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Dietary genistein supplementation in laying broiler breeder hens alters the development and metabolism of offspring embryos as revealed by hepatic transcriptome analysis

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ABSTRACT: Genistein (GEN) is a type of isoflavone mainly derived from soy products. In this experiment, we added 40 and 400 mg/kg GEN to the diet of laying broiler breeder hens to clarify the maternal effects of GEN on the development and metabolism of chick embryos. GEN treatment at 40 mg/kg increased embryonic length, weight, and liver index, as well as the width of the proliferative zone in the tibial growth plate of chick embryos. Gene ontology (GO) cluster analysis of the hepatic transcriptome showed that GEN treatment promoted embryonic development and cell proliferation. Low-dose GEN treatment increased insulin growth factor-binding protein (IGFBP)3 mRNA expression in the embryonic liver, whereas high-dose GEN treatment increased IGFBP5 expression and activated the apoptosis and protein tyrosine kinase signaling pathways. Furthermore, adding supplemental GEN to the diet of hens promoted the glycolysis process in the embryonic liver through the insulin-signaling pathway, upregulated target genes (phosphoglucomutase-2, hexokinase 1, dihydroxyacetone phosphate by aldolase, phosphofructokinase, platelet, and enolase 2), and enhanced the transport of carboxylic acids and cholesterol and the synthesis of unsaturated fatty acid (arachidonic acid) in the embryonic liver through upregulation of liver X receptor, sterol regulatory element-binding protein 1, and patatin-like phospholipase A. Additionally, GEN treatment increased fatty acid β-oxidation and Na^+/K^+-ATPase activity in the embryonic liver through activation of peroxisome proliferator-activated receptors (PPARs; PPARα and PPARδ) and the AMPK signaling pathway, which could provide energy for embryonic development. In addition, GEN treatment in hens increased superoxide dismutase activity and metallothionein expression in the chick embryonic liver and promoted lymphocyte proliferation through upregulation of mRNA expression of CDKN1A, IL12RB1, Sox11, PRKAR1A, PRKCQ, and TCF3. The improved immunity and antioxidant capacity, as a result of maternal GEN effects, was conducive to embryonic development. In summary, the addition of GEN to the diet of laying broiler breeder hens significantly promoted the development and metabolism of chick embryos.—Lv, Z., Fan, H., Zhang, B., Ning, C., Xing, K., Guo, Y. Dietary genistein supplementation in laying broiler breeder hens alters the development and metabolism of offspring embryos as revealed by hepatic transcriptome analysis. FASEB J. 32, 4214–4228 (2018). www.fasebj.org

KEY WORDS: genistein · embryo · transcriptome · metabolism · development

Genistein (GEN; 4′,5,7-trihydroxyisoflavone), the predominant isoflavone derived from soybeans, has been investigated for its hypolipidemic, antilipogenic, and antioxidant effects in biologic systems (1, 2). GEN is structurally similar to estrogen and can bind to vertebrate estrogen receptors (ESRs; ESR-α and ESR-β),

ABBREVIATIONS: AA, arachidonic acid; ABCG1, ATP-binding cassette transporter G1; ALDOC, fructose-biphosphate C aldolase; AOC, antioxidant capacity; CETP, cholesteryl ester transfer protein; CON, control; CSCM, corn-miscellaneous meal; CuZn-SOD, copper-zinc superoxide dismutase; DGE, differential gene expression; ELOVL, elongation of very long-chain fatty acid; ENO, enolase; ESR, estrogen receptor; FASN, fatty acid synthase; FDR, false discovery rate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GEN, genistein; GO, gene ontology; HE, hematoxylin and eosin; HGE, high genistein; HKDC, hexokinase domain containing; IGFBP, insulin growth factor-binding protein; KEGG, Kyoto Encyclopedia of Genes and Genomes; LGE, low genistein; LPL, lipoprotein lipase; LXR, liver X receptor; MHC, major histocompatibility complex; Mn-SOD, manganese superoxide dismutase; MT, metallothionein; PFKP, phosphofructokinase, platelet; PNPLA, patatin-like phospholipase A; PPAR, peroxisome proliferator-activated receptor; QC, quality control; qRT-PCR, quantitative RT-PCR; RNA-Seq, RNA sequencing; SDE, significantly differentially expressed; SLC, solute carrier; SOD, superoxide dismutase; SREBP, sterol regulatory element-binding protein; STARD5, StAR-related lipid transfer protein 5; T-AOC, total antioxidant capacity; T-SOD, total superoxide dismutase; VLDL, very low density lipoprotein

