

Exploration of glycogen deposition disorders in low-body-weight fetuses in a pig model

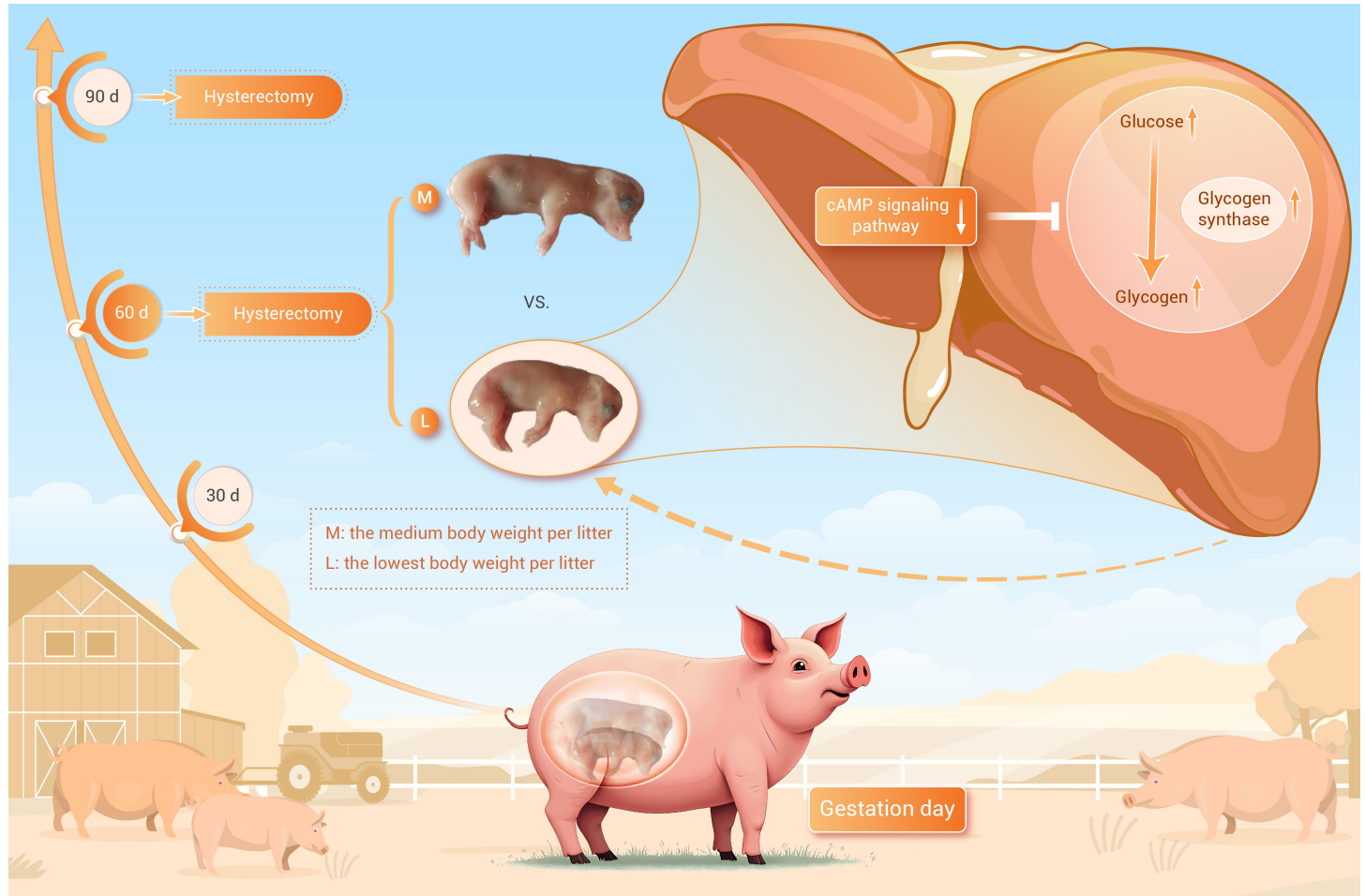
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GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- Hepatic glycogen deposition disorders happened in low-body-weight fetal pigs at mid-gestation but not at late gestation.
- Abnormal glucose deposition in the liver may impair the development of low-birth-weight fetal pigs at mid-gestation.
- The cAMP signaling pathway is crucial for regulating hepatic glycogen deposition in fetal pigs at mid-gestation.

Exploration of glycogen deposition disorders in low-body-weight fetuses in a pig model

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Glucose is a vital energy source for the fetus, storing any surplus as glycogen. Neonates experiencing intrauterine growth restriction exhibit decreased hepatic glycogen levels. Consequently, the regulation of glycogen deposition may hold immense significance in fetal development, yet the intricacies of glycogen deposition homeostasis remain poorly understood. Here, we aimed to explore the differences in fetal glycogen deposition and its potential mechanisms using fetal pigs with the lowest (L) and medium (M) body weights on gestation days 60 (GD 60) and 90 (GD 90). We observed higher hepatic glycogen concentrations in the L group on GD 60, which correlated with higher hepatic glucose levels and glycogen synthase activity. In addition, our investigation using RNA-seq and immortalized fetal pig liver cells revealed that the cAMP signaling pathway ranked prominently in the KEGG enrichment analysis of differentially expressed genes. Notably, this pathway was relatively suppressed in the livers of L group pigs on GD 60, compared to the M group. The cAMP signaling pathway suppression resulted in glycogen concentration upregulation in our *in vitro* model. Overall, our findings suggest that glucose needed for fetal development deposited abnormally in the liver may hinder the growth of low-body-weight fetuses at mid-gestation. Furthermore, we have identified the cAMP signaling pathway as a pivotal regulator of hepatic glycogen deposition homeostasis in fetuses at mid-gestation, offering a novel perspective on the factors contributing to delayed fetal growth.

INTRODUCTION

Fetuses that fail to reach their growth potential are recognized as intrauterine growth restriction (IUGR), and the incident rate of IUGR is approximately 10-15%.¹ Infants with IUGR have a higher mortality rate and are more susceptible to growth retardation, neurodevelopmental disorders, stroke, and metabolic syndrome later in life.¹ Maternal factors, including maturity, nutrition, lifestyle, and diabetic status, as well as the underdeveloped placenta, fetal genetic disease, and infection are all associated with IUGR.¹⁻⁴ The etiology of IUGR has not been fully clarified and the underlying molecular mechanisms of IUGR need to be further explored.

Glycogen storage plays a critical role in non-shivering thermogenesis and maintaining blood glucose levels in infants.⁵ However, neonates with IUGR exhibit reduced hepatic glycogen levels,⁶ and an inadequate energy supply is a significant contributor to impaired fetal development. Glucose serves as the primary energy source for the fetus, with excess glucose stored as glycogen. When glucose is scarce, the liver supplies energy through the glycogenolysis and gluconeogenesis pathways. Liver glycogen deposition homeostasis may be an important factor in fetal development, but the details are poorly understood.

