means ± SE. OGTT, oral glucose tolerance test; AUC, area under the curve. *P < 0.05; **P < 0.01, and ***P < 0.001 vs. control.

RESULTS

Breeding Insulin-Resistant Dams

Eight- to 10-week-old control females (IRlox/lox:alb-Cre⁻/⁻) and LIRKO females (IRlox/lox:alb-Cre⁻/⁻) were bred with aged matched control males (IRlox/lox:alb-Cre⁻/⁻). Three types of progeny resulted from these crosses: 1) control offspring (IRlox/lox:alb-Cre⁻/⁻) from control mothers (CC), 2) control offspring (IRlox/lox:alb-Cre⁻/⁻) from LIRKO mothers (CL), and 3) LIRKO offspring (IRlox/lox:alb-Cre⁻/⁻) from LIRKO mothers (LL) (Fig. 1). To study the contributions of genetically imposed insulin resistance in the mother per se to metabolic and endocrine phenotypes in the offspring, we compared the differences in phenotypes between control offspring born to insulin-resistant or control mothers (CL vs. CC).

Effects in the Mother

Changes in maternal body weight, blood glucose concentrations, and serum insulin concentrations. To assess the effects of genetic insulin resistance on weight gain patterns of dams during and after pregnancy, body weight was monitored from conception until lactation. All dams gained weight significantly during pregnancy, and body weight changes were comparable between control and LIRKO dams. Body weight gain of dams per pup was not significantly different between control and LIRKO dams (Table 1).

Control dams revealed lower blood glucose concentrations on gestational day 17.5 (G17.5) compared with the nongravid state (P < 0.05), whereas LIRKO dams did not. On the other hand, LIRKO dams exhibited elevated concentrations of blood glucose on G15.5 and G17.5 compared with the control group (Table 1).

Serum insulin concentrations were persistently high in LIRKO mothers compared with the control group throughout the entire study. In addition, both LIRKO and control dams displayed elevated concentrations of serum insulin on G15.5 compared with the nongravid state (P < 0.05) due to increased insulin demand during pregnancy (Table 1).

Development of gestational diabetes in pregnant LIRKO females. To determine whether LIRKO females could maintain glucose homeostasis during pregnancy, OGTT were performed

Table 3. Measurements of insulin tolerance test in control and LIRKO dams at different time points (G0, G15.5, L0, and L4)

<table>
<thead>
<tr>
<th>Initial Blood Glucose, %</th>
<th>Control (n = 6)</th>
<th>LIRKO (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time, min</td>
<td>Control (n = 6)</td>
<td>LIRKO (n = 6)</td>
</tr>
<tr>
<td>0</td>
<td>100.0 ± 0.0</td>
<td>100.0 ± 0.0</td>
</tr>
<tr>
<td>15</td>
<td>77.8 ± 4.6</td>
<td>79.2 ± 4.1</td>
</tr>
<tr>
<td>30</td>
<td>57.7 ± 4.7</td>
<td>65.2 ± 5.2</td>
</tr>
<tr>
<td>45</td>
<td>38.9 ± 4.3</td>
<td>59.8 ± 6.1</td>
</tr>
<tr>
<td>60</td>
<td>34.1 ± 4.8</td>
<td>69.4 ± 12.9*</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 and **P < 0.01 vs. control.
on G15.5. LIRKO females displayed impaired glucose tolerance at G15.5 compared with control females. In humans, gestational diabetes mellitus is diagnosed if two or more values are abnormal on a 2-h, 75-g OGTT. The normal cutoff values of the OGTT are <95 mg/dl at fasting, 180 mg/dl at 1 h, and 155 mg/dl at 2 h (35). According to our results, blood glucose levels of both control and LIRKO dams were <95 mg/dl at fasting but then increased 1 h after glucose challenge in both control and LIRKO females >180 mg/dl (362 ± 37 mg/dl in LIRKO vs. 255 ± 33 mg/dl in control, P < 0.05; Table 2). Although the blood glucose concentrations were within the normal range in control females 2 h after glucose challenge, they remained significantly high in LIRKO females (273 ± 24 mg/dl in LIRKO vs. 112 ± 24 mg/dl in control, P < 0.001), indicating glucose intolerance in the latter LIRKO females during pregnancy. The presence of impaired glucose tolerance and transient increase in blood glucose levels in LIRKO females during pregnancy suggests that this is a model of gestational diabetes (34).

