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Maternal Low-Protein Diet Induces Gender-Dependent Changes in Epigenetic Regulation of the Glucose-6-Phosphatase Gene in Newborn Piglet Liver[1–3]

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Abstract

Glucose-6-phosphatase (G6PC) plays an important role in glucose homeostasis because it catalyzes the final steps of gluconeogenesis and glycogenolysis. Maternal malnutrition during pregnancy affects G6PC activity, yet it is unknown whether epigenetic regulations of the *G6PC* gene are also affected. In this study, we fed primiparous, purebred Meishan sows either standard-protein (SP; 12% crude protein) or low-protein (LP; 6% crude protein) diets throughout gestation and analyzed hepatic *G6PC* expression in both male and female newborn piglets. The epigenetic regulation of *G6PC*, including DNA methylation, histone modifications, and micro RNA (miRNA), was determined to reveal potential mechanisms. Male, but not female, LP piglets had a significantly lower serum glucose concentration and greater hepatic *G6PC* mRNA expression and enzyme activity. Also, in LP males, glucocorticoid receptor binding to the *G6PC* promoter was lower compared with SP males, which was accompanied by hypomethylation of the *G6PC* promoter. Modifications in histones also were gender dependent; LP males had less histone H3 and histone H3 lysine 9 trimethylation and more histone H3 acetylation and histone H3 lysine 4 trimethylation on the *G6PC* promoter compared with the SP males, whereas LP females had more H3 and greater H3 methylation compared with their SP counterparts. Moreover, two miRNA, ssc-miR-339–5p and ssc-miR-532–3p, targeting the *G6PC* 3′ untranslated region were significantly upregulated by the LP diet only in females. These results suggest that a maternal LP diet during pregnancy causes hepatic activation of *G6PC* gene expression in male piglets, which possibly contributes to adult-onset hyperglycemia. J. Nutr. 142: 1659–1665, 2012.

Introduction

Maternal malnutrition during gestation may affect susceptibility to metabolic syndrome in adult offspring (1). Glucose homeostasis dysregulation is a key feature of metabolic syndrome. The liver plays an important role in maintaining glucose homeostasis through the sophisticated regulation of glucose production. Glucose production in the liver is particularly important for newborns who require adequate glucose to cope with parturition stress and to support tissue functions before suckling (2).

Glucose-6-phosphatase (G6PC) is a key enzyme that catalyzes the final step of gluconeogenesis and glycogenolysis and hydrolyzes glucose-6-phosphate to produce an inorganic phosphate and free glucose; therefore, it plays a key role in the homeostatic regulation of blood glucose concentrations. The overexpression of *G6PC* in liver disrupts glucose homeostasis in normal rats (3), whereas hyperglycemia enhances hepatic *G6PC* expression in diabetic rats (4).

Hepatic G6PC activity is under the control of fetal glucocorticoids, which rise dramatically near parturition. Glucocorticoids regulate *G6PC* gene transcription (5–7) via glucocorticoid receptor (GR) binding to its promoter. GR can either activate or repress gene transcription (8), depending on the subtype of the glucocorticoid response element (GRE)[7], which can be positive

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[3] Supplemental Tables 1–6 and Figure 1 are available from the ''Online Supporting Material'' link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
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[7] Abbreviations used: ChIP, chromatin immunoprecipitation; GRE, positive glucocorticoid response element; nGRE, negative GRE; H3Ac, histone H3 acetylation; H3K4me3, histone H3 lysine 4 trimethylation; H3K9me3, histone H3 lysine 9 trimethylation; H3K27me3, histone H3 lysine 27 trimethylation; IUGR, intrauterine growth retardation; LP, low protein; MeDIP, methylated DNA immunoprecipitation; miRNA, micro RNA; nGRE, negative glucocorticoid response element; SP, standard protein; UTR untranslated region.

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(GRE) or negative (nGRE). In rats, the *G6PC* gene promoter contains both GRE and nGRE (9). A number of studies indicate that intrauterine growth retardation (IUGR) and a maternal low-protein (LP) diet upregulate hepatic *G6PC* gene expression (10) and enzyme activity (11) in rat offspring, yet how maternal LP diet affects *G6PC* gene transcription regulation in the liver of newborn offspring is unknown.

Prenatal malnutrition has been reported to perturb epigenetic regulation, which results in long-term gene expression changes (12). To date, data regarding the effect of maternal malnutrition on epigenetic regulation of glucogenic genes are scarce. Moderate maternal nutrient restriction upregulates phosphoenolpyruvate carboxykinase 1 (PCK1) mRNA in the liver of baboon fetuses, which is associated with hypomethylation of the *PCK1* promoter in the liver (13). More recently, Strakovsky et al. (14) provided evidence that histone modifications are involved in the effect of a maternal high-fat diet on liver *PCK1* gene transcription in rats. Although glucose is reported to be involved in the post-transcriptional regulation of the *G6PC* gene (4) and computational prediction indicates that some micro RNA (miRNA) may target the human *G6PC* gene (15), no experimental data are available to support the role of miRNA in *G6PC* gene regulation.