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exerting either estrogenic or anti-estrogenic effects. The phenolic hydroxyl groups in the structure of soybean isoflavones can reportedly neutralize free radicals by acting as a hydrogen donor (3) and increase the total antioxidant capacity (T-AOC) in the livers of rats (4). Soy isoflavones, found in legumes and animal forages, can improve the growth and reproductive performance of livestock (5–7). Dietary GEN relieves lipid peroxidation, inflammatory responses, and fibrosis in the livers of mice fed choline-deficient diets (8). The addition of 20–80 mg/kg soy isoflavones to the diet enhances the immune response in broiler hens (9).

GEN treatment increases the concentrations of IGF-I and LH and the expression of the IGF receptor in the anterior pituitary of barrows, indicating that GEN affects the IGF-I system (10). The levels of insulin growth factor-binding protein (IGFBP)3 and IGF-I in the fetus determine the ability of cells to differentiate and proliferate, as well as to metabolize glucose and amino acids (11). GEN can also activate the peroxisome proliferator-activated receptors (PPARs; PPARα and PPARγ) as a ligand. Then, PPARs bind to the retinoid X receptor and form a heterodimer, which can bind to the PPAR response element in the promoter region to enhance fatty acid β-oxidation (12, 13). Research suggests that PPARδ can also promote fatty acid catabolism by enhancing fatty acid transport and β-oxidation (14). GEN has also been reported to increase the activity of $Na+/K^+$-ATPase in the livers of young rats (15). However, the mechanism of maternal influence of GEN on the development and metabolism of chick embryos remains unknown.

In our experiment, we aim to study the effects of GEN on embryonic development and metabolism from the perspective of maternal nutrition, thus providing a theoretical basis for regulating the growth and metabolism of offspring by adding bioactive compounds to the diet of matrilineal parent generations. Currently, studies on the maternal effects of isoflavones have mainly focused on mammals (16). Additionally, studies on the mechanism of maternal influence of GEN on the growth of offspring are rare. Research has shown that supplemental daidzein in the maternal diet during late gestation marginally affects the meat quality and skeletal muscle cellularity of the progeny (17). However, GEN treatment in maternal rats can cause subtle alterations in some sexually dimorphic behaviors of the offspring (18). Poultry eggs are recognized as carriers that provide nutrients for embryonic development and mediate maternal signal transmission to embryos. Dietary GEN for laying poultry can also be deposited into the yolks of the eggs (19). Dietary GEN (800 mg/kg) can reportedly improve egg quality and decrease the egg yolk malondialdehyde concentration in quail (20). Chick embryos have independent development and represent a good model for studying the effects of maternal nutrition on embryonic development and metabolism. According to the research cited above, we hypothesized that the addition of GEN to the diet of laying broiler breeder hens could promote embryonic development and modulate metabolism through the IGF system and PPAR pathway in embryos.

GEN has been applied in humans and monkeys as a nontoxic medicine (21, 22). However, the effects of GEN on reproductive performance may vary with sex, age, and hormonal status. High-dose GEN inhibits the activity of tyrosine-protein kinases and impairs the function of liver mitochondria (23). High-dose GEN can also activate apoptotic signaling pathways (24). Furthermore, serum GEN, found in soy-fed infants, may be capable of inducing thymic and immune abnormalities (25), prompting us to explore thoroughly the different effects between low- and high-dose GEN on embryonic development. Unlike mammals, the development and metabolism of chicken embryos within the eggshell are directly affected by their immunity and AOC. Transcriptomic analysis is an effective technique to determine the target genes and signaling pathways affected by environmental endocrine disruptors (26). Therefore, we used RNA-sequencing (RNA-Seq) to clarify the effects of feeding laying broiler breeder hens with low- and high-dose GEN on the development and metabolism of chick embryos through a combined analysis of immune and AOC.

