Hypoglycemia, hyperglucagonemia, and fetoplacental defects in glucagon receptor knockout mice: a role for glucagon action in pregnancy maintenance


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To assess the role of glucagon signaling in reproduction, a new set of controlled breedings using our laboratory’s Gcgr−/− mouse model was established. The phenotype of Gcgr−/− mice, which lack functional Gcgr, as shown by the lack of glucagon binding to the Gcgr−/− membranes, includes hypoglycemia, hyperglucagonemia, decreased adiposity, and pancreatic α-cell hyperplasia (26, 79).

To assess the role of glucagon signaling in reproduction, a systematic assessment of the reproductive axis (hypothalamic-pituitary-ovarian axis), implantation, and pregnancy maintenance was determined. Studies were performed in Gcgr−/−, Gcgr+/−, and Gcgr++/+ mice to determine whether pregnancy outcomes changed depending upon the combination of matings performed.

Disruption of the glucagon receptor (Gcgr) gene resulted in impaired reproduction, which is associated with maternal hypoglycemia and hyperglucagonemia, abnormal placentation, alteration in the expression of important placental genes, and late-gestation fetal demise. These results indicate that glucagon, similarly to insulin, plays an important role in normal female reproductive function.

MATERIALS AND METHODS

Mice. All studies were approved by the Institutional Animal Care and Use Committee at Albert Einstein College of Medicine, in accordance with Animal Welfare Act guidelines. We have described previously the generation of Gcgr−/− mice (17, 26). All animals were fed ad libitum and maintained in a murine hepatitis virus-free barrier facility on a 12:12-h light-dark cycle. Animals received a standard laboratory diet (Picolab Mouse Diet 20) consisting of 21.6% fat, 56.5% carbohydrates, and 21.9% protein. Crosses of F6 and F7 mice back-crossed onto the C57BL/6J background were used. Three-week-old animals were genotyped by Southern blotting, as described previously (17, 26).
Timed pregnancies. The following combinations of Gcgr mice were mated: Gcgr\(^{+/+}\) females with Gcgr\(^{+/-}\) males, Gcgr\(^{+/-}\) females with Gcgr\(^{-/-}\) males, Gcgr\(^{-/-}\) females with Gcgr\(^{+/-}\) males, and Gcgr\(^{-/-}\) females with Gcgr\(^{+/-}\) males (Table 1). To test for mating and possible pregnancy, female mice were placed in cages with male mice at 6 PM and monitored the next morning by 8 AM for the presence of a vaginal plug, as described previously (33, 79). To avoid differences in weight of the embryos and placentas due to small differences in gestational age, plug-positive and plug-negative mice were separated into two groups after the 12 h of contact with the males, and a wait time of 3 wk transpired before plug-negative females were mated again.

Placenta and embryo collection. Mice were euthanized on embryonic day (e)12.5, e15.5, and e18.5 of gestation. Embryos and placentas were examined, weighed, and either frozen or placed in 10% buffered formalin for histological analysis.

Placental glycogen analysis. Placental glycogen was determined using the method described previously by us (61, 77). Briefly, placental tissue was digested in 1 N NaOH and then spotted on 3 mm of Whatman paper. Amyloglucosidase was used to convert the glucagon to glucose. The glucose concentration was measured using Glucose Trinder (Sigma). Glycogen data are reported as micrograms of glucose per milligram of tissue.

Hormone assays. For hormone assays, serum was collected following retroorbital sinus bleeds and spun at 5,000 g. Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels were measured by RIA, as described previously (55, 56). Glucagon was determined with RIAs from Linco Research Immunossay (St. Charles, MO).

Histology and immunostaining. Ovaries and pituitaries were removed from adult animals and placed in 10% buffered formalin. Tissues were embedded in paraffin, and 5-μm sections were prepared. Pituitary sections were immunostained with antibodies to LH and FSH (rabbit anti-rat LH and rabbit anti-rat FSH, respectively). Detection was carried out using goat anti-rabbit IgG conjugated to CY3 at a dilution of 1:1,000 (Jackson ImmunoResearch, West Grove, PA). Placentas were stained with hematoxylin and eosin. Sections were viewed using a Zeiss Axioskop microscope and a Spot RT color camera for immunostaining (Diagnostic Instruments, Sterling Heights, MI) as well as a Zeiss Axioscope-2 for light microscopy (Zeiss, Thornwood, NY). Each section was covered systematically by accumulating nonoverlapping fields of 1.3 × 10^6 μm². Morphological analysis was performed using OpenLab Image Analysis software (Improvision Imaging, Lexington, MA). The pituitary volume was assessed using three-dimensional axiometric analysis with Image Pro (Media Cybernetics, Silver Spring, MD).

Superovulation and oocyte retrieval. Ovulation was induced in 4- to 6-wk-old virgin mice. Animals were injected intraperitoneally with 5 units of pregnant mare serum gonadotropin (Hoffmann-LaRoche, Nutley, NJ) in sterile saline and 46 h later with 5 units of human chorionic gonadotropin (Sigma, St. Louis, MO) to induce ovulation. Oocytes were collected from oviducts the following morning and counted under a Nikon dissecting microscope.