Most dietary manipulation studies use rats or sheep as models to gain insight into human metabolic regulation. Compared with rats and sheep, pigs are considered a better model for metabolic studies, because they are more similar to humans in morphology, physiology, metabolism, and omnivorous habits (16,17). However, study of nutritional programming of hepatic *G6PC* regulation in pigs is lacking.

Therefore, the present study was designed to answer 2 questions: first, to assess the impact of maternal dietary protein on hepatic *G6PC* gene expression in neonatal offspring piglets and second, to determine whether such an effect involves *G6PC* transcriptional regulation, including GR binding and epigenetic modifications of the *G6PC* promoter.

Materials and Methods

Animals and experimental design. The animal experiment was conducted at the National Meishan Pig Preservation and Breeding Farm at Jiangsu Polytechnic College of Agriculture and Forestry, Jurong, Jiangsu Province, P. R. China. Fourteen primiparous, purebred Meishan gilts (body weight, 36.1 ± 1.8 kg) were randomly assigned to standard- (SP) and low- (LP) protein groups. The SP sows were fed diets containing 12% crude protein during gestation and those in the LP group were fed diets containing 6% crude protein (**Supplemental Table 1**). The dietary treatment began 1 mo before artificial insemination at the first observation of estrus. All the gilts used in the study were artificially inseminated with a mixture of semen samples obtained from 2 littermate boars. Maternal dietary treatment did not affect the fertilization rate. Sows were fed twice daily (0800 and 1400 h) with rations of 1.8 kg/d during gestation. Newborn piglets were individually weighed immediately after parturition. The farrowing duration ranged from 3 to 4 h and was not affected by the maternal diet. The piglets of the same litter were gathered and confined in the warm creep area. One male and one female piglet of the mean body weight (±10%) were selected from each litter and exsanguinated before suckling. Blood was collected immediately and the liver (without the gall bladder) was harvested within 20 min, snap-frozen in liquid nitrogen, and stored at −80°C for further analysis.

The experiment was undertaken following the guidelines of the Animal Ethics Committee of Nanjing Agricultural University.

Hormone and metabolite assays. Serum concentrations of cortisol and glucagon were measured using respective commercial RIA kits (nos. D10PZB and F03PZB, Beijing North Institute of Biological Technology) with assay sensitivities of 2 μg/L and 16.1 ng/L, respectively. The intra- and inter-assay variations were 10 and 15%, respectively, for both kits. Serum glucose, TG, lactic acid, and nonesterified fatty acid concentrations were determined with enzymatic colorimetric methods using commercial kits for glucose (no. 6006, Shanghai Rongsheng Biotech), TG (No. 6011, Shanghai Rongsheng Biotech), lactic acid (no. A019, Nanjing Jiancheng Bioengineering Institute), and nonesterified fatty acid (no. A042, Nanjing Jiancheng Bioengineering Institute).

Liver glycogen content. The hepatic glycogen content was determined as previously described (18). The results are expressed as mg glycogen/g of liver (wet weight).

RNA isolation, cDNA synthesis, and real-time PCR. Total RNA was isolated from liver samples using TRIzol Reagent (no. 15596026, Invitrogen) according to the manufacturer's instructions and reverse transcribed with the PrimeScript 1st Strand cDNA Synthesis kit (no. D6110A, Takara). Two microliters of diluted cDNA (1:50) was used in each real-time PCR assay with Mx3000P (Stratagene). All primers (**Supplemental Table 2**) were synthesized by Generay Biotech. Several reference genes were tested and peptidylprolyl isomerase A was chosen as a reference gene, because it was expressed in liver at a similar level to the genes of interest and is not affected by the experimental factors (diet and gender).

G6PC enzyme activity assay. Liver G6PC activity was determined according to a modified version of previously described protocols (19,20). A microsomal preparation (volume fraction of sample = 0.06) was pipetted into an assay mixture (pH 6.5) containing 26.5 mmol/L glucose-6-phosphate and 1.8 mmol/L EDTA, incubated at 30°C for 10 min and quenched with a final concentration of 4% perchloric acid. Inorganic phosphate in the supernatants was determined with a Pi detection kit (no. C006–1, Nanjing Jiancheng Bioengineering Institute). One unit of activity represents 1 μmol/L of inorganic phosphate released per minute.