TABLE 1. *Ingredients and analyzed and calculated chemical composition of the experimental diets*

Ingredient or parameter	Value
Composition	
Corn	68.99
Soybean meal	4.00
Corn protein	9.15
Gossypol-free cottonseed protein	6.00
Limestone	7.76
Soybean oil	0.50
Dicalcium phosphate	2.09
NaCl	0.35
Trace mineral premix[a]	0.30
Choline chloride (50%)	0.12
Mycotoxin adsorbent	0.10
DL-Methionine	0.0515
Vitamin premix[b]	0.035
Santoquin	0.03
Phytase	0.016
4% Flavomycin	0.015
Lysine · HCl (78%)	0.373
Threonine	0.0664
Tryptophan	0.0481
Total	100
Analysis	
Available metabolic energy (Mcal/kg)	2.83
Crude protein	16.1
Calcium	3.48
Total phosphorus	0.678
Available phosphorus	0.47
Methionine	0.34
Lysine	0.805
Methionine + cysteine	0.626
Threonine	0.60
Tryptophan	0.18

Units: % or as noted. [a]Supplied the following per kilogram complete diet: Cu, 8 mg; Zn, 75 mg; Fe, 80 mg; Mn, 100 mg; Se, 0.15 mg; I, 0.35 mg. [b]Supplied the following per kilogram complete diet: vitamin A, 12,500 IU; vitamin D_3, 2500 IU; vitamin E, 30 IU; vitamin K_3, 2.65 mg; thiamine, 2 mg; riboflavin, 6 mg; vitamin B_{12}, 0.025 mg; biotin, 0.0325 mg; folic acid, 1.25 mg; pantothenic acid, 12 mg; niacin, 50 mg.

TABLE 2. *Effects of maternal GEN on the liver index of chick embryos (n = 8)*

Treatment	Liver index
CON	2.18 ± 0.07b
LGE	2.27 ± 0.07a
HGE	2.20 ± 0.05b
P	0.024

Liver index (%), liver weight/embryo weight. Mean values without a common identifier (a, b) differ significantly ($P < 0.05$).

MATERIALS AND METHODS

Animals and experimental design

All procedures performed in the present experiment were approved by the Institutional Animal Care and Use Committee of China Agricultural University. A total of 720 Ross 308 laying broiler breeder hens, all aged 55 wk, were allocated to 3 treatment groups [control (CON), low GEN (LGE), and high GEN (HGE)], with 8 replicates of 30 hens each, and were fed with diets containing different levels of GEN. The CON group was fed a cornmiscellaneous meal diet (CSCM) without GEN supplementation (**Table 1**). As is shown in **Table 2**, the CSCM was formulated to meet the nutritional requirements of laying broiler breeders according to the Nutrient Requirements of Poultry (1994). The LGE and HGE groups were fed the CSCM, supplemented with GEN, at 40 and 400 mg/kg, respectively. The supplemental GEN was a synthetic product from Kai Meng Chemical Plant (Xi An, China) with a purity of 99.9%. The levels of GEN in the diets were determined by analysis (CON = 2.97 mg/kg, LGE = 43.21 mg/kg, and HGE = 402.61 mg/kg). All of the caged hens were raised in a ventilated henhouse in which the light regimen was 16-h light:8-h dark. Before the start of the experiment, all hens were fed with a basal diet for 2 wk to deplete GEN reserves in the body. During the 8-wk experiment, each hen was allotted 155 g feed at 6:00 AM every day and had free access to water. Male Ross 308 broiler breeders were given a commercial diet under the same feeding condition as the hens. On the last day of the experiment, hatching eggs (15 eggs per group) from each replicate were hatched. Incubation was conducted under standard conditions of 70–80% humidity and 37.8°C with intermittent rotation. On embryonic d 19, we randomly selected 2 embryos from each replicate and measured their body size (embryo length, embryo weight, and tibia length) and liver organ index. We also selected 1 embryo from each replicate and collected liver samples in liquid nitrogen, which were stored at −80°C for RNA-Seq and biochemical detection. In addition, we collected the growth plate from 1 tibia of the same embryo and then rapidly froze it in 4% paraformaldehyde for hematoxylin and eosin (HE) staining.