RNA isolation and quantitative real-time PCR analysis. Total RNA was prepared from placenta, kidney (positive controls), and gastrocnemius muscle (negative control) (n = 6–10 mice per litter per group) of C57BL/6J mice, using TRIzol reagent (Invitrogen, Life Technologies), treated with DNase (Invitrogen protocol) to prevent DNA contamination (Invitrogen, Life Technologies). The RNA was checked for DNA contamination, using PCR with control primers as described previously (79). Quantitative real-time PCR (qRT-PCR) was the method of choice to determine the expression of genes of interest in the placenta. qRT-PCR analysis was performed from Gcgr\(^{+/-}\) or Gcgr\(^{-/-}\) placenta (n = 6–10/group) using the Taqman 7900 HT Sequence Detection System (ABI Prism) apparatus with SYBR Green (Applied Biosystems) reagent and sequence-specific primer pairs (73, 79). The cycling conditions for RT-PCR were as follows: 48°C for 30 min and 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 1 min. The PCR was run on ABI prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Quantitative values were obtained as threshold PCR cycle number (C\(_T\)) when the increase in fluorescent signal of PCR product showed exponential amplification. The target gene mRNA level was normalized to the housekeeping gene in the same sample as that described previously (73, 79). The ratio of relative expression of the target gene in Gcgr\(^{+/-}\) placentas to that in Gcgr\(^{-/-}\) placentas was then calculated as 2^\((\Delta\Delta C_{\text{T}})\) (73, 79). These reference genes were selected on the basis of their stable expression, as determined by previous studies. Furthermore, no housekeeping gene from the same pathway was used to prevent false-positive results. Sequence-specific primer pairs are provided in Tables 3 and Table 4.

Statistical analysis. Data were analyzed using the SPSS software (SPSS, Chicago, IL), and ANOVA statistical tests were applied (Stat View, Abacus). Significance was defined by P < 0.05.

RESULTS

Lack of glucagon signaling does not alter hypothalamic-pituitary-ovarian axis. We have reported previously that lack of glucagon signaling affects litter size, fetal growth, and survival, suggesting that glucagon signaling plays an important role during mating and pregnancy maintenance (79). To better characterize the reproductive deficit associated with the lack of glucagon signaling during mating and pregnancy, controlled breedings using various maternal and paternal Gcgr genotypes were established (Table 1). To control for timed matings, 125 mating pairs were placed in individual cages for 12 h, after which the males were removed and females examined for the presence of copulation plugs. Gross anatomic examination of 6-wk-old female Gcgr\(^{-/-}\) mice revealed normal development of external genitalia and the reproductive tract. No differences in the percentage of the copulation plugs were noted in Gcgr\(^{-/-}\) females mated to either Gcgr\(^{+/-}\) or Gcgr\(^{-/-}\) males or Gcgr\(^{-/-}\) females mated to either Gcgr\(^{+/-}\) or Gcgr\(^{-/-}\) males during the course of the study (25.9% in Gcgr\(^{-/-}\) females vs. 22.7% of Gcgr\(^{-/-}\) females, P = not significant [NS]), suggesting that lack of glucagon signaling does not affect copulation (Table 1).

Pituitary morphology demonstrated that Gcgr\(^{-/-}\) females had a modestly enlarged anterior lobe (30% increase in surface area compared to Gcgr\(^{+/-}\) females). The anterior pituitary lobes were separated into two groups after the 12-h contact with the males, and a wait time of 3 wk transpired before plug-negative females were mated again.
area measured in pixels, \( P = 0.008 \)) and a similar intermediate lobe surface area compared with Gcgr\(^{+/+}\) females. Immunofluorescence microscopy showed normal distribution of LH and FSH cells in pituitaries of Gcgr\(^{-/-}\) females (Fig. 1). Normal serum LH (7 ± 1 vs. 6 ± 1 vs. 8 ± 2 ng/ml in Gcgr\(^{-/-}\), Gcgr\(^{+/+}\), and Gcgr\(^{+/+}\), respectively, \( P = \text{NS} \)) and FSH levels (51 ± 6 vs. 43 ± 3 vs. 55 ± 5 ng/ml in Gcgr\(^{-/-}\), Gcgr\(^{+/+}\), and Gcgr\(^{+/+}\), respectively, \( P = \text{NS} \)) further suggested normal pituitary function.

To assess ovarian response, 6-wk-old virgin females were superovulated, and oocytes were retrieved. Gcgr\(^{-/-}\) mice displayed a similar number of oocytes compared with Gcgr\(^{+/+}\) and Gcgr\(^{+/+}\) counterparts, suggesting a normal response to superovulation (11 ± 6 vs. 12 ± 7 vs. 13 ± 4 in Gcgr\(^{-/-}\), Gcgr\(^{+/+}\), and Gcgr\(^{+/+}\), respectively, \( P = \text{NS} \)).