Exacerbation of insulin resistance in pregnant LIRKO females. Insulin tolerance tests revealed that LIRKO dams were mildly resistant to the blood glucose-lowering effects of exogenous insulin before pregnancy and are severely resistant on day 15.5 of pregnancy (Table 3). One interpretation of these data is that pregnancy exacerbates the preexisting insulin resistance in LIRKO dams.

Changes in hormone concentrations. To determine the changes in pregnancy hormones in our model, plasma levels of prolactin, progesterone, estradiol, and leptin were measured in LIRKO and control dams. The production of prolactin, progesterone, and estrogens increases exponentially in normal pregnancy and declines during lactation (14). Interestingly, plasma prolactin levels remained low in LIRKO females throughout gestation, whereas they increased in control females. The levels of prolactin observed in our experiments are consistent with a previous study in mice (28). Plasma progesterone levels were elevated at G15.5 in both control and LIRKO females but were significantly lower in LIRKO compared with control females. In contrast, plasma estradiol levels on G15.5 and leptin levels on G17.5 were significantly higher in LIRKO compared with control females (Table 4).

Effects in the Offspring

Effects of maternal insulin resistance on litter size and neonatal death. The mean litter sizes for the control (6.69 ± 0.46 pups/litter) and the LIRKO groups (5.85 ± 0.63 pups/litter) was not significantly different (P = 0.09). The newborn deaths in litters from either group were also not significantly different (control, 0.92 ± 0.47 pups/litter vs. LIRKO, 1.69 ± 0.52 pups/litter), although there was a tendency to increase in the latter group (P = 0.14) (Table 5). These results suggest that maternal insulin resistance was not obviously detrimental to fetal life in this model.

Low birth weight and rapid catchup growth in offspring born to insulin-resistant mothers. To investigate whether maternal insulin resistance affects growth of the progeny, we monitored body weights of offspring from E13.5 to P10 (Fig. 2, A and B). Offspring in both groups and both sexes gained body weight significantly over the period from E13.5 to P10. The birth weights of male offspring born to insulin-resistant mothers were below the mean value of the birth weights of the male control group, and these offspring recovered their lower body weight shortly after birth by gaining more weight compared with CC group. The CL offspring not only recovered their body weight deficiency but in fact exhibited faster weight gain and outpaced the controls in absolute weight gain in the postnatal period. Similarly, female pups from LIRKO mothers exhibited reduced birth weight and catchup growth at P10. Together, these data indicated that maternal insulin resistance results in low birth weight followed by catchup growth in offspring in both sexes.

Higher blood glucose and insulin concentrations in offspring born to insulin-resistant mothers shortly after birth. To evaluate the effects of maternal insulin resistance on glucose homeostasis in the offspring, blood glucose and plasma insulin concentrations were analyzed. As expected, blood glucose increased gradually in all offspring as they aged and gained weight. No significant difference in blood glucose levels was observed between groups either before or at the time of birth in either sex (Fig. 2, C and D). However, fluctuations in the concentrations of plasma insulin were observed in both CL and CC groups before birth due to the alterations in the islet cell population in the late fetal period. Plasma insulin concentrations of CC fetuses peaked at E17.5 (P < 0.05, E17.5 vs. E15.5), followed by a decrease at birth (P < 0.05, E17.5 vs. P0) in both sexes (Fig. 2, E and F). Similarly, plasma insulin concentrations of CL fetuses increased at E17.5 (P < 0.05, E17.5 vs. E15.5), but this increase remained lower compared