Pigs share genetic and physiological traits with humans,⁷ including similar hepatic cell subpopulations and transcriptional regulators,⁸ making them valuable models for studying fetal physiology and metabolism. Newborn

piglets with low birth weight exhibit significantly lower hepatic glycogen concentrations than those with normal birth weight,^{9,10} highlighting the relevance of pigs as a promising model for the study of fetal hepatic glycogen. During gestation, hepatic glycogen levels rise, which is primarily fueled by glucose,^{11,12} with fetal hepatic glycogenolytic and gluconeogenic enzymes exhibiting low activity levels.¹³ However, there is limited knowledge on hepatic glycogen deposition changes in low-body-weight fetuses.

Here, we aimed to explore the difference in glycogen deposition and its potential mechanisms. To this end, we investigated fetal pigs with different body weights on gestation days (GDs) 60 (GD 60; mid-gestation) and 90 (GD 90; late gestation). Our results contribute to valuable insights into human fetal developmental disorders.

MATERIALS AND METHODS

Experimental design and sample collection

Primiparous sows were selected in this study because they produce a higher proportion of low-birth-weight piglets.¹⁴ Primiparous sows (Landrace × Yorkshire) from Longhua Animal Husbandry (China) were mated using identical semen samples. Pregnant sows had free access to drinking water and were fed 2.5 kg/day from days 0 to 70 of gestation, and then 2.2 kg/day from days 71 to 90 of gestation. A corn and soybean meal-based diet that met the nutrition requirements recommended by the National Research Council was administered.¹⁵ On GD 60 and 90, eight healthy sows with similar body conditions were chosen at each time point. After overnight fasting, sows were hysterectomized under anesthesia. The number and weight of each fetus were recorded (Table S1). The fetus with the lowest body weight (L) in the litter and one fetus with the medium body weight (M), which is near the average weight of the litter, were picked out in each litter. The livers of the selected fetuses were separated and weighed. Liver and *longissimus dorsi* muscle samples were flushed with saline, snap-frozen in liquid nitrogen, and stored at -80°C. Liver segments were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde.

Cell culture and treatment

Immortalized fetal pig primary hepatocytes were a gift from Professor Li (China Agricultural University). Cells were isolated from a fetal Chinese experimental mini-pig on GD 60 and immortalized. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco), 50 µg/ml penicillin, and 50 µg/ml streptomycin (Gibco) in a 5% CO₂ incubator at 37°C. When the cells reached 70% confluence, they were treated with different concentrations of H89 (MCE, USA) in DMEM. After treatment for 24 h, the cells were collected for glycogen content analysis.

Body condition and liver index

Crown-rump length (the supine length of the piglet from the crown of its

head to the base of its tail) and chest circumference of the fetus were measured.¹⁶ The liver index was calculated as a percentage of body weight.

Determination of hepatic biochemical and enzyme activity parameters

The glycogen content in tissues and cells was determined using a commercial glycogen concentration assay kit (Solarbio, China) following the manufacturer's instructions. Hepatic glucose, triglyceride, and cholesterol levels were determined using the Cobas C311 Analyser (Roche Diagnostics, Basel, Switzerland). The concentrations of hepatic glucose-6-phosphate (G6P) and glucose-1-phosphate (G1P), as well as the activities of hepatic glucokinase (GK), glucose-6-phosphatase (G6Pase), glycogen synthase (GS), and the phosphorylated glycogen phosphorylase (GP_a, the active form of glycogen phosphorylase, abbreviated as GP), were measured using commercial assay kits (Suzhou Grace Biotechnology Co., Ltd, China) according to the manufacturer's instructions. Hepatic biochemical and enzyme activity parameters were normalized to total protein concentration. Protein concentration was determined using a BCA protein assay kit (Beyotime Institute of Biotechnology, China).

Periodic acid-Schiff (PAS) staining

PAS staining was performed as described previously.⁹ Briefly, fixed samples were dehydrated, paraffin-embedded, and sectioned at 4 µm. After washing with distilled water, the sections were incubated in 0.5% periodic acid for 5 min, followed by incubation in Schiff's reagent for 15 min. The samples were counterstained, sealed, and photographed under an Olympus BX51 microscope (Olympus, Japan) at 100× magnification.

Transmission electron microscopy (TEM) analysis

TEM analysis was performed as described previously.¹⁷ Briefly, the liver samples were fixed in 3% glutaraldehyde and post-fixed in 1% osmium tetroxide. After dehydration with an increasing gradient of acetone, the samples were embedded in Epon 812. Ultra-thin sections were cut, stained with uranyl acetate and lead citrate, and analyzed using a JEM-1400-FLASH Transmission Electron Microscope (JEOL, Japan).

Determination of hepatic DNA and protein concentrations

The DNA concentration in the liver tissue was determined following the method described previously.¹⁸ Briefly, tissue was homogenized in a buffer (pH 7.4) consisting of 50 mM sodium phosphate, 2 M NaCl, and 2 mM EDTA. After brief sonication, 2 µg/ml Hoechst 33258 (Sigma, USA) was added, and the fluorescence intensity was detected in a spectrofluorometer using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. DNA concentration was calculated using calf thymus DNA (Sigma) as standard. Hepatic protein concentrations were determined using a BCA Protein Assay Kit (Beyotime Institute of Biotechnology).

Determination of hepatic uridine-diphosphate-glucose (UDPG) concentration

The liver tissue was homogenated in methanol, sonicated for 30 min, and incubated at 4°C for 1 h. After centrifugation at 13,899 ×g at 4°C for 15 min, the supernatant was obtained for ultra-performance liquid chromatography (LC) with triple quadrupole mass spectrometric (UPLC-QqQ-MS) analysis. The LC separation was performed using the Atlantis Premier BEH Z-HILIC column (1.7 µm, 2.1 mm × 100 mm; Waters, USA) with a column temperature of 40°C and a flow rate of 0.30 mL/min. The mobile phases A and B consisted of water and acetonitrile, respectively. The operation time was 5 min and the injection volume was 6 µL. The MS parameters were as follows: ion source; electrospray ionization; curtain gas: 35 arbitrary units (arb); collision gas: 9 arb; ion spray voltage: 4500 V; temperature: 450°C; ion source gas 1: 45 arb; and ion source gas 2: 45 arb. The UDPG (Shanghai yuan ye Bio-Technology, China) standard solution was prepared to identify and quantify the UDPG peaks at $R_t = 2.75$ min. The UDPG concentration was normalized to the total protein concentration.