Lack of glucagon signaling alters the maternal metabolic milieu, leading to intrauterine growth restriction and fetal demise. The lack of abnormalities in the hypothalamic-pituitary-gonadal axis of the Gcgr\(^{-/-}\) mice suggested that abnormal implantation could be the source of impaired reproduction. We have shown previously that lack of Gcgr is associated with a significant decrease in litter size at e18.5 (79). In addition to the hypoglycemia noted during pregnancy (79), Gcgr\(^{-/-}\) pregnant female mice were significantly hyperglucagonemic compared with Gcgr\(^{+/+}\) and Gcgr\(^{+/+}\) pregnant controls [Gcgr\(^{-/-}\); 23,500 ± 590 vs. 76 ± 8 (Gcgr\(^{+/+}\)) and 60 ± 6 pg/ml (Gcgr\(^{+/+}\), \( P < 0.001 \)). These data suggest that Gcgr signaling may be required for normal pregnancy maintenance. To address this possibility, a new set of controlled timed pregnancies comparing Gcgr\(^{-/-}\), Gcgr\(^{+/+}\), and Gcgr\(^{+/+}\) female matings were performed, and mice were euthanized at an earlier time point, e12.5, as well as at e15.5 and e18.5 of gestation (early, midpregnancy, and 1 day prior to delivery, respectively).

To determine whether partial deletion of the Gcgr will affect fetal growth and development, Gcgr\(^{+/+}\) fetuses were studied. At e12.5, Gcgr\(^{-/-}\), Gcgr\(^{+/+}\), and Gcgr\(^{+/+}\) pregnancies had comparable fetal (0.09 ± 0.01 vs. 0.14 ± 0.03 vs. 0.12 ± 0.02 g in Gcgr\(^{-/-}\), Gcgr\(^{+/+}\), and Gcgr\(^{+/+}\), respectively, \( P = \text{NS} \)) and placental weights (0.18 ± 0.02 vs. 0.15 ± 0.02 vs. 0.16 ± 0.02 g in Gcgr\(^{-/-}\), Gcgr\(^{+/+}\), and Gcgr\(^{+/+}\), respectively, \( P = \text{NS} \)). Independent of maternal genotype, body weight and crown-to-rump length were similar at e15 (1.5 ± 0.3 vs. 1.5 ± 0.2 vs. 1.2 ± 0.1 cm in Gcgr\(^{-/-}\), Gcgr\(^{+/+}\), and Gcgr\(^{+/+}\), respectively, \( P = \text{NS} \)). As reported previously (79), at e18.5, Gcgr\(^{-/-}\) fetuses were significantly smaller and Gcgr\(^{-/-}\) placentas significantly larger compared with Gcgr\(^{+/+}\) pregnancies (Gcgr\(^{+/+}\) females mated to Gcgr\(^{+/+}\) males). Further analyses showed that Gcgr\(^{-/-}\) females displayed multiple resorption sites (Table 2). Interestingly, Gcgr\(^{+/+}\) pregnancies were segregated depending on whether the genotype of the mother was Gcgr\(^{-/-}\) or Gcgr\(^{+/+}\).

In these cases, although all pups were Gcgr\(^{-/-}\), their number

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**Fig. 1.** Anterior pituitary FSH and LH immunoreactivity of Gcgr\(^{-/-}\) and Gcgr\(^{+/+}\) animals. Representative pituitary sections are from 3 different animals from each genotype. Samples incubated with preimmune IgG are also shown.
vascular channels and a thickened interstitium. Thirty percent were from the same litter and exhibited moderate narrowing of
respectively,
grossly abnormal with areas of hyperemia and edema (Fig. 2,
Resorption sites 4.6
placenta (1, 48), the marked histological abnormalities in
Gcgr
placentas may play a role in the histological abnormalities
and diffuse interstitial trophoblast hyperplasia associated with
necrosis of vessels, increased vacuolization of syncytial cells,
avings included moderate to pronounced mineralization, fibrinoid
account for the observed placental abnormalities in Gcgr
mice.

Controversy about the role of glucagon in the placental
glycogen stores exists. Some evidence suggests that glucagon
may induce the breakdown of placental glycogen to provide
glucose to the fetuses (19). Conversely, others suggest that
placental glycogenolysis is not activated by glucagon (9, 34).
In support of the latter, no difference in placental glycogen
content was measured in Gcgr
placentas compared with Gcgr
placentas (82 ± 10 vs. 79 ± 8 μg/mg in Gcgr
vs. Gcgr
, respectively; n = 6/group, P = NS). This observation suggests
that placental glycogen metabolism is regulated by other ma-
ternal or fetal hormones (22).