Western-blot analysis. Cytoplasmic and nuclear extracts from 100 mg of frozen liver tissue were prepared as previously described with some modifications (21). Protein concentrations were determined with a Pierce BCA Protein Assay kit (no. 23225, Thermo). Western-blot analysis for GR (no. sc-1004, Santa Cruz) and phospho-Ser^{211}GR (no. 4161, Cell Signaling Technology) were carried out according to the protocols provided by the manufacturers. Lamin A (no. BS3774, Bioworlde) and β-actin (no. AP0060, Bioworlde) were used to verify the purity of cytoplasmic and nuclear proteins, respectively. GAPDH (no. AP0063, Bioworlde) and histone H1 (no. BS1655, Bioworlde) were used as loading controls in Western-blot analyses.

Quantitation of miRNA predicted to target 3′ untranslated region of G6PC. Six micrograms of high-quality total RNA was polyadenylated by poly (A) polymerase at 37°C for 1 h in 20 μL of reaction mixture using a Poly (A) Tailing kit (No. 1350, Ambion) according to the manufacturer's instructions. Treated RNA was then dissolved and reverse-transcribed using a poly (T) adapter. qPCR was performed using SYBR (No. DRR081A, TaKaRa) with a miRNA-specific forward primer and a universal reverse primer complementary to part of the poly (T) adapter sequence. Because no validated reference gene was available for pig miRNA, a random DNA oligonucleotide was added to total RNA samples before polyadenylation as an exogenous reference to normalize miRNA expression. The sequences for all primers, the poly (T) adapter, and the exogenous reference are listed in Supplemental Table 2.

Cloning the promoter and 3′ untranslated region sequences of the G6PC gene. The G6PC gene promoter was cloned using the GenomeWalker Universal Genome Walker kit (no. 638904, Clontech). Using adaptor primer as the upstream primer and G6PC-specific primers as downstream primers (F: 5′-CACAGCTACCCAGAGGAGTTTGAT-3′, R1: 5′-CAAGATGAACCAATCCTGGGAGTCC-3′, R2: 5′-AGTC-TAAATCCACGCTGGTTCTTGC-3′), the PCR reaction was performed according to the kit instruction. PCR products were analyzed on 1.5%

agarose gels and the resulting DNA bands were recovered from the gels. All the recovered DNA samples were cloned into pMD 19-T Vector (no. D102A, Takara) and sequenced. As shown in Figure 3*A*, the porcine *G6PC* promoter sequence (1212 bp), which contains 2 repeated sequences from −1005 to −801 and from −755 to −503, was predicted using the RepeatMasker Web server (22). Also, a CpG island (−89 to +112) was predicted in the porcine *G6PC* promoter by Sequence Manipulation Suite (23) and GRE sites were predicted by TRANSFAC 6.0. Three primer pairs were designed to amplify the 3 fragments of the proximal promoter sequence for G6PC transcription regulation.

G6PC 3′ untranslated region (UTR) was cloned by 3′ RACE methods using Tailed cDNA. PCR reactions were performed with TaKaRa LA Taq (no. DRR002A) using a universal primer (5′-AGTGTCATCCCCTACTGCCT-3′) as the upstream primer and the adaptor primer as the downstream primer (F: 5′-TAGAGTGAGTG-TAGCGAGCA-3′). Forty-five cycles of amplification were performed, consisting of 30 s at 94°C, 30 s at 55°C, 1 min at 72°C, and a final 5-min extension at 72°C. PCR products were analyzed and cloned as described above.

Methylated DNA immunoprecipitation analysis. Methylated DNA immunoprecipitation (MeDIP) analysis was performed as previously described (24) with some modifications. High-quality genomic DNA isolated from liver was sonicated to produce small fragments (300–1000 bp). Two micrograms of fragmented DNA was heat denatured to produce single-stranded DNA and a portion of the denatured DNA was stored as control (input) DNA. A mouse monoclonal antibody against 5-methyl cytidine (no. ab10805, Abcam) was used to immunoprecipitate methylated DNA fragments. The immune complexes were captured with protein G agarose beads (80 μL, 50% slurry, pretreated with denatured salmon sperm DNA and BSA, no. P2009, Beyotime Institute of Biotechnology). The beads bound to immune complexes were washed to eliminate nonspecific binding and resuspended in 250 μL digestion buffer containing proteinase K. Finally, the MeDIP DNA was purified. A small aliquot of MeDIP DNA and control input DNA was used to amplify the G6PC proximal promoter sequences by real-time PCR with specific primers designed with Primer 5 software (Supplemental Table 2).