Real-time quantitative polymerase chain reaction (RT-qPCR)

RNA extraction and RT-qPCR were conducted as described previously.¹⁹ Briefly, total RNA was prepared using AG RNAex Pro Reagent (Accurate

Biotechnology [Hunan] Co., Ltd.) following the manufacturer's instructions. Complementary DNA was synthesized using an Evo M-MLV RT Kit with gDNA Clean for qPCR (Accurate Biotechnology [Hunan] Co., Ltd.). Primers (Table S2) were designed using the Primer-BLAST tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The RT-qPCR was performed according to the manufacturer's instruction of the SYBR Green Premix Pro Taq HS qPCR Kit (Accurate Biotechnology [Hunan] Co., Ltd.). Several reference genes were tested and analyzed using NormFinder,²⁰ and the combination of peptidylprolyl isomerase A (PPIA) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ) was the most stable (Table S3). The relative gene expression was normalized by the combination of PPIA and YWHAZ using the $2^{-\Delta\Delta Ct}$ method.²¹ Data were displayed as relative values to the M group.

Capillary-based immunoassay

Protein expression in the liver tissue was determined using the Wes automated western blotting system and correlative reagent kit (ProteinSimple, USA). Proteins from liver tissue were extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology) and the total protein concentration was determined using a BCA protein quantitation assay kit (Beyotime Institute of Biotechnology). The immunoassays were performed according to the manufacturer's instructions. The antibodies used in this study are listed in Table S4. The protein expression was normalized to α -tubulin, and data were displayed as relative values to the M group. Samples were not boiled to detect G6Pase expression levels.

RNA-seq analysis

Hepatic transcriptome analysis was performed as described in a previous study.²² Total RNA was extracted using TRIzol (Invitrogen, USA), the library was prepared using Truseq™ RNA sample prep Kit (Illumina, USA) following the manufacturer's instructions, and sequencing was performed using the Illumina Novaseq 6000 platform (Illumina). Sequencing data were submitted to the NCBI Sequence Read Archive (SRA, No. PRJNA907772). Sequencing data were analyzed and plotted using the Majorbio Cloud Platform (<https://cloud.majorbio.com/>). Genes with a P -value < 0.05, fold change > 1.50 or fold change < 0.67 in comparison between groups L and M were recognized as differentially expressed genes (DEGs). The DEGs were validated using RT-qPCR. Briefly, 10 DEGs were randomly selected for RT-qPCR analysis. The results were compared with RNA-seq data (Figure S1).

Statistical analyses

All statistical analyses were performed using GraphPad Prism (V8.0.2). Diagram displays were made using GraphPad Prism (V8.0.2) and Adobe Illustrator (V24.0.1). Statistical analysis of the L and M groups on the same gestation day was performed using a Student's unpaired T -test (for normally distributed data with equal variance) or Mann-Whitney test (for non-normally distributed data or data with unequal variance) to assess differences between the two groups after the potential outliers identified by ROUT analysis were removed.²³ Data of immortalized fetal pig primary hepatocytes were analyzed using one-way analysis of variance (ANOVA) with *post-hoc* comparisons made by the least significant difference (LSD) test. Data are expressed as means ± standard error of the mean (SEM). $P < 0.05$ was considered statistically significant, and $P < 0.01$ was considered extremely significant.

RESULTS

Hepatic glycogen deposition disorders occur in low-body-weight fetal pigs at mid-gestation

To determine the critical period of glycogen deposition disorders in low-body-weight fetal pigs, we selected the L and M groups on GD 60 and GD 90 (Figure 1A). Comparisons of body weight, crown-rump length, and chest circumference revealed significantly smaller measurements in the L group on both GD 60 and GD 90 than in the M group (Figure 1B).

Next, we analyzed glycogen deposition in the liver and *longissimus dorsi* muscles using a glycogen concentration assay. Notably, differences in glycogen levels were observed only in the liver (Figure 1C). Intriguingly, the L group showed a significantly higher hepatic glycogen concentration on GD 60 (Figure 1C).