Lack of glucagon signaling alters placental gene expression.
Some of the pathological findings in Gcgr
placentas sug-
gested that lack of Gcg signaling might be accompanied by
significant changes in the expression of genes that regulate
fetal growth, adrenergic signaling, glucose transport, and pla-
cental vascularization (Table 3). Lack of Gcg was associ-
ated with a significant downregulation of genes that regulate 1
growth: insulin like growth factor-I (IGF-I; -3.06x), IGF-I
receptor (−3.2x), IGF-II (−2.66x), IGF-binding protein 1
(−4.56x), leptin receptor (−4.1x), and insulin receptor (−3.41x);
2 adrenergic signaling: adrenoceptor (ADR) subtypes
ADR1B (−5.5x), ADRA2A (−4.86x), ADRA2B (−6.51x), ADRB1
(−4.11x), ADRB2 (−5.45x), and ADRB3 (−10.6x); 3 vas-
cularization and angiogenesis: hypoxia-inducible factor-1α
subunit (HIF-1α; −1.4x), vascular endothelial growth factor
(VEGF; −2.73x), and chemokine receptor (CXCRA4; −5.4x)
(71, 74); 4 retinol transport: retinol-binding protein 1 (RBP-1;
−3.37x) (38); 5 cell proliferation and differentiation: V-fos
murine osteosarcoma viral oncogene homolog (FOS; −2.5x)
(6); 6 G protein-coupled receptors glucagon receptor (Gcg;
−7.5x) and glucagon-like peptide 1 (GLP-1; −5.47x) (26) (it
is important to note that the Gcg primers designed for this
study were able to detect residual Gcg expression in Gcgr
placentas; the primers designed to amplify the Gcg in this
study encompassed the exons not deleted by the knockout
construct, and therefore, the resultant amplicon contained
the exons at the 3’ end of the Gcg gene that are outside of the
knockout construct); and 7 oxidative stress: glutathione
peroxidase (GPX1; −3.8x), Dnaj (Hsp40) homolog, subfamily B,
member 5 (DNAJB5, −4.19x) (49, 54). Unexpectedly, the lack
of Gcg signaling did not alter glucose [glucose transporters
(GLUT1), 3, and 8] or Na
–coupled neutral amino acid trans-
porter 4 transporter expression (SLC38A4) (21, 35), glucoc-
tcoid receptor (NR3C1) (44), or 11β-hydroxysteroid dehy-

table 2. Embryonic day 18.5 gestational characteristics

| Genotype  | Gcg
-/- X Gcg
-/- | Gcg
-/- X Gcg
+/+ | Gcg
+/+ X Gcg
-/- | Gcg
+/+ X Gcg
+/+ |
<table>
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<tr>
<td>No. of litters</td>
<td>7</td>
<td>7</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>No. of pups</td>
<td>4.3 ± 0.8</td>
<td>5.7 ± 0.8</td>
<td>7.3 ± 1.0</td>
<td>8.5 ± 1.0*</td>
</tr>
<tr>
<td>No. of live pups</td>
<td>3.6 ± 0.8</td>
<td>5.6 ± 0.8</td>
<td>7.3 ± 1.0</td>
<td>8.5 ± 1.0*#</td>
</tr>
<tr>
<td>Resorption sites</td>
<td>4.6 ± 1.1</td>
<td>1.9 ± 0.5</td>
<td>0.7 ± 0.6*</td>
<td>1.3 ± 0.8*</td>
</tr>
</tbody>
</table>

Data are means ± SE. * P < 0.05 compared with Gcg
-/- female X Gcg
-/- male; # P < 0.05 compared with Gcg
-/- female X Gcg
+/+ male.
drogenase 1 and 2 (HSD11B1 and -2) gene expression (3). Lack of Gcgr signaling significantly upregulated tumor necrosis factor (ligand) superfamily member 6 (FASL; 1.65x), which is typically associated with an increase in apoptosis.

**DISCUSSION**

Glucagon is a central mediator of glycemic control and is released by pancreatic α-cells in response to hypoglycemia. Thus, glucagon and the Gcgr are critical in maintaining postprandial and fasting blood glucose levels (30). In utero, glucagon-sensitive cAMP production is markedly suppressed until immediately after birth (24). The fetus relies on maternal glucose for its nutritional needs. Late in gestation, when there is rapid growth, the fetus may be particularly vulnerable to the maternal hypoglycemia in Gcgr<sup>−/−</sup> mice (79). Glucose is the main substrate for energy metabolism in the human fetus and placenta. We have demonstrated previously that Gcgr<sup>+/+</sup>, Gcgr<sup>+/−</sup>, and Gcgr<sup>−/−</sup> embryos derived from Gcgr<sup>+/+</sup> mothers display normal blood glucose levels, whereas Gcgr<sup>−/−</sup> embryos derived from Gcgr<sup>−/−</sup> mothers display significant hypoglycemia (79) and hyperglucagonemia compared with Gcgr<sup>+/−</sup> and Gcgr<sup>+/+</sup> mice (26). Thus, the severe hypoglycemia experienced by Gcgr<sup>−/−</sup> embryos from Gcgr<sup>−/−</sup> mothers may reflect the long-term exposure to a lower glucose environment. The hypoglycemic milieu may also compromise the amino acid transport activity, further decreasing fetal nutrient supply (39, 64). This possible lack of nutrients could explain the IUGR in fetuses from Gcgr<sup>−/−</sup> mothers and account for the differences between e12, e15.5 (normal fetal weights), and e18.5 (IUGR). The etiological roles of lower glycemia and hyperglucagonemia are further supported by differential outcomes in the Gcgr<sup>+/−</sup> pregnancies. Gcgr<sup>+/+</sup> females mated...
Table 3. List of differentially expressed placental genes between Gcgr<sup>-/-</sup> and Gcgr<sup> +/-</sup> placentas