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) analysis was performed according to our previous publication (25) with some modifications. Briefly, 200-mg frozen liver samples were ground in liquid nitrogen and washed with PBS containing protease inhibitor cocktail (no. 11697498001, Roche). After cross-linking in 1% formaldehyde, the reaction was stopped with 2.5 mol/L glycine. The pellets were washed with PBS and lysed with SDS lysis buffer containing protease inhibitors. Chromatin was sonicated to an average length of ~500 bp and the protein-DNA complex was diluted in ChIP dilution buffer precleared with salmon sperm DNA/protein G agarose beads (60 μL, 50% slurry). The precleared chromatin preparations were incubated with 2 μg of primary antibody overnight at 4°C (antibody information is shown in **Supplemental Table 3**). A negative control was included with normal rat IgG or no antibody added. Protein G agarose beads (120 μL, 50% slurry) were added to capture the immunoprecipitated chromatin complexes. Finally, reverse cross-linking was performed to release DNA fragments from the immunoprecipitated complex at 65°C for 5 h and the DNA was purified. Immunoprecipitated DNA was used as a template for real-time PCR using specific primers to amplify genomic sequences at the promoter region of G6PC gene (Supplemental Table 2).

Statistical analysis. All data are presented as the mean ± SEM. For body weight, liver weight, serum biochemical variables, liver glycogen, and G6PC enzyme activity, 2-way ANOVA using the general linear model procedure of SPSS 17.0 for Windows was used to assess the main effects of diet, gender, and their interaction. When significant main effects or interactions were observed, the LSD post hoc test was conducted to evaluate differences between the groups. If the data were not normally distributed or the variance was not equal, then log10-transformed data were analyzed. For relative quantitative data of gene/protein expression or epigenetic modifications, a *t* test for independent samples was applied to analyze the effect of maternal diet in male or female piglets, separately, because we were not able to include triplicates of all samples from both sexes in the same assays. The $2^{-\Delta\Delta Ct}$ method was used to analyze real-time PCR data. Expression of mRNA or protein was expressed as the fold of the mean of the male or female SP group. Differences were considered significant at $P < 0.05$.

Results

The reproductive performance of sows and serum biochemical variables of piglets. The mean birth weight of all the piglets born to LP sows was lower than that of their SP counterparts ($P < 0.01$), whereas the mean litter size and litter weight were not affected (**Supplemental Table 4**). The LP piglets used in the study also had lower body weights ($P < 0.05$) and liver weights ($P < 0.05$) compared with SP piglets. The same diet effect was shown for the serum concentrations of glucose ($P < 0.01$) and lactic acid ($P < 0.05$). Body weight ($P < 0.05$) was higher in males compared with females, and serum cortisol ($P = 0.07$) and glucose ($P = 0.06$) tended to be higher in males. Although the main effect for the overall interactions between diet and gender were not significant, post hoc analysis revealed a diet effect ($P < 0.05$) on glucose in only males.

Hepatic glycogen, G6PC enzyme activity, and gluconeogenic gene expression. The liver glycogen content tended to be higher ($P = 0.06$) in LP piglets compared with that of SP piglets (**Supplemental Table 5**). Hepatic G6PC enzyme activity was also higher ($P < 0.05$) in LP piglets than in SP piglets (**Fig. 1***A*). Post hoc analysis revealed a diet effect ($P < 0.05$) on hepatic G6PC enzyme activity in LP males compared with SP males (Fig. 1*A*).

The mRNA abundances of 8 genes involved in hepatic gluconeogenesis were quantitated with real-time PCR, and a sexual dimorphic response of these genes to maternal LP diet was observed in the liver of newborn piglets. Among these genes, 2 rate-limiting enzymes, fructose-1,6-bisphosphatase 1 ($P < 0.05$) and G6PC ($P < 0.01$), were significantly upregulated in the liver of LP males compared with SP males (Fig. 1*B*), whereas no differences were found in females (data not shown). In agreement with mRNA expression results, hepatic G6PC activity was significantly enhanced in male piglets born to sows fed LP diets.

Hepatic GR expression and protein content. The maternal LP diet affected hepatic *GR* expression at the level of both mRNA and phosphorylated protein. *GR* mRNA expression increased ($P < 0.05$) (**Fig. 2***A*) in both male and female LP piglet liver. Although the cytoplasmic content of total GR did not differ between the LP and SP piglets (Fig. 2*B*), the nuclear content of phospho-Ser^{211} GR was upregulated ($P < 0.01$) (Fig. 2*C*) in LP males compared with the SP males. The purity of cytoplasmic and nuclear proteins was verified with β-actin and Lamin A, respectively (**Supplemental Fig. 1**).