Hepatic glycogen differences were further confirmed by PAS staining and

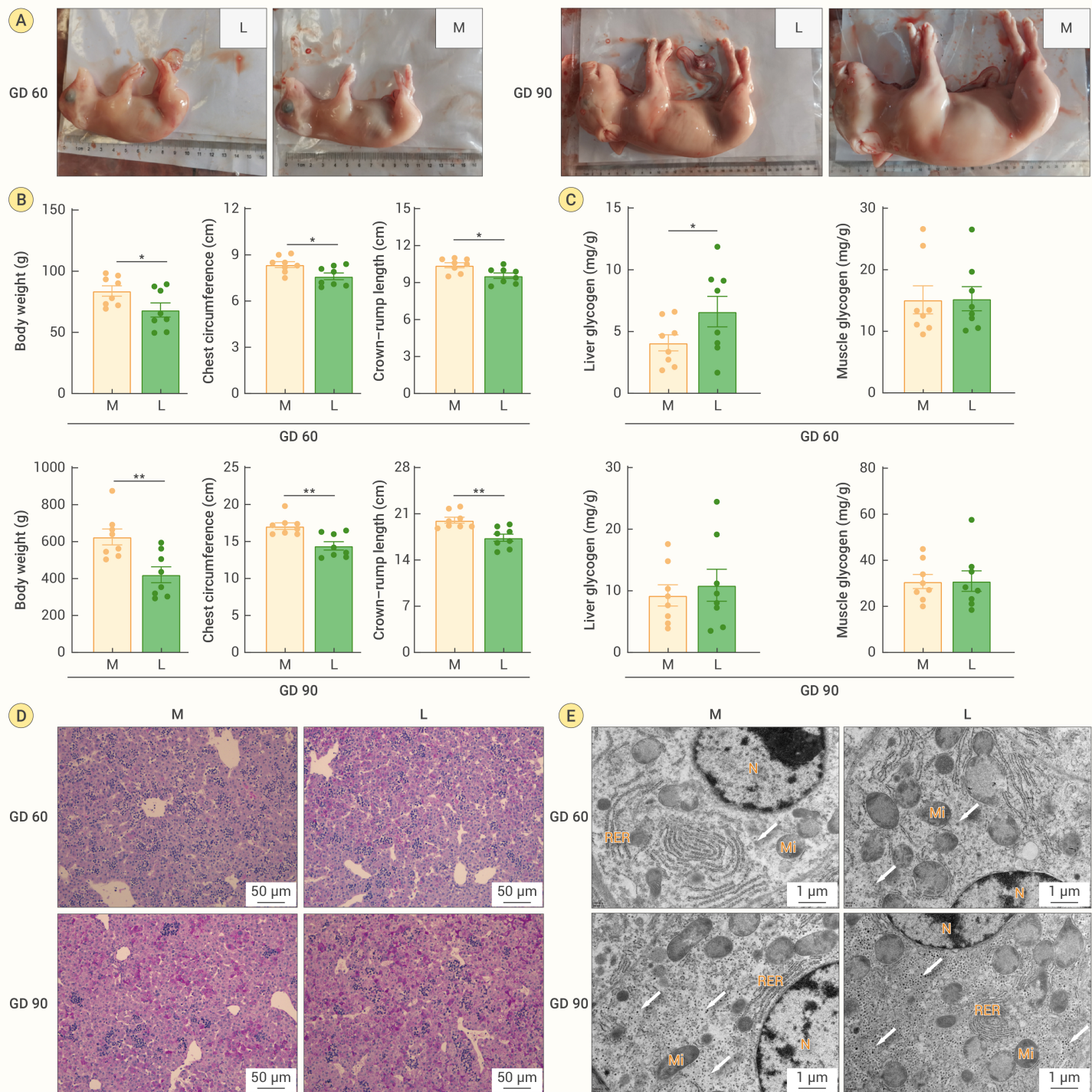


Figure 1. Low-body-weight fetal pigs have higher hepatic glycogen concentration (A) Representative images of the L and M group fetal pigs on GD 60 and GD 90. (B) Comparisons of fetal pig characteristics on GD 60 and GD 90. (C) Glycogen concentration differences in hepatic and *longissimus dorsi* muscle samples in fetal pigs. (D) Representative images of periodic acid-Schiff stain (PAS) samples from the liver. Scale bar = 50 μ m. (E) Representative transmission electron microscopy images of the liver. Glycogen granules in the cytoplasm are shown (black arrows). Scale bar = 1 μ m. * P < 0.05, ** P < 0.01. Data are presented as means $\pm$ SEM (n = 8). GD: gestation day, M: medium body weight, L: the lowest body weight. N: nucleus, Mi: mitochondria, RER: rough endoplasmic reticulum

transmission electron microscopy. Notably, compared to those in the M group, the positive signal was remarkably stronger in the L group (Figure 1D), and more glycogen granules were observed in the L group on GD 60 (Figure 1E). However, minimal variation was noted between the two groups on GD 90 (Figures 1D–E). Taken together, these results indicate that glycogen deposition disorders happened in the L group on GD 60.

Higher hepatic glycogen concentration is not related to liver development and intrauterine position

Tissue development is closely related to its metabolic homeostasis, and

fetal growth is influenced by intrauterine factors such as position.²⁴ Therefore, we assessed liver development to explore its potential impact on hepatic glycogen concentration. No significant difference was observed in the liver index between the two groups on GD 60 and GD 90 (Figure 2A). Similarly, there were no significant differences in developmental indicators, such as DNA concentration, protein concentration, and protein/DNA ratio between the two groups (Figure 2B).

We also found that the L group had a similar intrauterine position, compared with the M group, on both GD 60 and GD 90 (Figure 2C). These findings suggest that a higher hepatic glycogen concentration is not related

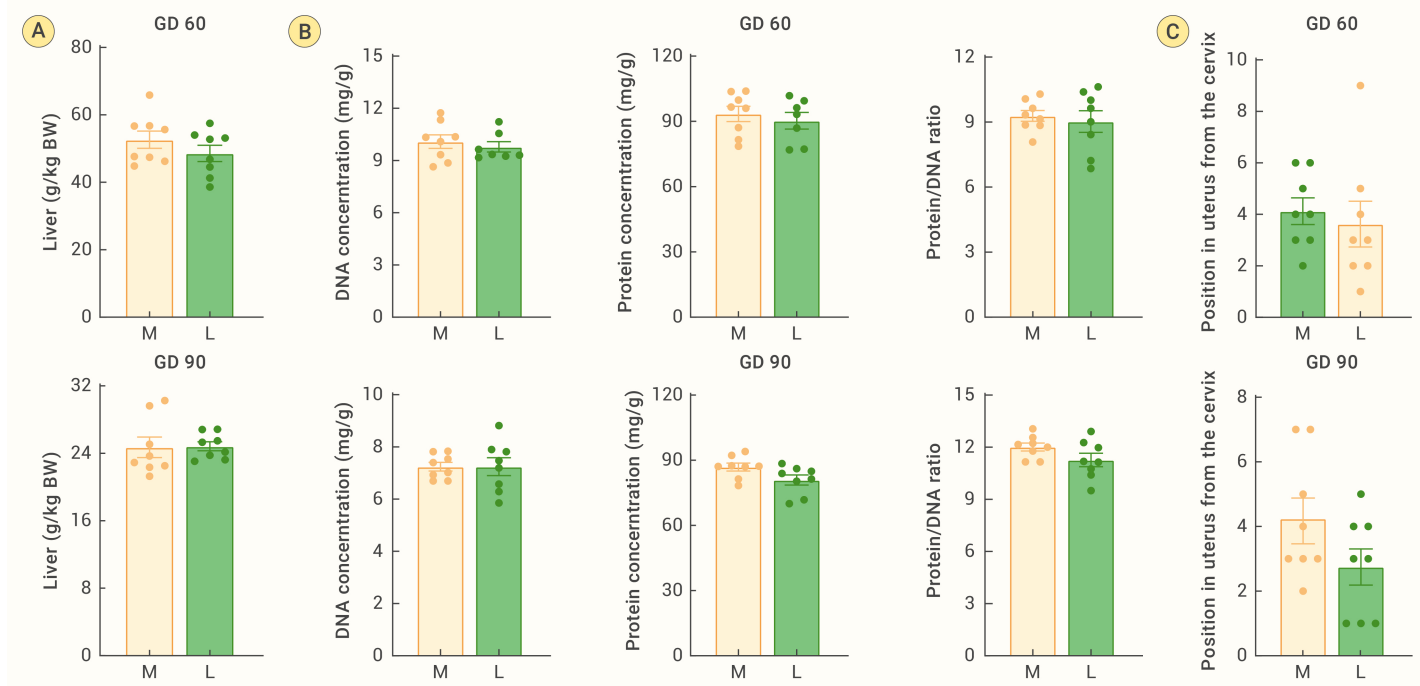


Figure 2. Higher hepatic glycogen concentration is not related to liver development and intrauterine position (A) Liver index of fetal pigs. (B) DNA concentration, protein concentration, and protein/DNA ratio in the liver. (C) The intrauterine position of fetal pigs. Data are presented as means ± SEM ($n = 8$). GD: gestation day, M: medium body weight, L: the lowest body weight.

to liver development or intrauterine position.