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Symbol</th>
<th>Gene Sequence (Forward and Reverse)</th>
<th>P Value</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth factors</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Insulin-like growth factor I</td>
<td>IGF-I</td>
<td>AACAAGCCCCACAGGTATAGG (forward)</td>
<td>0.01</td>
<td>-3.0</td>
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<tr>
<td></td>
<td></td>
<td>AAAGCAACACTCACTCACAATGC (reverse)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin-like growth factor I receptor</td>
<td>IGF-IR</td>
<td>GAGAAGACCACTCACTCAACCTAGAG (forward)</td>
<td>0.04</td>
<td>-3.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTGGCTTCGGCCAGACA (reverse)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin-like growth factor II</td>
<td>IGF-II</td>
<td>CATGCGCTGGATGTGGTGTT (forward)</td>
<td>0.02</td>
<td>-2.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CAGAAAGTTGGATGGTGGTGG (reverse)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin like growth factor binding protein-1</td>
<td>IGFBP-1</td>
<td>TCTCTTGGAACCCGATGACG (forward)</td>
<td>0.02</td>
<td>-4.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TTCTTGGACTCAGGATGCTC (reverse)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leptin receptor</td>
<td>LEPR</td>
<td>CAGCTGTGAATGGAGAGTCGT (forward)</td>
<td>0.001</td>
<td>-4.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TTGGCTCGAGATTTCCTCAGCCT (reverse)</td>
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<td></td>
</tr>
<tr>
<td>Insulin receptor</td>
<td>INS R</td>
<td>CTCAGAAGATCATCCGGTTCT (forward)</td>
<td>0.001</td>
<td>-3.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TTCAAAATAGGCACTCCAGAC (reverse)</td>
<td></td>
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<tr>
<td>Adrenergic receptors</td>
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<tr>
<td>Adrenergic α1B-receptor</td>
<td>ADRA1B</td>
<td>TGCCGACCCGGTTGATCT (forward)</td>
<td>0.04</td>
<td>-5.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCCCATTGAGGGCTAATG (reverse)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adrenergic α2A-receptor</td>
<td>ADRA2A</td>
<td>GCCCCGCGCAGACTCTC (forward)</td>
<td>0.04</td>
<td>-4.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CATACCAGGCTGGCGGAA (reverse)</td>
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</tr>
<tr>
<td>Adrenergic α2B-receptor</td>
<td>ADRA2B</td>
<td>GTGCCACAGAAGCCCTACT (forward)</td>
<td>0.04</td>
<td>-6.5</td>
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<tr>
<td></td>
<td></td>
<td>CTTGCCGAAATAGTGAAGA (reverse)</td>
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</tr>
<tr>
<td>Adrenergic β1-receptor</td>
<td>ADRB1</td>
<td>CGGAGCCGCTGTCTGCTGCT (forward)</td>
<td>0.02</td>
<td>-4.1</td>
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<tr>
<td></td>
<td></td>
<td>ACGAAACCTCTTGAGGCAGAAA (reverse)</td>
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<tr>
<td>Adrenergic β2-receptor</td>
<td>ADRB2</td>
<td>CATATCATAGGGCCGCGT (forward)</td>
<td>0.02</td>
<td>-5.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GTCCCTGATAAGGGCCGAGAT (forward)</td>
<td></td>
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</tr>
<tr>
<td>Adrenergic β3-receptor</td>
<td>ADRB3</td>
<td>TCCTCGGGTGTCTTGTGAGTACG (forward)</td>
<td>0.006</td>
<td>-10.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CACTGGTATGGAATGACG (reverse)</td>
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<td></td>
</tr>
<tr>
<td>Vascularization</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Hypoxia-inducible factor 1, α-subunit</td>
<td>HIF-1α</td>
<td>CAAACTGAGAGCTGGTGGTGG (forward)</td>
<td>0.006</td>
<td>-1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTGGAGTGGTACTGGTAGTATG (reverse)</td>
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<tr>
<td>Vascular endothelial growth factor</td>
<td>VEGF</td>
<td>CACCCAGGGCCTGCTGT (forward)</td>
<td>0.02</td>
<td>-2.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCTCTATCTATGGACGACTTTT (reverse)</td>
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<tr>
<td>Chemokine (C-X-C motif) receptor 4</td>
<td>CXCR4</td>
<td>ACTACGCTGGAGCGGATACGT (forward)</td>
<td>0.02</td>
<td>-5.4</td>
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<tr>
<td></td>
<td></td>
<td>GGTACGCCAGGCGGACATAG (reverse)</td>
<td></td>
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<tr>
<td>Cell proliferation</td>
<td></td>
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<tr>
<td>FBIJ marine osteosarcoma viral oncogene homolog</td>
<td>FOS</td>
<td>GAGAAGCTCTGACTGACGAGGG (forward)</td>
<td>0.04</td>
<td>-2.5</td>
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<tr>
<td></td>
<td></td>
<td>ATCCCAATATGAGCCCAATAGTTATGTA (reverse)</td>
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<tr>
<td>Glucagon receptor</td>
<td>GGCR</td>
<td>AACATGGTGATCTCTGCTGT (forward)</td>
<td>0.0001</td>
<td>-7.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GTGGAGGCTAGAAAGATGTTATATG (reverse)</td>
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<tr>
<td>Glucagon-like peptide 1 receptor</td>
<td>GLP-1R</td>
<td>GCTTCAGCTGCTGGTGGT (forward)</td>
<td>0.001</td>
<td>-5.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CAAAGACGCTCAGGATGATG (reverse)</td>
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<td>Apoptosis</td>
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<td></td>
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<tr>
<td>Fas ligand (TNF superfamily, member 6)</td>
<td>FASLG</td>
<td>CTGTGCGCTGCGCTGGGA (forward)</td>
<td>0.05</td>
<td>+1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTACCGAGATGTCTCAGCTGAG (reverse)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retinol transport</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retinol-binding protein 1, cellular</td>
<td>CRBP1</td>
<td>GATGGGGAAGAAACTTGGTGC (forward)</td>
<td>0.002</td>
<td>-3.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCCAGGAAAGCAGCAG (reverse)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxidative stress</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutathione peroxidase 1</td>
<td>GPX1</td>
<td>AGTCCGCGGTTATGCTGCTTTC (forward)</td>
<td>0.02</td>
<td>-3.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAGAGCGACGATCTCTCAGATGA (reverse)</td>
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<td></td>
</tr>
<tr>
<td>DnaJ (Hsp40) homolog, subfamily B, member 5</td>
<td>DNAJB5</td>
<td>ACTTCGCCGGAATGATTAACCA (forward)</td>
<td>0.0111</td>
<td>-4.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGTTATGACTGCGTCTGAG (reverse)</td>
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</tr>
</tbody>
</table>