GR binding and epigenetic modifications of G6PC. The ChIP assay revealed lower ($P < 0.01$) (**Fig. 3***B*) GR binding to fragments 1 (−396 to −299, GP primer 1) and 2 (−293 to −84, GP primer 2) in male LP piglet liver, but no difference was detected in females (Fig. 3*C*). In contrast, GR binding to fragment 3 (−5 to +155, GP primer 3), which is located in the coding region, was higher ($P < 0.05$) (Fig. 3*B*,*C*) in both male and female LP piglets.

MeDIP analysis using GP primer 3 to amplify the fragment spanning most of the CpG island (Fig. 3*A*) detected hypome-

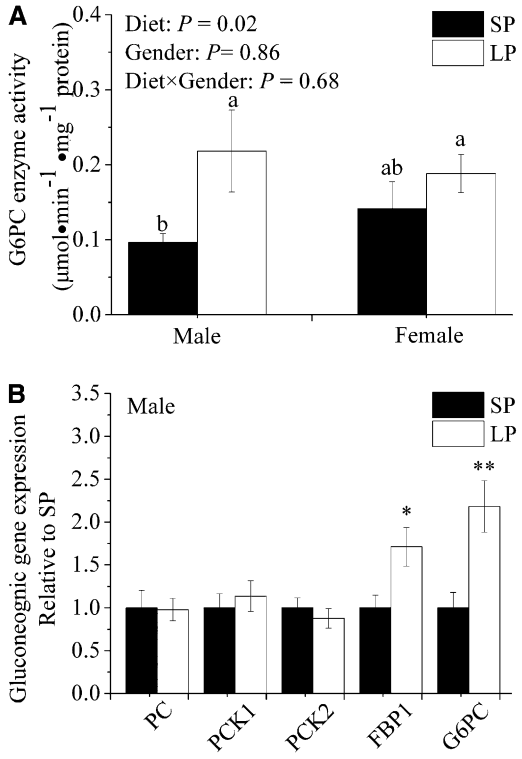

FIGURE 1 Hepatic G6PC activity (*A*) and gluconeogenic gene expression (*B*) of piglets born to sows fed an SP or LP diet. Peptidylprolyl isomerase A was used as the reference gene. Values are mean ± SEM, $n = 7$. For G6PC enzyme activity, means without a common letter differ, $P < 0.05$; for gene expression, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. SP of the same gender. LP, low protein; SP, standard protein.

thylation ($P < 0.01$) (Fig. 3*D*) of the *G6PC* promoter in male LP piglet liver, corresponding to augmented G6PC mRNA expression CpG methylation of the G6PC gene promoter did not differ in the female piglets (Fig. 3*D*).

The enrichment of 4 histone modification marks on the proximal promoter of *G6PC* was determined by ChIP, followed by real-time PCR using GP primer 3. The gender-dependent alterations in histone modification surrounding the *G6PC* promoter were observed in response to maternal LP diet ($P < 0.01$). Histone H3 acetylation (H3Ac) ($P < 0.01$) and histone H3 lysine 4 trimethylation (H3K4me3) ($P < 0.01$) were higher, whereas histone H3 ($P < 0.01$) and histone H3 lysine 9 trimethylation (H3K9me3) ($P < 0.01$) were lower in LP males compared with SP males (Fig. 3*E*), corresponding to hypomethylation of the hepatic *G6PC* promoter and upregulated G6PC expression. However, histone H3 ($P < 0.01$), H3K4me3 ($P < 0.01$), H3K9me3 ($P < 0.01$), and histone H3 lysine 27 trimethylation (H3K27me3) ($P < 0.01$) surrounding the *G6PC* promoter were all significantly higher in LP females compared with SP females (Fig. 4*F*).

Expression of miRNA predicted to target the G6PC gene. A 960-bp 3′UTR of the *G6PC* gene was cloned and 33 miRNA were predicted to target the 3′UTR of *G6PC* according to probability of interaction by target accessibility, an online miRNA prediction tool (26). Among the 33 predicted miRNA, 12 were verified in the liver by real-time PCR. None of the 12 miRNA determined in male newborn piglet liver were affected by maternal LP diet, whereas hepatic ssc-miR-339–5p ($P < 0.05$) and ssc-miR-532–3p ($P < 0.01$) were upregulated in the liver of female piglets born to sows fed an LP diet (**Supplemental Table 6**).