Higher hepatic glucose levels contribute to hepatic glycogen accumulation in low-body-weight fetal pigs at mid-gestation

To investigate the mechanism underlying hepatic glycogen changes, glucose and lipid metabolite levels in the umbilical cord plasma and liver were measured. On GD 90, no significant differences were observed in metabolite and hormone levels in the umbilical cord plasma, nor hepatic levels of glucose, cholesterol, and triglyceride between the L and M groups (Figures S2A–B).

Hepatic cholesterol and triglyceride levels were comparable between groups L and M on GD 60 (Figure 3A). However, the glucose, G1P, and UDPG levels were significantly higher in the L group than in the M group on GD 60 (Figure 3A). These results indicate an elevated carbohydrate flux into the glycogen synthesis pathway in the liver of the L group on GD 60.

Next, the activities of critical enzymes involved in carbohydrate metabolism were assessed to determine the underlying mechanisms. On GD 60, GK activity in the liver was significantly higher in the L group than in the M group, whereas G6Pase activity remained unchanged (Figure 3B). The mRNA and protein expression levels of pyruvate kinase L (PKL) were significantly lower in the L group than in the M group on GD 60 (Figures 3C–D).

The protein levels of critical gluconeogenic enzymes such as G6Pase and phosphoenolpyruvate carboxykinase (PEPCK) remained consistent in the L group on GD 60 (Figure 3D). *G6PC1* mRNA expression was significantly upregulated, while *PCK1* mRNA expression remained consistent in the L group on GD 60 (Figure 3C). The mRNA and protein expression of lactate dehydrogenase A (LDHA), glucose-6-phosphate dehydrogenase (G6PD), and phosphogluconate dehydrogenase (PGD) were comparable between the two groups on GD 60 (Figures 3C–D). These changes contributed to a balance in G6P levels (Figure 3A). Taken together, these findings indicate that elevated hepatic glucose flowed into the glycogen synthesis pathway, and contributed to glycogen accumulation in the L group on GD 60 (Figure 3E); a similar hepatic glucose level may lead to a consistent hepatic glycogen concentration in the L group on GD 90.

Higher hepatic GS activity contributes to hepatic glycogen accumulation in the low-body-weight fetal pigs at mid-gestation

The activities of GS and GP directly influence glycogen deposition. The

activities of GS and GP were comparable between the two groups on GD 90 (Figure 4A). Notably, the GS and GP activities were significantly higher in the L group than in the M group on GD 60 (Figure 4A). Correspondingly, lower GS phosphorylation and higher GP phosphorylation were observed in the L group on GD 60 (Figures 4B–C).

To elucidate the mechanism underlying the changes in GS and GP activity on GD 60, we examined the protein levels of their regulators. In the L group, the expression levels of protein phosphatase 1 regulating subunit, PTG, and phosphorylase kinase β -subunit (PHKB) were significantly higher than those in the M group on GD 60 (Figures 4D–E). Furthermore, GSK3 β phosphorylation was significantly higher in the L group than in the M group, while the expression level of the protein phosphatase 1 regulating subunit, G_L , remained unchanged (Figures 4D–F). Taken together, these findings suggest that higher GS activity contributed to hepatic glycogen accumulation in the L group on GD 60, and similar GS activity might lead to a consistent hepatic glycogen concentration in the L group on GD 90.

cAMP signaling pathway plays a crucial role in hepatic glycogen deposition disorders

RNA-seq analysis was performed to explore key pathways involved in hepatic glycogen deposition. A high correlation between samples within the group indicated high parallelism (Figures 5A–B). Compared to those in the M group, 189 genes were upregulated and 154 genes were downregulated in the L group on GD 60 (Figure 5C). The cAMP signaling pathway was noticed for its essential role in carbohydrate metabolism²⁵ in the KEGG enrichment analysis (Figure 5D). Western blot analysis confirmed the suppression of the cAMP signaling pathway in the L group fetal liver on GD 60 (Figures 5E–F).

Next, an *in vitro* experiment was performed using H89 (a cAMP signaling pathway inhibitor) to further explore the influence of the cAMP signaling pathway on glycogen concentration. H89 was found to significantly promote the glycogen concentration in primary hepatocytes from fetal pigs (Figure 5G). Collectively, our results demonstrate that the cAMP signaling pathway plays a critical role in hepatic glycogen deposition homeostasis in the L group on GD 60.

DISCUSSIONS

The present study revealed hepatic glycogen deposition disorders in low-body-weight fetal pigs at mid-gestation. We also identified the critical