Up- and downregulated genes are shown by fold changes in Gcgr<sup>-/-</sup> compared with Gcgr<sup> +/-</sup> placentas (n = 5–10/group; P values show statistically significant differences among the groups).

with either Gcgr<sup> +/-</sup> males or Gcgr<sup>-/-</sup> males have normal gestations. However, Gcgr<sup>-/-</sup> females mated with either Gcgr<sup> +/-</sup> males or Gcgr<sup>-/-</sup> males display abnormal gestations that are characterized by large numbers of resorption sites, IUGR, and some fetal death. Aside from the lower glycermia and hyperglucagonemia effect on pregnancy outcome, one may speculate that fetal glucagonemia, suggested by the α-cell hyperplasia found in fetal pancreatic islets as early as embryonic day 15 (79), could also have an effect on fetal growth and development. Interestingly, Gcgr<sup>-/-</sup> fetuses from Gcgr<sup> +/-</sup> pregnancies, which also displayed α-cell hyperplasia (79), exhibit normal growth and development, suggesting that abnormal gestations seen in Gcgr<sup>-/-</sup> mice are not a native defect of the Gcgr<sup>-/-</sup> fetuses.

Maternal hyperglucagonemia may play a role in the differential outcomes of the Gcgr<sup> +/-</sup> pregnancies. Although glucagon does not cross the placenta (42), the expression of glucagon receptor mRNA as shown by us (please refer to RESULTS) and/or the presence of glucagon in placenta by others (19, 31, 57) suggests a possible paracrine effect of glucagon. Glucagon stimulates the generation of cAMP and estradiol secretion in human term trophoblast and may (19, 31, 57) or may not (34,
mobilize placental glycogen stores from glycogen cells. The mouse placenta consists of two fetal compartments, the labyrinthine zone and junctional zone, and a maternal decidual component. The junctional zone is a cellular compartment consisting of at least two trophoblast subtypes: spongiform trophoblast and glycogen cells. Glycogen cells may play a role in degradation of the extracellular matrix surrounding maternal arteries that facilitates arterial dilation and improves placental vascularization (19). Additionally, glycogen cells may provide a substantial source of energy to the fetus at the end of gestation (9, 19, 25, 45). Interestingly, glucagon staining has been shown on plasma membranes of placental glycogen cells, suggesting stimulation of glucose release by glucagon (19). Placental glycogen metabolism seems to be regulated by cAMP (9) and IGF-II levels (22). Furthermore, insulin, which plays a major role in regulating glycogen metabolism during the postnatal period, has no effect on placental glycogenesis (68). Hyperglycemia (7), as well as diabetes (8), has been shown to increase placental glycogen stores. Thus, one may speculate that hypoglycemia will decrease placental glycogen stores. In our model, the presence of normal glycogen stores in Gcgr−/− compared with Gcgr+/+ placentas in a low maternal and fetal glucose milieu may indicate that either a lack of glucagon signaling or a decrease in IGF-II or catecholamine receptor expression may contribute to the altered release of glycogen. Thus, abnormal glycogen degradation could explain the enlarged placentas observed in Gcgr−/− females.

Proglucagon is normally processed to GLP-1 in the intestinal L cell by a prohormone convertase 1/3. The actions of GLPs are mediated by members of the glucagon receptor superfamily of G protein-coupled receptors that share similar structural and functional properties with respect to ligand binding and signal transduction (15). Glucagon can bind and activate the GLP-1 receptor with reduced affinity and efficacy (26). In addition, hyperglucagonemia seen in Gcgr−/− mice is associated with a three- to 10-fold increase in circulating GLP-1 amide levels (26). Thus, the Gcgr−/− placental phenotype could be the result of the cross-affinity for GLP-1 receptor by glucagon and/or the increase in GLP-1 levels. GLP-1 receptor expression is downregulated in Gcgr−/− placentas. Interestingly, nothing is known about the action of GLP-1 on placental tissue. It is plausible that GLP-1, which has been found to have direct vascular action, may improve placental vascularization by relaxing blood vessels (53). Thus, the reduction in expression of the incretin receptors may determine, at least in part, the placental phenotype in Gcgr−/− mice. Several outstanding questions remain. For example, is the reduction in GLP-1 receptor expression a counterregulatory response to exposure to the elevated levels of GLP-1 and/or glucagon? Furthermore, is signaling through the GLP-1 receptor necessary for the GLP-1 effect in the placental tissue? Recently, it has been shown that GLP-1 can interact directly with extracellular tissues through a glycosylphosphatidylinositol/inositolphosphoglycan-coupled receptor (52). Based on our data, it is possible that GLP-1 may contribute to placental vascularization through a cAMP-coupled GLP-1 receptor.