### Table 4. Measurements of hormone levels in control and LIRKO dams during pregnancy

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 7)</th>
<th>LIRKO (n = 7)</th>
<th>Control (n = 7)</th>
<th>LIRKO (n = 7)</th>
<th>Control (n = 7)</th>
<th>LIRKO (n = 7)</th>
<th>Control (n = 7)</th>
<th>LIRKO (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prolactin, ng/ml</td>
<td>81.3 ± 24.1</td>
<td>33.1 ± 12.7</td>
<td>2.98 ± 0.6</td>
<td>2.20 ± 0.3</td>
<td>102.3 ± 5.0</td>
<td>121.8 ± 6.2</td>
<td>157.4 ± 24.8</td>
<td>252.8 ± 14.5</td>
</tr>
<tr>
<td>Progesterone, ng/ml</td>
<td>143.8 ± 39.3</td>
<td>11.9 ± 3.4†</td>
<td>22.62 ± 4.7**</td>
<td>9.90 ± 1.1***</td>
<td>110.2 ± 6.5</td>
<td>170.3 ± 17.8†</td>
<td>229.0 ± 33.8</td>
<td>272.3 ± 33.9</td>
</tr>
<tr>
<td>Estradiol, pg/ml</td>
<td>187.4 ± 24.9*</td>
<td>45.0 ± 15.0†</td>
<td>9.75 ± 2.7*</td>
<td>5.43 ± 1.1*</td>
<td>227.2 ± 59.0</td>
<td>155.4 ± 8.9*</td>
<td>162.2 ± 19.0</td>
<td>427.2 ± 71.2†</td>
</tr>
<tr>
<td>Leptin, pg/ml</td>
<td>83.0 ± 25.8</td>
<td>15.2 ± 6.6†</td>
<td>3.07 ± 0.6</td>
<td>2.89 ± 1.4</td>
<td>120.6 ± 16.7</td>
<td>108.6 ± 12.8</td>
<td>203.9 ± 46.4</td>
<td>149.5 ± 20.4**</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 vs. G0; **P < 0.01 vs. G0; ***P < 0.001 vs. G0; †P < 0.05 vs. control; ‡P < 0.01 vs. control.

### Table 5. Litter size and newborn death

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Newborn No.</th>
<th>Newborns That Died After Birth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total no.</td>
<td>No./litter</td>
</tr>
<tr>
<td>Control (13)</td>
<td>87</td>
<td>6.69 ± 0.46</td>
</tr>
<tr>
<td>LIRKO (13)</td>
<td>76</td>
<td>5.85 ± 0.63</td>
</tr>
<tr>
<td>P value</td>
<td>0.09</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE.
with the CC group. Low levels of insulin at E17.5 might indicate abnormalities in the development of fetal β-cells in offspring born to insulin-resistant mothers. Shortly after birth, male offspring born to LIRKO mothers showed significant increases in both blood glucose and plasma insulin concentrations compared with male CC offspring. These effects were more prominent at P10 in the female CL offspring.

C-peptide analysis in CL males. To determine whether the increased plasma insulin levels in 4-day-old and 10-day-old CL males were a consequence of increased secretion from β-cells, we also measured plasma C-peptide levels. C-peptide, which is cosecreted with insulin from β-cells at a 1:1 molar ratio, is a reliable measurement of secretion (44). Although C-peptide levels tended to increase in 4-day-old CL males compared with CC, the difference did not reach statistical significance (Fig. 3A). To differentiate insulin secretion vs. insulin resistance, the mean for C-peptide/insulin (C/I) ratio was calculated as individual C-peptide concentration divided by the insulin concentration (39). Although there were no significant differences in the C-peptide levels between the CL and CC pups on either P4 (Fig. 3A) or P10 (Fig. 3B), the C/I ratio tended to decrease in 4-day-old CL males compared with controls. These results suggested that the altered insulin levels are likely due to development of insulin resistance in CL pups (Fig. 3C).