Body-size measurement and organ indices

On d 11, 13, 15, 17, and 19 after fertilization, 1 embryo from each replicate was used for measurements of wet weight and body length (27). The relative embryo weight was defined as wet embryo weight/egg weight, relative embryo length was defined as embryo body length/egg weight, and the liver index was defined as liver weight/embryo weight. Each tibial growth plate specimen was cut into paraffin sections and stained with HE. Then, we measured the width of the proliferative zone in the growth plate using the Leica Microsystems DMi8 S Platform (Wetzlar, Germany). The proliferative zone is defined as extending from the first flattened chondrocyte in the column to the first shape-changed chondrocytes. Proliferative cells were flattened, with their width much greater than their height (ratio of width to height >2).

Antioxidant and metabolic indices

The concentrations of metallothionein (MT) and arachidonic acid (AA) in the embryonic liver samples were analyzed using ELISA kits (Beijing JinHaiKeYu Biologic Technology Development Co. Ltd., Beijing, China). The T-AOC and levels of antioxidant enzymes [total superoxide dismutase (T-SOD), copper-zinc SOD (CuZn-SOD), and manganese SOD (Mn-SOD)] and Na^+/K^+-ATPase in the liver were detected using assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The protein content of the supernatant was measured by Coomassie Brilliant Blue G-250 reagent with bovine serum albumin as a standard.

RNA isolation, library preparation, and sequencing

Total RNA was extracted and purified from frozen liver tissues using Trizol (Transgene, Strasbourg, France), according to the manufacturer's protocols. Total RNA was then quantified using a nucleic acid/protein quantitative measuring instrument (Bio-Rad, Hercules, CA, USA), and then the samples were pooled into 1 sample per group at a standardized concentration. Next, 4 RNA samples from each of the 4 replicates per treatment were packed in dry ice and sent to Macrogen Millennium Genomics

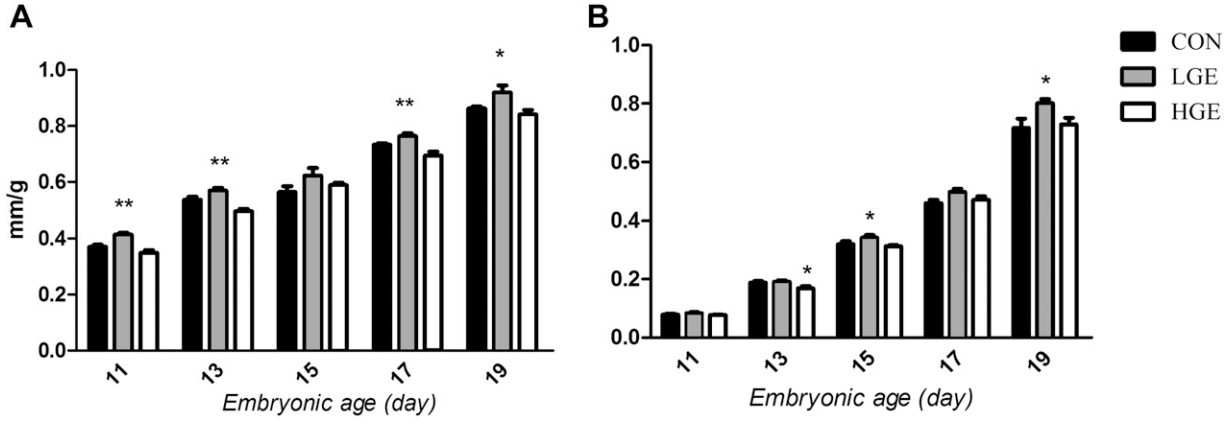

Figure 1. The effects of maternal GEN on the body sizes of embryos at d 11, 13, 15, 17, and 19 ($n = 8$, means ± SD). *A*) Relative embryo length (body length/egg weight, mm/g); *B*) relative embryo weight (wet body weight/egg weight). *$P < 0.05$, **$P < 0.01$.