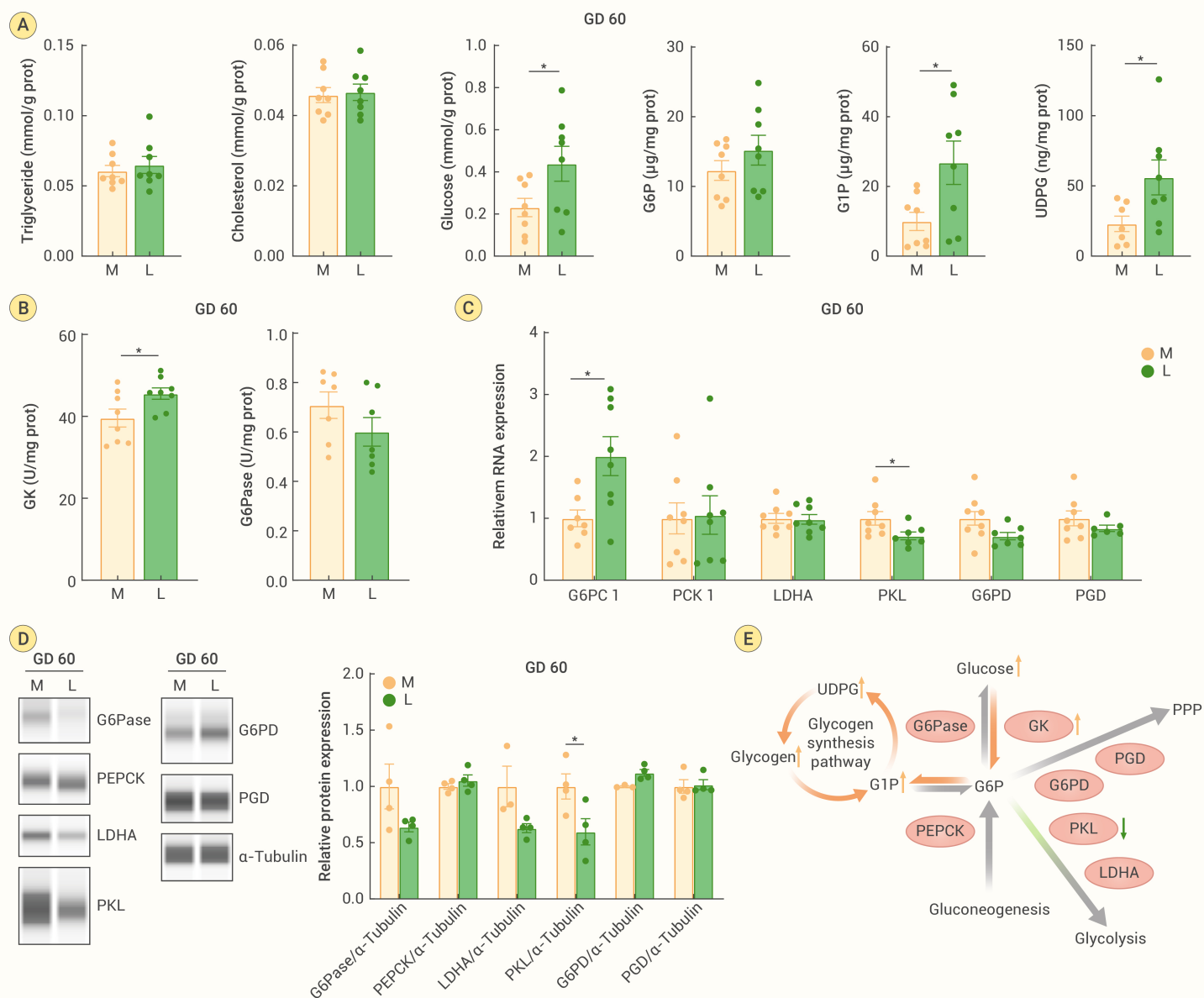


Figure 3. Increased hepatic glucose flux into the glycogen synthesis pathway contributes to glycogen accumulation in the L group on GD 60 (A) The concentrations of hepatic glucose-lipid metabolites in fetal pigs, $n \geq 7$. (B) Enzymatic activity of GK and G6Pase in the liver, $n \geq 7$. (C) Relative mRNA expression of genes involved in glucose metabolism, $n \geq 6$. Data are presented as relative values to the M group. (D) Relative protein expression of genes involved in glucose metabolism, $n \geq 3$, data are presented as the relative values to the M group. (E) Overview of glucose metabolism pathways. Orange arrows indicate upregulation and green arrows indicate downregulation. * $P < 0.05$, ** $P < 0.01$. Data are presented as means $\pm$ SEM. GD: gestation day; M: medium body weight; L: the lowest body weight; G6P: glucose-6-phosphate; G1P: glucose-1-phosphate; UDPG: uridine-diphosphate-glucose; GK: glucokinase; G6Pase: glucose-6-phosphatase; PEPCCK: phosphoenolpyruvate carboxykinase; LDHA: lactate dehydrogenase A; PKL: pyruvate kinase L; G6PD: glucose-6-phosphate dehydrogenase; PGD: phosphogluconate dehydrogenase; PPP: pentose phosphate pathway.

metabolites, enzymes, and pathways involved in glycogen deposition disorders. We propose that abnormal deposition of glucose in the liver, which is required for fetal development at mid-gestation, may lead to impaired fetal development.

The total gestation period for pigs is approximately 114 days. Placental development in pigs, which is crucial for fetal growth, is completed between GD 60 and GD 70.²⁶ By GD 90, the fetal pig's body weight begins to increase rapidly.²⁷ Thus, fetal pigs on GD 60 and GD 90 were selected for this study.

Notably, fetal hepatic glycogen concentration changes in low-body-weight pigs at mid-gestation are opposite of those at birth.⁹ This result is consistent with unpublished data from our lab that the hepatic glycogen concentration is also higher in low-birth-weight fetal Huanjiang mini-pigs on GD 45 and GD 75, indicating that different breeds may have an abnormal deposition of hepatic glycogen in low-body-weight fetal pigs at mid-gestation.

In our study, no significant difference was observed in hepatic glycogen on GD 90 between the L and M groups. Reportedly, the hepatic glycogen of IUGR fetal sheep is comparable to that of the control group on GD 125.²⁸ Thus, we

hypothesize that the hepatic glycogen in normal-body-weight fetal pigs may catch up in late gestation, and overtake the low-body-weight fetal pigs at terminal pregnancy. This catch-up may be related to the energy allocation pattern of low-birth-weight fetuses during late gestation. Reportedly, the low-birth-weight newborn piglets exhibited significantly higher relative weights of vital organs, including the brain and the heart.¹⁰ Low-birth-weight fetuses allocate more energy towards the development of these organs,²⁹ potentially resulting in a decrease in hepatic glycogen and a consequently lower glycogen concentration at birth. Collectively, hepatic glycogen deposition disorder was observed in low-body-weight fetal pigs at mid-gestation.

To explore the mechanism underlying hepatic glycogen concentration differences, we evaluated liver development. DNA concentration reflects hyperplasia, and the protein/DNA ratio is an indicator of cell size.³⁰ Our results showed similar levels in these indices and comparable liver indices between the two groups on GD 60 and GD 90, which excluded the influence of liver development on glycogen concentration differences.