Higher plasma leptin, glucagon, and GLP-1 concentrations in offspring born to insulin-resistant mothers shortly after birth. To further determine changes in metabolism of offspring, metabolic markers (leptin, glucagon, and active GLP-1) were measured.
measured in plasma samples obtained from 4-day-old pups, when catch up growth was detected in the LIRKO group (Fig. 2, G and H). Plasma leptin concentrations were elevated more than twofold in male CL compared with male CC offspring. Similarly, female pups from LIRKO mothers had elevated levels of leptin compared with female CC pups, but this did not reach statistical significance. An increase in leptin concentrations, which is an adiposity marker (32), in offspring born to insulin-resistant mothers might indicate an increase in adipose mass. CL pups exhibited significantly higher levels of plasma glucagon and GLP-1 than CC pups in both sexes. Together, these results suggest that maternal insulin resistance induces multiple metabolic alterations in the offspring, as shown by elevated levels of leptin, glucagon, and GLP-1.

Adipocyte size is increased in 10-day-old CL pups. To examine whether the increased plasma leptin levels in CL males were due to an increase in adipose tissue, adipocyte size was measured. Hematoxylin and eosin staining revealed that mean adipocyte diameters were similar in CL and in CC pups in both sexes (Fig. 4, C and D). When adipocytes were plotted according to their size, we noted a marked decrease in the number of small adipocytes (20–30 and 30–40 μm) and an increase in adipocytes >50 μm in CL compared with CC offspring, indicating a rightward shift in the CL group (Fig. 4, E and F). Thus, the increase in adipocyte size might be one factor that contributes to higher body weights and metabolic phenotypes in CL mice.

To determine whether the increase in adipocyte size was accompanied by alterations in gene expression in adipocyte differentiation processes, we performed quantitative RT-PCR of RNA from adipose tissue of 10-day-old CL offspring. As shown in Fig. 5, a significant increase in expression of the adipogenic transcription factor C/EBPα was observed in both sexes. PPARα gene expression was significantly increased in CL females and tended to be higher in CL males ($P = 0.08$). Expression of genes involved in lipogenesis (Fasn, Acc, and Chrebp) or the differentiated adipocyte marker adip-2 tended to be higher in the CL group but did not reach statistical significance, and the expression of Srebf1 did not show a change (primer sets are listed in Table 6).

Reduced β-cell area and islet number in CL offspring. To elucidate how insulin resistance in the mother could affect the development of fetal endocrine pancreas, pancreatic sections were analyzed for β-cell morphology both before and after birth. Although morphologically similar islets were observed in both CL and CC offspring (Fig. 6A), the β-cell mass tended to decrease in CL compared with CC throughout the study in male offspring (Fig. 6B). In females, β-cell mass was comparable between CC and CL offspring throughout the study and tended to decrease when they were 10 days old. The percentage of β-cell area in CL offspring was significantly lower in males at P4 and P10 (Fig. 6C) and relatively small in CL females at P10 ($P = 0.09$). The total number of islets was fewer in both CL males and females than CC at P10 (Fig. 6D).

Diminished β-cell proliferation in CL offspring during early postnatal life. To determine the contribution of β-cell proliferation to the observed changes in β-cell mass and β-cell area in offspring, pancreatic sections were immunostained with the Ki-67 antibody, a marker for proliferating cells (Fig. 6E) and insulin. The percentage of Ki-67+ β-cells tended to decrease in CL offspring during the perinatal period and exhibited a significant reduction on P10 compared with CC pups (Fig. 6F). This suggests that a low β-cell proliferation capacity contributes in part to the significantly reduced β-cell area in the CL group. Despite reduction in their β-cell area, offspring born to insulin-resistant mothers were mildly hyperinsulinemic. This may indicate a higher insulin content per β-cell. Hypersecretion of insulin of islets observed in offspring of undernutrition pregnancies (18) and neonatal β-cell hyperactivity observed in non obese diabetic mice neonates (43) indicate that maternal environment might affect β-cell secretory function in offspring.

To determine whether formation of new islets could contribute to the observed changes in β-cell mass and β-cell area in offspring, the number of small islet clusters was evaluated (Fig. 7A). Reduction in the number of small islet clusters of 10-day-old offspring in CL compared with CC offspring was observed in both sexes (Fig. 7B). In females, the number of small islet clusters was evaluated (Fig. 7C). Reduction in the number of small islet clusters of 10-day-old offspring in CL compared with CC offspring was observed in both sexes (Fig. 7C).
old CL pups vs. CC was observed (Fig. 7\textsuperscript{B}). This suggests that the appearance of small islet cell clusters contributes in part to the significantly reduced $\beta$-cell area in the CL group.