TABLE 3. *Effects of maternal GEN on the levels of AA and Na^+/K^+-ATPase in the livers of chick embryos (n = 8)*

Treatment	AA	Na^+/K^+-ATPase
CON	7.37 ± 0.59b	1.12 ± 0.08b
LGE	8.67 ± 0.63a	1.25 ± 0.20b
HGE	8.81 ± 0.99a	1.54 ± 0.14a
P	0.010	0.004

Units: AA, ng/ml; Na^+/K^+-ATPase, μmol/mg. Mean values without a common identifier (a, b) differ significantly ($P < 0.05$).

(Shenzhen, China) for further library preparation and sequencing. RNA-Seq was performed on the Illumina HiSeq 2500 platform using pair-end sequencing with a read length of 126 bp.

Transcriptomic construction

All reads were mapped to the *Gallus gallus* genome (assembly Gallus_gallus5.0; National Center for Biotechnology Information, *https://www.ncbi.nlm.nih.gov/genome/111*), using TopHat v.2.0.9 (28) with the newest *G. gallus* annotation file. Quality control (QC) and statistics on the reads were confirmed with FASTQC (Babraham Bioinformatics; Babraham Institute, *http://www.bioinformatics.Babraham.ac.uk/projects/fastqc/*). Transcripts were assembled and quantified by Cufflinks 2.0.2. Isoforms assembled by Cufflinks from the 2 samples were then sent through the Cuffcompare utility, along with the gene annotation file, to generate an integrated combined Gene Transfer Format annotation file. To minimize annotation artifacts, we excluded all single-exon transcripts shorter than 200 bp that were mapped to known genes. Transcripts labeled with class code "j" by the Cuffcompare utility were considered new isoforms of known genes and added to the *G. gallus* annotation file, and the resulting new annotation file was used as the reference file and sent through Cuffcompare to generate the following events: "annotated exons," "unknown, generic overlap with reference," "potentially novel isoforms of genes," "intergenic transcripts," "intron retention events," and "exonic overlap with reference on the opposite strand."

Identification of differentially expressed genes; gene ontology terms

Cuffdiff software was used to calculate the "fragments per kilobase of exon per million fragments mapped" values of each gene in all samples using the new reference file. Differentially expressed genes (DGEs) and their corresponding *P* values were determined using tests based on a negative binomial distribution, which reflects their properties. All obtained *P* values were adjusted for the false discovery rate (FDR), with multiple testing procedures used to control for type I errors (29). The genes whose expression levels showed a $|\text{fold change}| > 1.5$ at an FDR < 0.10 between 2 samples were defined as DGEs. The Database for Annotation, Visualization and Integrated Discovery (*https://david.Ncifcrf.gov/*) and OmicsBean (*http://www.omicsbean.cn*) were used to perform gene function enrichment analyses based on gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation for the significantly DGEs (SDE) (30). The variable PAS_Value, representing pathway activation strength, was used to measure the activation profiles of the signaling pathways from the expression of individual genes.

Protein–protein interaction analysis

Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) is a widely used biologic database and web resource of known and predicted protein–protein interactions. A network model was generated with the Cytoscape web application using information gained from 4 levels of functional analysis: fold changes of genes/proteins, protein–protein interactions, KEGG pathway enrichment, and biologic process enrichment. A default confidence cutoff of 400 was used: interactions with confidence scores above that threshold are shown as solid lines between genes/proteins, and the remaining interactions are shown as dashed lines.

Confirmation of RNA-seq results with qRT-PCR

To confirm the sequencing results, we performed quantitative RT-PCR (qRT-PCR) on 20 randomly selected DGEs. A total of 6 RNA samples from each group was reverse transcribed to cDNA using a PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Kyoto, Japan), according to the manufacturer's instructions. Primers were designed *via* Primer Express 3.0.1 software (Applied Biosystems, Foster City, CA, USA) and are shown in Supplemental Data 4. qRT-PCR was carried out in triplicate with the LightCycler 480 SYBR Green I Master Kit (Roche, Basel, Switzerland) in a 15 μl reaction on a LightCycler 480 (Roche Applied Science, Penzberg, Germany) using the following program: 95°C for 10 min; 45 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s; and 72°C for 6 min. The relative gene expression values were calculated using the $2^{-\Delta\Delta Ct}$ method. The mRNA levels of the DGEs were normalized against an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistical analysis

The results are expressed as the means ± SD or the means ± SEM (for gene expression), and differences were considered significant when $P < 0.05$, as calculated by ANOVA with SPSS 11.0 for Windows.