Another cause of the growth restriction seen in the e18.5 Gcgr−/− and Gcgr+/- fetuses derived from Gcgr−/− mothers could be their abnormal placentas. Many of the morphological changes seen in Gcgr−/− placentas coincide with a decline in trophoblastic functional activity that may be due to a relatively mild, sustained uteroplacental ischemia. Uteroplacental ischemic changes can result in increased mineralization (59) and fibrinoid necrosis of vessels (10), as seen in the abnormal placentas from Gcgr−/− mothers. These defects would further compromise fetal perfusion through the placenta, resulting in decreased materno-fetal nutrient transfer (39, 40).

Recently, a number of stressors that alter expression of genes involved in angiogenesis and metabolic response in placenta have been identified (28, 29, 69, 75). Similarly, in Gcgr−/− mice, lack of glucagon signaling repressed the transcription and translation of a significant proportion of genes (81). In response to stress, some of the most highly downregulated genes were those related to fetal growth, adrenergic signaling, angiogenesis, cell proliferation, and oxidative stress. Among the downregulated genes associated with fetal and placental growth were the IGF axis (14) and the insulin and leptin receptor (76). IGF-II has been shown to influence the supply of maternal nutrients to the fetus (20) and regulates glycogen metabolism (22). In addition, as shown in Table 4, a selection of genes was not differentially expressed in Gcgr−/− placenta compared with control. Furthermore, Fas ligand is upregulated in Gcgr−/− placentas, suggesting that lack of glucagon signaling does not universally decrease the transcriptional machinery. A possible explanation of the findings is that the selection of genes was biased to genes that have been shown to be downregulated in different placental pathologies. Yang et al. (81) reported recently that lack of glucagon signaling widely downregulated transcripts involved in gluconeogenesis, amino acid catabolism, and fatty acid oxidation. Interestingly, the downregulation of the genes was accompanied by a similar decrease in translation ($r = 0.88$). Thus, Gcgr−/− pregnancies have a complex phenotype, and negative effects on one pathway may lead to compensatory adjustments in other pathways. At this point, further studies may be needed to determine whether lack of glucagon signaling overall decreases the transcriptional machinery.

An increase in epinephrine signaling observed in other tissues (liver and white adipose tissue) (26) may lead to the downregulation of the adrenergic receptors seen in Gcgr−/− placentas (32, 70). Among the downregulated genes associated with adrenergic signaling were the ADRA1B, ADRB2, and ADRAB3, which have been involved in the regulation of the contractility of the placental vessels (62, 66), and the ADRA2, which has been found to be essential at the placental interface between mother and embryo in establishing the circulatory system (5, 50, 58). Alteration in placental blood flow mediated by these ADRAAs could be further exacerbated by the downregulation of important factors associated with angiogenesis, vasculogenesis, and endothelial cell growth such as VEGF (18) and CXCR (74). Alterations in placental vascularization could also lead to an increase in placental apoptosis, as suggested by the upregulation of FASL expression (12), and placental oxidative stress (downregulation of Gpx1 and Hsp40) (63, 65), further exacerbating fetal growth and development.

An interesting and unexpected finding was the impaired expression of HIF-1α, key player in the hypoxic response, by inducing the expression of specific hypoxic-regulated genes such as VEGF (37). Possible explanations could be that hypoxic induction of HIF-1α protein has been shown to occur with no change or moderate increase in mRNA levels, consistent with a transcriptional activation and an increase in protein
placental barrier, which is decreased in Gcgr
fer will be dependent on the concentration gradient across the
facilitated nature of transplacental glucose transport, net trans-
expression across the plasma membranes. Because of the
delayed growth and development of the fetus (79) was that lack of glucagon signaling
not alter the expression of glucose or neutral amino acid
transporters in placenta. GLUTs are the main regulators of the
maternal fetal glucose transfer (11). GLUT1 has been shown to
be inversely regulated by glucose concentrations and positively
regulated by IGF-1 and hypoxia (11). In growth-retarded neo-
placentas. Placental IGF has