To determine the effect of maternal insulin resistance on the development of endocrine cells other than $\beta$-cells, we measured $\alpha$-cell area and $\alpha$-cell mass of the offspring. $\alpha$-Cell area tended to decrease in the CL group after birth, and the difference between CL and CC group became significant in males on P10 (Fig. 7\textsuperscript{C}). Similarly, $\beta$-cell mass was significantly smaller in CL compared with the CC pups on P0 and was slightly smaller, albeit insignificant, on P4 and P10 (Fig. 7\textsuperscript{D}).

**DISCUSSION**

The impairment of maternal glucose homeostasis has clearly defined effects on the development of the fetus, and especially on the development and function of its endocrine pancreas (45). However, the effects of maternal insulin resistance on fetal metabolism and the consequences on fetal pancreas development have not been fully explored. To examine the hypothesis that an insulin-resistant intrauterine environment influences metabolism in the offspring and development of the endocrine pancreas, we used LIRKO females as an insulin-resistant mouse model.

Human pregnancy is characterized by a series of metabolic changes to meet the demands of the growing fetus. For example, an increase in serum insulin levels, a slight decrease in blood glucose levels, and development of peripheral insulin resistance all occur during pregnancy, and these changes trigger adaptive responses in $\beta$-cells to increase both insulin secretion and mass (41). Similarly, in our study, control mothers exhibited enhanced serum insulin and reduced blood glucose during late gestation, whereas LIRKO females, who already exhibit hyperinsulinemia (27), showed a further increase in insulin levels and an increase in blood glucose during pregnancy. Glucose and insulin tolerance tests on G15.5 showed a decrease in insulin sensitivity in LIRKO mice compared with controls. LIRKO females developed pronounced diabetic phenotypes during pregnancy and returned to pregestational levels after parturition in terms of blood glucose concentrations and insulin sensitivity. Considering that normal pregnancy itself induces a physiological insulin-resistant state (1), especially during late gestation, it is conceivable that LIRKO dams were more glucose intolerant and displayed a transient increase in blood glucose levels on G15.5 compared with control dams. These phenotypic changes observed in LIRKO females prompted us to use them as a potential model for studying the effects of gestational diabetes and insulin resistance in the mother on progeny.

Control offspring born to insulin-resistant mothers had reduced birth weight compared with offspring born to control...
mothers, and this is consistent with the results from studies using hyperinsulinemic pregnant rats created by exogenous insulin treatment (without causing hypoglycemia) (5, 38). In the latter studies, fetuses of hyperinsulinemic rats were smaller than those of control mothers. Although maternal insulin has been reported not to cross the placental barrier to reach the fetus, excessive amounts of insulin in maternal circulation can alter placental gene expression to affect growth and function of placenta (9). Khamaisi et al. (21) and Skarzinski et al. (40) reported altered expression of endothelin-converting enzyme-1 and nitric oxide synthase expression in the placenta of hyperinsulinemic dams compared with normal pregnant dams and found an association between these alterations in the placental gene expression and intrauterine growth restriction in rats with maternal hyperinsulinemia. In accord with these findings, elevated insulin concentrations in LIRKO dams could result in various alterations in the placenta to influence fetal growth and development. However, the presence of transient hyperglycemia along with hyperinsulinemia in our model indicates that both elevated blood glucose and insulin levels potentially contribute to the fetal phenotype.

Consistent with previous reports, we observed that loss of insulin signaling in the liver of LIRKO mouse leads to an increase in serum leptin concentrations (7) and reduction in serum triacylglycerol and free fatty acid concentrations (27). Furthermore, during pregnancy, plasma levels of prolactin,
progesterone, estradiol, and leptin were also altered in LIRKO dams compared with control dams. Although changes in placental hormone levels are known to occur during maternal adaptation to pregnancy to allow for optimal fetal growth, the roles of placental hormone expression in regulating fetal growth remain poorly understood (14). Thus, we cannot rule out the possibility that changes in one or more of these hormones and/or metabolites contribute to reduced birth weight or other abnormalities in control offspring born to LIRKO mothers (13).