RESULTS

Maternal GEN alters the body size of chick embryos

As shown in **Fig. 1**, we selected body parameters to measure embryonic development. Feeding laying broiler

TABLE 4. *Effects of maternal GEN on the antioxidant indices in the livers of chick embryos (n = 8)*

Treatment	T-AOC	T-SOD	CuZn-SOD	Mn-SOD	MT
CON	5.65 ± 0.30c	120.4 ± 6.7b	72.7 ± 5.5b	47.7 ± 2.7c	7.34 ± 0.59b
LGE	6.40 ± 0.42b	138.9 ± 6.5a	83.7 ± 2.2a	55.2 ± 5.7b	8.67 ± 0.63a
HGE	7.10 ± 0.68a	147.7 ± 13.5a	79.9 ± 10.5ab	67.8 ± 5.6a	8.81 ± 0.99a
P	<0.001	0.001	0.001	<0.001	0.100

Units: T-SOD, CuZn-SOD, and Mn-SOD, U/mg; MT, ng/ml. Mean values without a common identifier (a, b, c) differ significantly ($P < 0.05$).

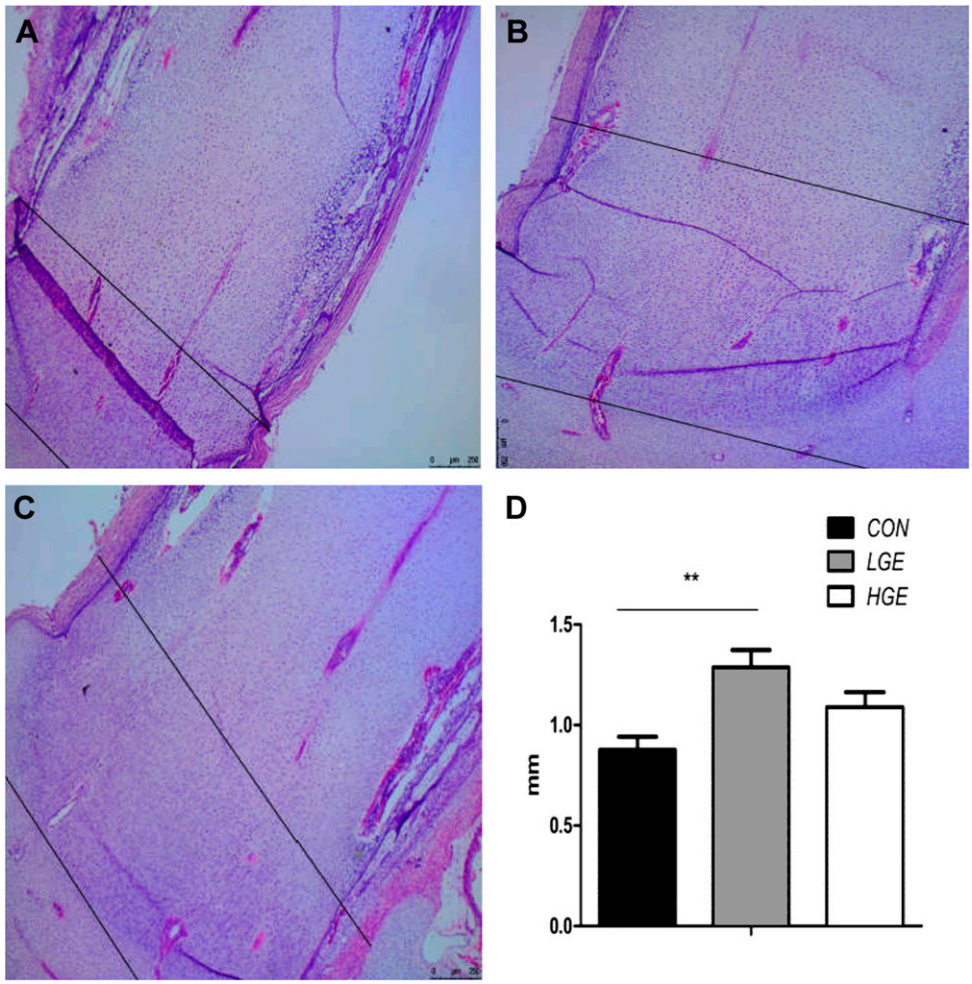

Figure 2. The width of the proliferative zone in the growth plate of the embryonic tibia. HE-stained paraffin sections were measured under original magnification at ×80. The proliferative zone is defined as extending from the first flattened chondrocyte in the column to the first shape-changed chondrocytes. The proliferative cells were flattened, with their width much greater than their height (ratio of width to height >2). *A–C*) Samples in the 3 groups (CON, LGE, and HGE), respectively. *D*) Significant differences among the 3 groups (n = 8, means ± SD). $^{**}P < 0.01$.

breeder hens with 40 mg/kg GEN significantly increased the relative length of their embryos compared with that of the embryos of the CON and HGE groups at d 11, 13, 15, 17, and 19 ($P < 0.05$). Meanwhile, the relative embryo weight was increased at d 15 ($P < 0.05$), 17 ($P = 0.088$), and 19 ($P < 0.05$). GEN treatment at 400 mg/kg decreased the relative embryo weight at d 13 ($P = 0.041$). However, no significant differences were observed between the CON and HGE groups at d 11, 15, 17, or 19. Furthermore, we found that 40 mg/kg dietary GEN in hens increased the width of the proliferative zone in the growth plate of the embryonic tibia (Fig. 1), as well as the embryonic liver index at d 19 ($P < 0.05$; Table 2). Therefore, low-dose GEN supplementation in the diet of laying broiler breeder hens could promote embryonic development (**Table 3**).