The conjoint increase in G1P, UDPG, and glycogen indicated an increased

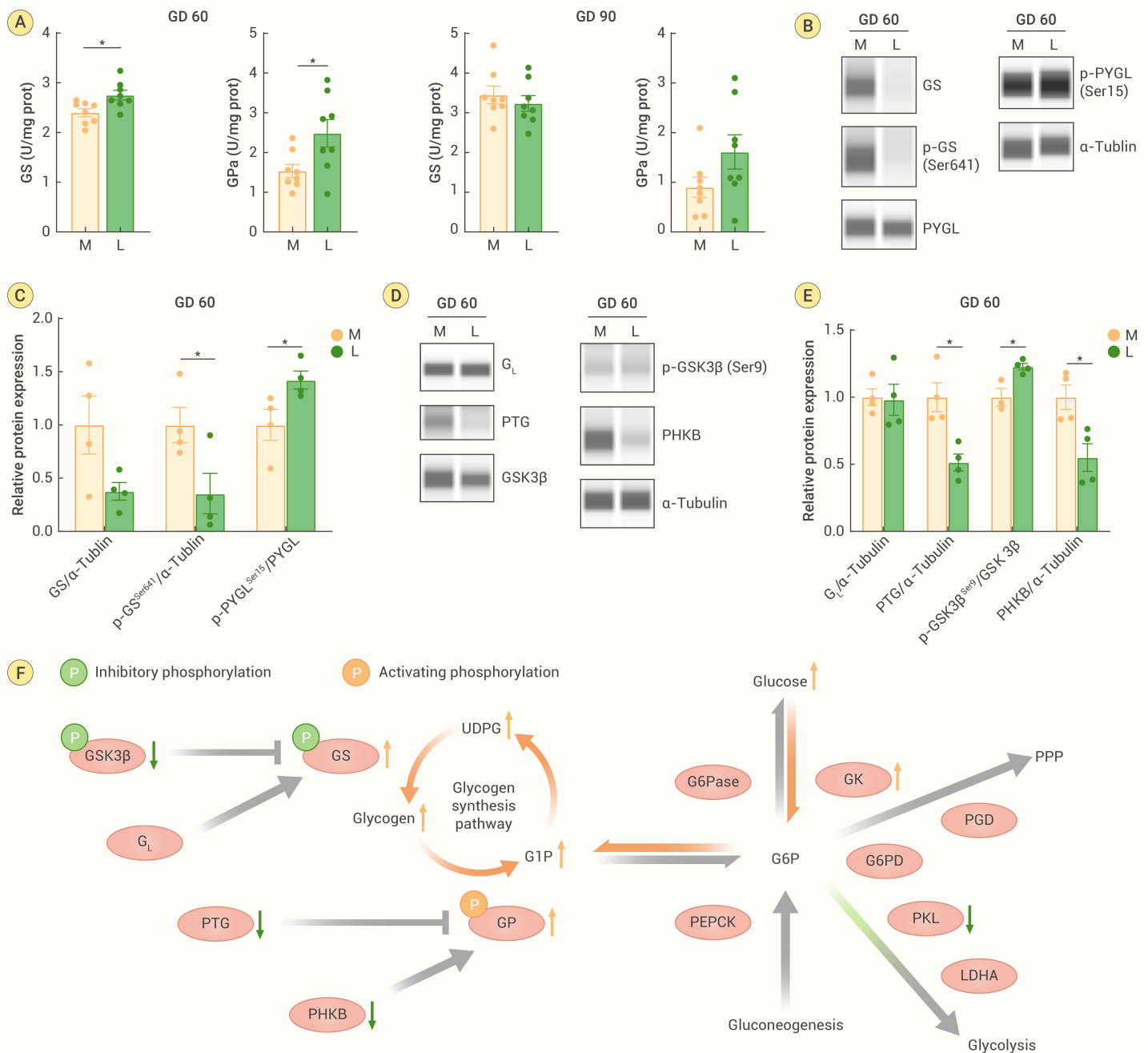


Figure 4. Protein expression levels of regulators involved in modulating GS and GP activity on GD 60 (A) GS and GP activity in fetal pig liver. Data are presented as means $\pm$ SEM and displayed as relative values to the M group ($n = 8$). (B, C) Protein expression levels of total and phosphorylated GS and GP in fetal pig liver tissues. Data are presented as means $\pm$ SEM and displayed as relative values to the M group ($n = 4$). (D, E) Protein expression levels of regulators involved in modulating GS and GP activity in fetal pig liver tissues. Data are presented as means $\pm$ SEM and displayed as relative values to the M group ($n \geq 3$). * $P < 0.05$. (F) Overview of glucose metabolism pathways. Orange arrows indicate upregulation and green arrows indicate downregulation. GD: gestation day, M: medium body weight, L: the lowest body weight, GS: glycogen synthase, GP: glycogen phosphorylase, GPa: phosphorylated GP, PYGL: liver isoenzyme of glycogen phosphorylase, PTG, G_L: protein phosphatase 1 regulating subunit, GSK3: glycogen synthase kinase 3, PHKB: phosphorylase kinase β -subunit, G6P: glucose-6-phosphate, G1P: glucose-1-phosphate, UDPG: uridine-diphosphate-glucose, GK: glucokinase, G6Pase: glucose-6-phosphatase, PEPCK: phosphoenolpyruvate carboxykinase, LDHA: lactate dehydrogenase A, PKL: pyruvate kinase L, G6PD: glucose-6-phosphate dehydrogenase, PGD: phosphogluconate dehydrogenase, PPP: pentose phosphate pathway.

carbohydrate flux into the glycogen synthesis pathway on GD 60. As a central molecule in multiple metabolic pathways, hepatic G6P comes from glucose or gluconeogenesis, and also flows into glycolysis and the pentose phosphate pathway (PPP).³¹ In the present study, higher intracellular glucose and GK activity, and lower PKL expression levels were found in the L group than in the M group on GD 60. No significant difference was observed in the expression of critical enzymes in gluconeogenesis and PPP between the L and M groups on GD 60. These results indicate that elevated hepatic glucose flows into the glycogen synthesis pathway, contributing to higher hepatic glycogen deposition in low-body-weight fetal pigs at mid-gestation. Insufficient glucose supply is one of the major causes of retarded fetal growth.³² Thus,

abnormal deposition of glucose in the liver, which is required for growth, might lead to impaired fetal development.

GS activity is negatively correlated with phosphorylation, whereas GP activity is positively correlated with phosphorylation. The protein phosphatase 1 regulating subunit G_L and PTG are regulators of glycogen metabolic enzymes.^{33,34} Both PTG and G_L participate in the regulation of GS and GP, but their regulations are selective; G_L primarily serves as a GS phosphorylase and PTG primarily serves as a GP phosphorylase.^{35,36} Phk catalyzes the phosphorylation and the activation of GP.³⁷ The lower Phk and PTG protein expression levels in this study appeared to have contradictory effects on GP phosphorylation in the L group on GD 60. Reportedly, PTG may coun-