Discussion

In the present study, a maternal LP diet during pregnancy resulted in IUGR, which is indicated by significantly lower birth and liver weights in both the male and female offspring sampled. This finding agrees with early reports that maternal protein deprivation during pregnancy reduces piglet birth weight and

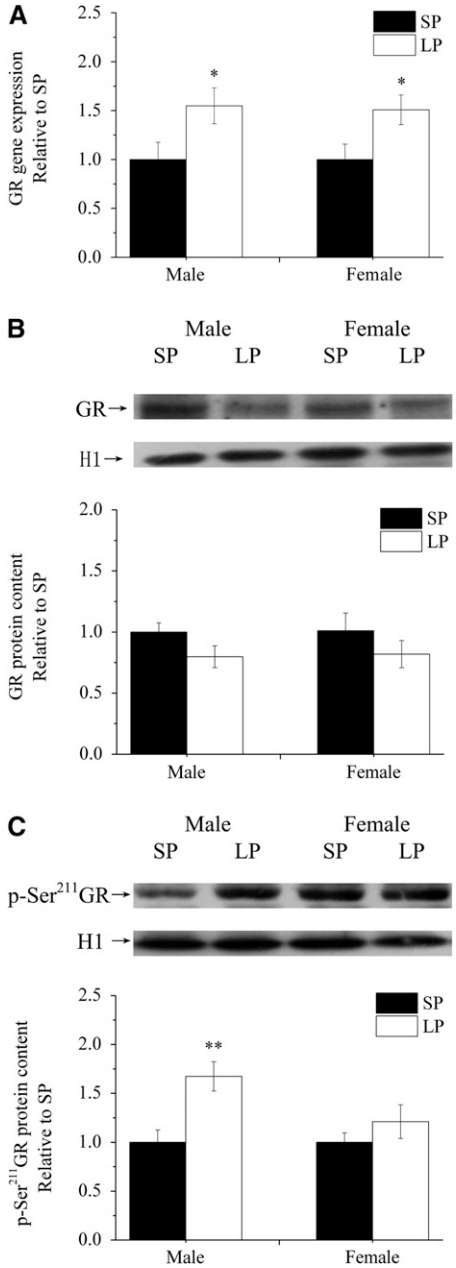

FIGURE 2 *GR* gene expression (*A*), cytoplasmic GR protein content (*B*), and nuclear phospho-Ser^{211}GR protein content (*C*) in the liver of piglets born to sows fed an SP or LP diet. For gene expression, peptidylprolyl isomerase A was used as the reference gene. For Western blot, H1 was used as the internal standard. Values are mean ± SEM, $n = 7$. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. SP of the same gender. GR, glucocorticoid receptor; H1, histone H1; LP, low protein; SP, standard protein.