Offspring born with low birth weight displayed a rapid catchup growth after birth and surpassed the weight of the
controls in early postnatal days. The finding of an association between accelerated catchup growth and increased adiposity (17) suggested that these offspring showed overgrowth due to the increase in their adipose mass. The elevated levels of plasma leptin, a marker of adiposity, and enlarged adipocytes associated with altered expression patterns of genes involved in adipocyte differentiation and lipogenesis supported these data. The finding of an association between accelerated catchup growth in early postnatal days and increased risk for insulin resistance and obesity in later life (33) could indicate an increased susceptibility to development of metabolic disease in the CL offspring during adulthood.

Shortly after birth, control offspring born to LIRKO mothers displayed higher plasma concentrations of both glucose and insulin than offspring born to control mothers, indicating early development of insulin resistance. In humans, early development of adiposity and insulin resistance after catchup growth supports this concept (17). Together with hyperinsulinemia and hyperglycemia, we observed higher plasma glucagon concentrations in CL pups. It is possible that α-cells of CL pups were insulin resistant, and therefore, they were poorly responsive to the suppressive effects of insulin (20) and glucose (31, 48). A similar explanation could underlie the higher concentrations of glucagon in CL males on day 4 after birth, even though their α-cell mass was similar to CC pups. Four-day-old CL pups also had elevated levels of GLP-1 that might be upregulated in response to the hyperglycemia. Further studies are warranted to identify mechanisms underlying hyperglucagonemia and elevated levels of GLP-1 observed in control pups of LIRKO mothers.

Normally, plasma insulin concentrations increase rapidly during the late fetal period, followed by a decrease immediately after birth (26, 30) due to the alterations in fetal β-cell mass in rats (19, 25). The lower insulin concentrations and relatively small β-cell mass in the control fetuses of LIRKO mothers compared with those of control mothers on E17.5 might indicate that maternal insulin resistance impairs the development of fetal β-cells. Following birth, CL pups exhibited reduced β-cell area and islet number and relatively reduced β-cell mass; however, they completely recovered their low body weights, supporting the possibility of a selective impairment in pancreas development by maternal insulin resistance. Reduced β-cell proliferation at P10 in CL pups compared with CC pups makes it likely that maternal insulin resistance affected β-cell proliferation and consequent reduction in β-cell mass in CL offspring. Consistent with our findings, previous studies have reported that abnormal intrauterine milieu could affect the development of the fetal endocrine pancreas by inducing gene expression modification permanently in pancreatic β-cells, leading to the development of diabetes in adulthood. Epigenetic alterations involved in the reduced β-cell mass could be one underlying molecular mechanism (10, 36, 37, 42).

The most profound differences in metabolic parameters between control offspring born to control and insulin-resistant mothers were evident during the early postnatal days, a stage that is approximately equivalent to postnatal human infancy or human childhood in mice. These results have potential implications for humans if maternal insulin resistance increases the risk of insulin resistance and obesity in children.

Early nutrition both in utero and after birth is known to be critical for the development of the offspring. Breast milk composition has been shown to influence infant growth and accrual of fat and lean body mass (12). Therefore, postnatal
consumption of breast milk produced by LIRKO mothers might be a contributor to the metabolic phenotype observed in CL offspring. The impact of lactational nutrition on offspring by cross-fostering pups onto control mothers or insulin-resistant mothers warrants further investigation.

In the present study, we specifically assessed the effects of maternal insulin resistance on metabolic and endocrine phenotypes of offspring independently from the effects of maternal obesity. Since obesity is associated with a multitude of metabolic impairments, the exact cause of abnormalities in the metabolism of offspring and endocrine pancreas development would be confounding when studying obese models. In utero exposure to an insulin-resistant environment impairs adequate development of the endocrine pancreas, which fails to recuperate after birth, leading to decreased β-cell reserve and a potential predisposition to type 2 diabetes. Further studies are necessary to investigate the underlying mechanisms of reduced β-cell mass and β-cell proliferation in the progeny of insulin-resistant mothers.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


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