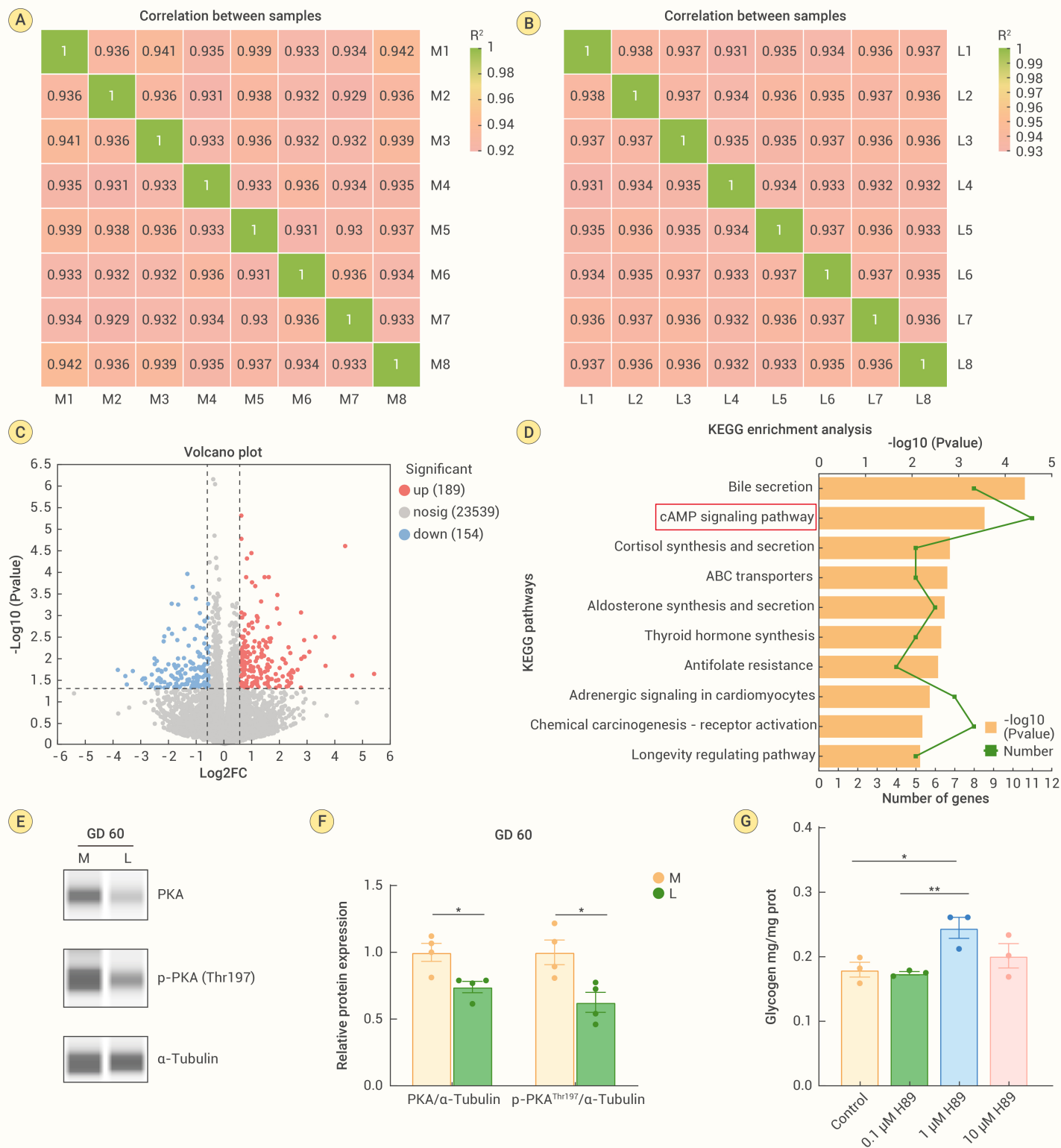


Figure 5. RNA-seq analysis reveals potential targets involved in hepatic glycogen deposition regulation on GD 60 (A-B) Spearman correlation analysis between samples on GD 60, $n = 8$. (C) Volcano plots of differentially expressed genes between the L and M groups on GD 60, $n = 8$. Red and blue dots represent upregulated and downregulated genes, respectively. (D) KEGG enrichment analysis of DEGs. (E, F) Western blot analysis of proteins involved in the cAMP signaling pathway in fetal pig livers on GD 60, $n \geq 3$. Data are presented as means $\pm$ SEM and protein data are displayed as the relative values to the M group. (G) The effect of H89 (a cAMP signaling pathway inhibitor) on glycogen concentration in immortalized fetal pig primary hepatocytes, $n = 3$. Data are presented as means $\pm$ SEM. Experiments were performed in triplicate. * $P < 0.05$, ** $P < 0.01$. GD: gestation day, M: medium body weight, L: the lowest body weight, DEG: differentially expressed gene, PKA: cAMP-dependent protein kinase.

teract the activation of GP by glucagon, perhaps via the inhibition of Phk.³⁵ Thus, lower PTG expression levels may have led to higher GP activity in the L group on GD 60.

GSK3 plays a critical role in the covalent regulation of GS. Inactivated GSK3 inhibits the phosphorylation of GS, leading to higher GS activity which results

in elevated glycogen synthesis.^{36,38} In this study, the G_L expression levels were comparable between the two groups and inhibited GSK3 β induced higher GS activity in the L group on GD 60. As for fetal pigs on GD 90, no significant difference was observed in GS activity between the two groups, which might have contributed to the comparable hepatic glycogen concentration. Overall,

these findings support the idea that GS activity plays a key role in hepatic glycogen deposition homeostasis in fetal pigs during mid- and late gestation.

The cAMP signaling pathway was identified as a critical pathway involved in glycogen deposition homeostasis using RNA-seq analysis combined with *in vitro* verification. The cAMP signaling pathway participates in the regulation of many glucose metabolic enzymes including GK, phosphorylase kinase, GS, PEPCK, and G6Pase.^{25,39-41} However, its role in the modulation of these enzymes in the fetal pig liver requires further exploration.

This study has some limitations. First, the role of the placenta and hormones in the umbilical cord plasma in glycogen deposition disorders at mid-gestation remains to be elucidated. In addition, hepatic glycogen changes in low-body-weight fetal pigs at early and final gestation need to be further clarified, which will help to illustrate the dynamic changes in hepatic glycogen deposition homeostasis in low-body-weight fetal pigs during the entire period.

In conclusion, our findings show that higher glycogen deposition occurs in the liver but not in the muscle on GD 60 in low-body-weight fetuses. Abnormal deposition of glucose in the liver, which is required for fetal development at mid-gestation, may lead to retarded fetal growth. The homeostasis of hepatic glycogen deposition in fetal pigs during mid- and late gestation is intimately tied to the hepatic glucose concentration and the activity of glycogen synthase. The cAMP signaling pathway plays a key role in hepatic glycogen homeostatic regulation in mid-gestation fetuses. This study provides a new perspective on the etiology of IUGR.

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AUTHOR CONTRIBUTIONS

H.L. and Q.L. provided funding. H.L., Y.Y., and Q.L. designed the study. H.L., M.B., M.Q., L.W., and X.X. edited the manuscript. Y.S., S.W., Y.W., Y.Z., and C.P. performed the experiments. Y.S. analyzed the data, drew the pictures, and prepared the manuscript. All authors contributed to the manuscript and approved the final version.

DECLARATION OF INTERESTS

Y.Y. is an Editorial Board member of The Innovation Life. He was blinded from review-

ing or making final decisions on the manuscript. The article was subject to the journal's standard procedures, with peer review handled independently of this Editorial Board member and their research groups. The other authors declare that they have no conflicts of interest.

ETHICAL STATEMENT AND PATIENT CONSENT

This study followed the guidelines for animal research and was approved by the Animal Welfare Committee of the Institute of Subtropical Agriculture, Chinese Academy of Science (2013020).

DATA AND CODE AVAILABILITY

All of the data necessary to evaluate the conclusions of the article are included in the article or the supplemental information.

SUPPLEMENTAL INFORMATION

It can be found online at <https://doi.org/10.59717/j.xinn-life.2024.100119>