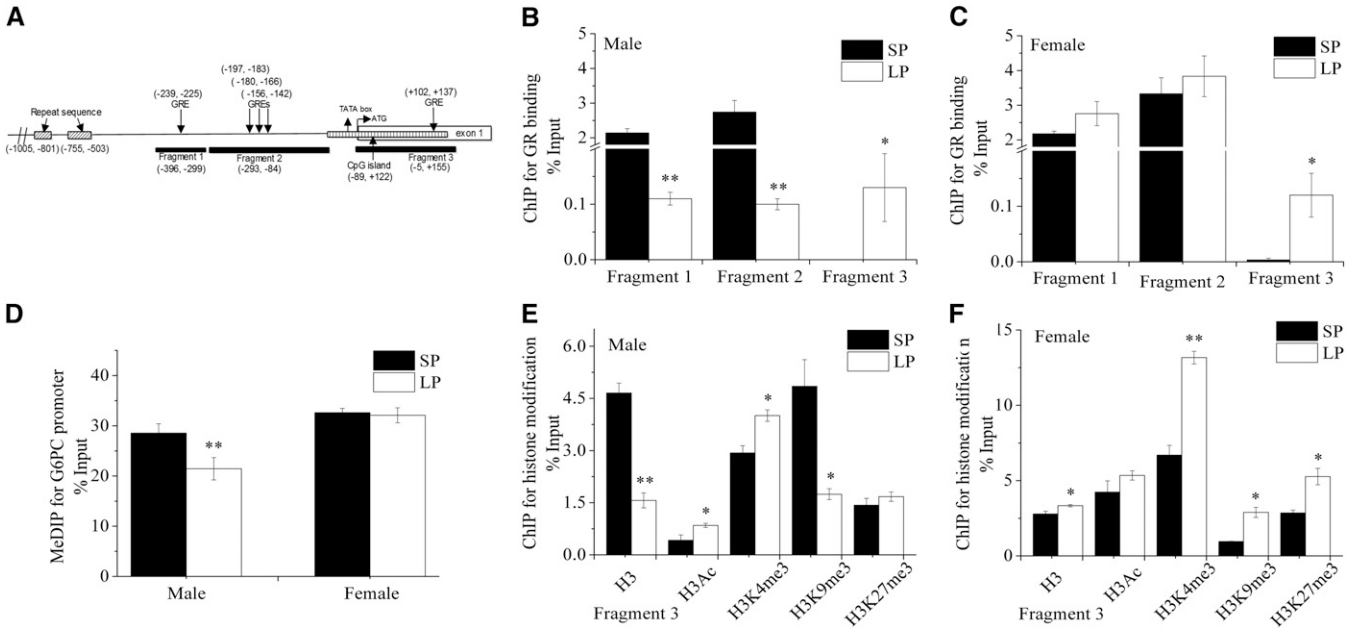

FIGURE 3 Schematic structure of the pig G6PC gene promoter (*A*), GR binding (*B,C*), DNA methylation (*D*), and histone modifications (*E,F*) on the *G6PC* promoter in the liver of piglets born to sows fed an SP or LP diet. Input DNA was used to normalize the data. Values are mean ± SEM, $n = 7$. $^*P < 0.05$, $^{**}P < 0.01$ vs. SP of the same gender. GR, glucocorticoid receptor; H3Ac, histone H3 acetylation; H3K4me3, histone H3 lysine 4 trimethylation; H3K9me3, histone H3 lysine 9 trimethylation; H3K27me3, histone H3 lysine 27 trimethylation; LP, low protein; SP, standard protein.

decreases liver, brain, heart, and kidney weights (27,28). Furthermore, we found that growth retardation in the LP piglets was associated with lower serum glucose and high hepatic glycogen at birth, which is in line with previous publications stating that moderate maternal nutrient restriction increases hepatic glycogen storage in fetal baboons (29) and unilateral ligation of the uterine artery causes IUGR in fetal guinea pigs, which is associated with increased hepatic glycogen content (30). The increased hepatic glycogen content suggests an adaptive mechanism of energy conservation by reduced glycolysis or enhanced gluconeogenesis in response to fetal nutritional deficiency.

Gluconeogenesis in newborns is activated by fetal cortisol, which rises dramatically near term (31,32). Maternal undernutrition (50% food restriction) during late gestation causes a significant increase in plasma corticosterone in newborn rats (33), yet serum cortisol did not differ between the LP and SP newborn piglets. This disparity between species may be attributable to differential prepartum cortisol surges (32,34); the serum cortisol concentration in newborn piglets is 3- to 4-fold higher than the adult concentration in this study and others (35,36). Therefore, it is possible that a prepartum cortisol surge may have masked differences caused by the maternal LP diet.

Cortisol exerts its biological activity through GR. Although we did not detect a difference in the serum cortisol concentration between LP and SP piglets, the GR mRNA abundance and nuclear phospho-Ser^{211}GR content were significantly upregulated in LP piglets, suggesting augmented cortisol signaling in response to the maternal LP diet. There is evidence that dexamethasone exposure in late gestation (37) and a maternal LP diet (37,38) both increase *GR* mRNA concentrations in rat liver. Furthermore, GR phosphorylation at Ser(211) is reported to be a biomarker for dexamethasone-induced GR activation in vivo (39).

G6PC, a target gene of GR, is a gatekeeper of hepatic glucose production. Previous studies indicate that fetal pigs treated with cortisol in late gestation demonstrate enhanced G6PC enzyme activity in liver at birth (32). Maternal protein restriction during gestation increases hepatic G6PC enzyme activity in rats (11). We also detected increased hepatic *G6PC* mRNA expression and enzyme activity in LP piglets, but only in males. Interestingly, hepatic activation of G6PC was associated with a significantly lower serum glucose concentration in male LP piglets. The lower serum glucose was unlikely the consequence of hepatic G6PC activation but rather a triggering factor for enhanced hepatic gluconeogenesis in newborn piglets. To our knowledge, this is the first evidence that a maternal LP diet during gestation induces a gender-specific response of *G6PC* expression in the liver. A similar gender-specific effect was reported in rat fetuses, where a maternal LP diet during the preimplantation period upregulated hepatic PCK expression only in males (40). The gender disparity may be attributable to fetal growth trajectory rates and the plasticity in response to the hormonal milieu such as sex hormones, adipokines, and cytokines (41–43).