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Symbol</th>
<th>Gene Sequence (Forward and Reverse)</th>
<th>P Value</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin like growth factor-binding protein 3</td>
<td>IGFBP-3</td>
<td>GCTGTGTTCACTCCATATGACAC (forward) GGGTACCTAGTTCTTCCTCC (reverse)</td>
<td>NS</td>
<td>−1.34</td>
</tr>
<tr>
<td>Solute carrier family 2, member 1</td>
<td>SLC2A1</td>
<td>GATGACGAGCAGCTGAGT (forward) CACAGTGAAAGGCGCTTGTTA (reverse)</td>
<td>NS</td>
<td>−1.91</td>
</tr>
<tr>
<td>Solute carrier family 2, member 3</td>
<td>SLC2A3</td>
<td>TTCTGGGCTCTGAAAGACTG (forward) GCCGTTTGCAAGATACTG (reverse)</td>
<td>NS</td>
<td>−1.93</td>
</tr>
<tr>
<td>Solute carrier family 2, member 8</td>
<td>SLC2A8</td>
<td>GGCGGCTGGGATAGCT (forward) TGAAAGCAGGATAGAAAT (reverse)</td>
<td>NS</td>
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</tr>
<tr>
<td>Solute carrier family 38, member 4</td>
<td>SLC38A4</td>
<td>GAAGCCATGGCTTATTATCTC (forward) CCCCCGGGTTAAGTGGTGT (reverse)</td>
<td>NS</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Genes are shown by fold changes in Gcgr<sup>−/−</sup> compared with Gcgr<sup>+/+</sup> placentas (n = 5–10/group). NS, no significant differences were found among the groups.

stability (80), and during prolonged hypoxia, HIF-1α proteins
downregulate HIF-1α mRNA expression (78).

Another perplexing finding in growth-retarded hypoglycemic Gcgr<sup>−/−</sup> fetuses (79) was that lack of glucagon signaling
did not alter the expression of glucose or neutral amino acid
transporters in placenta. GLUTs are the main regulators of the
maternal fetal glucose transfer (11). GLUT1 has been shown to
be inversely regulated by glucose concentrations and positively
regulated by IGF-1 and hypoxia (11). In growth-retarded neo-
placentas, no changes in GLUT or neutral amino acid transport
activity were present when compared with age-matched, nor-
mally grown controls (36). Thus, alterations in fetal growth as
well as the hypoglycemia observed in Gcgr<sup>−/−</sup> fetuses are not
cased by a decreased glucose or neutral amino acid transport
expression across the plasma membranes. Because of the
facilitated nature of transplacental glucose transport, net trans-
fer will be dependent on the concentration gradient across the
placental barrier, which is decreased in Gcgr<sup>−/−</sup> mothers (79).

Therefore, fetal hypoglycemia may be an adaptation to mater-
nal hypoglycemia and/or reduced placental area sec-
ondary to pathological changes seen in Gcgr<sup>−/−</sup> placentas.

Alterations in fetal growth and placental function may not be
explained by changes in cortisol levels, as demonstrated pre-
viously in female Gcgr<sup>−/−</sup> mice (10). This interpretation is
supported by the results of placental gene expression (Table 4)
(26, 67).

Our findings suggest that the changes in gene expression
found in Gcgr<sup>−/−</sup> placentas are either an adaptive response to a relatively mild, sustained uteroplacental ischemia or that changes in gene expression are an altered response, secondary to the lack of glucagon signaling, that will lead to a decline in trophoblastic functional activity. As a consequence, physiological
demands are not met in a significant number of fetuses,
leading to increased numbers of resorption sites in the placen-
tas from Gcgr<sup>−/−</sup> mothers.

One caveat not addressed in this study is that alterations in fetal growth and placental function could also be explained by
uterine factors such as the IGF system, which has autocrine and
paracrine functions (72). A compelling result is the downregu-
lation of the IGF axis in Gcgr<sup>−/−</sup> placentas. Placental IGF has
been shown to be transcriptionally regulated by nutrients, growth factors, and cAMP to stimulate growth (13, 23). Inter-
estingly, similar to the placentas, Gcgr<sup>−/−</sup> mice have a small
decrease in serum IGF-I levels (26), further altering fetal
growth. Thus, one may speculate that glucagon signaling may
have a role in the transcriptional regulation of IGF-I possibly
through the activation of adenylate cyclase and the rise in
cAMP. Further studies are needed to assess the relationship
between glucagon signaling and regulation of the IGF axis.

In summary, the results of this study show that Gcgr<sup>−/−</sup>
mice display impaired reproduction associated with maternal
hypoglycemia and hyperglucagonemia throughout pregnancy.
The source of fertility impairment lies in late pregnancy demise
associated with gross placental anomalies in mice lacking
functional Gcgr. To our knowledge, this is the first reported
confirmation of Gcgr gene expression in mouse placenta,
suggesting the potential for a direct mechanism of glucagon
action at the level of the placenta. This finding could likely
have important ramifications for our understanding of counter-
regulatory hormone action and physiology during pregnancy
and development. Overall, our findings suggest a novel role for

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.
AUTHOR CONTRIBUTIONS
S.O., P.V., L.C., X.-Q.D., R.W.G., S.E.R., R.R., A.F.P., M.J.C., and C.K. performed the experiments; S.O. and P.V. analyzed the data; S.O., M.J.C., and P.V. interpreted the results of the experiments; S.O. and P.V. prepared the figures; S.O., P.V., R.W.G., N.S., and M.J.C. edited and revised the manuscript; S.O., P.V., L.C., X.-Q.D., R.R., A.F.P., C.K., N.S., and M.J.C. approved the final version of the manuscript; S.O., P.V., and M.J.C. did the conception and design of the research; P.V. and S.O. drafted the manuscript.

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