The promoter of the mouse *G6PC* gene contains 3 positive GRE (−197 to −183, −180 to −166, −156 to −142) and one nGRE (−239 to −225) based on the in vitro analysis (9). However, our results implicate the presence of 4 nGRE (−321 to −306, −272 to −259, −256 to −242, −232 to −218) in the porcine *G6PC* promoter, because the GR binding to all these 4 putative GRE is inversely correlated to *G6PC* mRNA expression. Further functional analyses, both in vivo and in vitro, are required to verify the nature of these GRE on the porcine *G6PC* promoter. The functional nature of a GRE is determined by the interaction of GR with other transcription factors. For example, a positive GRE effect is observed in the presence of c-Jun, whereas an nGRE effect is exhibited in the presence of relatively high levels of c-Fos (44). It is noted that significantly enhanced GR binding to a half-site GRE in exon 1 of the *G6PC* gene was detected in the LP piglets, which is positively correlated to *G6PC*

gene expression. An androgen response element located in the exon 1-coding sequence of cystain-related protein gene crp2 was verified as an enhancer (45). However, whether this exonic GRE on the porcine *G6PC* coding sequence serves as an enhancer awaits further investigation.

Epigenetic regulation plays an important role in maternal nutritional programming. Maternal dietary protein restriction throughout gestation induces hypomethylation of GR and/or PPARα gene promoters in male offspring rats both at weaning (46) and in adults (47), and a 30% food restriction in pregnant baboons causes hypomethylation of the *PCK* promoter in fetal liver (13). In agreement with these findings, we report for the first time, to our knowledge, the hypomethylation of the *G6PC* gene promoter induced by a maternal LP diet in male piglets. *G6PC* gene promoter hypomethylation was associated with enhanced gene transcription, suggesting an inhibitory role of CpG methylation on *G6PC* gene transcription.

Histone modifications are also subject to nutritional programming and subsequently regulate gene transcription in concert with DNA methylation. In the present study, enhanced *G6PC* expression in the liver of male LP piglets was accompanied by enriched activation marks (H3Ac and H3K4me3) and diminished repression mark (H3K9me3). Our results agree with previous findings in rat liver that enhanced *GR* expression is associated with *GR* promoter hypomethylation as well as more H3Ac and less H3K9me2/3 (48). Interestingly, the alterations of histone modifications were different in females, with the LP females showing more H3 trimethylation marks on K4, K9, and K27 compared with their SP counterparts, probably due to a higher enrichment of H3 in general. Nevertheless, concerning the ChIP analysis of histone methylation marks, the specificity of the antibodies is of great importance. The antibodies for H3K4me3 and H3K9me3 are experimentally validated by the company to specifically recognize trimethylation, but not mono- or di-methylations. However, the antibody against H3K27me3 was not experimentally validated for its specificity; therefore, caution should be taken in the data interpretation.

Because the patterns of DNA methylation and histone modifications on a specific locus are closely associated (49), it is likely that the histone modification alterations observed in our study were induced at least partly by changes in DNA methylation. Interestingly, although *G6PC* promoter methylation was not altered in female LP piglet liver, the histone modifications differed from those in male LP piglets. All 3 detected histone marks, H3K4me3, H3K9me3 and H3K27me3, were higher. In addition, the enrichment of total H3 on *G6PC* promoter decreased in males and increased in females. The lower density of H3 observed in males might indicate more relaxed chromatin, which allows transcription factors to bind the promoter and activate gene transcription. Conversely, higher H3 enrichment detected in females may imply condensed nucleosomes, which are associated with transcriptional repression.

The mechanism underlying gender disparity in the epigenetic regulation of *G6PC* transcription in response to a maternal LP diet in pigs is unknown. Pre- and postnatal dietary exposure to phytoestrogens in mice causes gender-specific epigenetic regulation in the skeletal α-actin gene due to disrupted fetal endocrine environment (50). In this study, male and female embryos are presumably exposed to the same uterine environment and the gender differences may be due to the divergent intracellular environment during sexual differentiation. Certainly, we cannot exclude the influence of the fetal endocrine environment, which occurs later in development when the gonads begin to function. Further studies are necessary to elucidate the molecular mechanisms of gender differences.

The gender-specific regulation of *G6PC* expression also seems to occur at the post-transcriptional level. We detected increased expression of ssc-miR-339–5p and ssc-miR-532–3p in female LP piglets. These 2 miRNA are predicted to target the 3′UTR of the porcine *G6PC* gene to degrade *G6PC* mRNA or repress *G6PC* gene translation. How maternal nutritional intervention regulates mRNA expression and how epigenetic mechanisms including DNA methylation, histone modifications, and miRNA interact with other regulatory factors like GR to cause gender-specific responses of the *G6PC* gene are still unclear.

In conclusion, our results suggest that a maternal LP diet during gestation affects hepatic gluconeogenesis in newborn piglets in a gender-specific manner through the combined action of GR binding and epigenetic mechanisms on the *G6PC* gene promoter. Neonatal changes in hepatic gluconeogenesis may persist to adulthood, with long-term consequences on glucose homeostasis. Our findings may shed light on the gender-biased fetal programming of many human metabolic diseases.

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