Maternal GEN increases the AOC, MT, and AA levels and the activity of Na^+/K^+-ATPase in the embryonic liver

As shown in **Table 4**, GEN supplementation in the diet of laying broiler breeder hens increased the activity of T-SOD ($P = 0.001$) and the protein level of MT in the embryonic liver ($P < 0.05$). Supplementation at 40 mg/kg significantly increased the activity of CuZn-SOD in the livers of offspring embryos ($P = 0.001$); 40 and 400 mg/kg supplementation levels both increased the activity of Mn-SOD in the embryonic liver ($P < 0.001$; **Fig. 2**). Accordingly, GEN treatment significantly increased the T-AOC of the embryonic liver ($P < 0.001$), and the 400 mg/kg supplementation level had an even greater effect on this index. Meanwhile, the addition of GEN to the diet of laying broiler breeder hens increased the level of AA and the activity of Na^+/K^+-ATPase in the embryonic liver ($P < 0.05$; **Table 5**).

QC of RNA-Seq data and identification of transcripts expressed in the embryonic liver

In this study, we established 12 cDNA libraries from the livers of embryos in the CON, LGE, and HGE groups, with

TABLE 5. *Effects of maternal GEN on the levels of AA and Na^+/K^+-ATPase in the livers of chick embryos (n = 8)*

Treatment	AA	Na^+/K^+-ATPase
CON	7.37 ± 0.59b	1.12 ± 0.08b
LGE	8.67 ± 0.63a	1.25 ± 0.20b
HGE	8.81 ± 0.99a	1.54 ± 1.54a
P	0.010	0.004

Units: AA, ng/ml; Na^+/K^+-ATPase, μmol/mg protein. Mean values without a common identifier (a, b) differ significantly ($P < 0.05$).

TABLE 6. *Characteristics of the reads from 12 embryo liver libraries*

Sample ID	Q20	GC content	Raw reads	Clean reads	Mapped reads	Mapping ratio[a]	Concordant pair alignment rate[b]
A1	95.3	52.57	43,005,094	40,581,495	33,304,804	82.1	77.3
A2	95.6	51.55	45,530,132	42,946,166	35,247,126	82.1	76.8
A3	95.1	53.46	41,915,271	39,322,365	32,094,829	81.6	73.6
A4	95.6	51.56	49,825,991	46,962,829	39,984,421	85.1	85.1
B1	95.8	49.69	43,242,077	40,664,190	34,744,021	85.4	80.3
B2	95.7	49.37	40,142,534	37,846,737	32,614,076	86.2	81.5
B3	94.9	51.84	45,819,328	43,241,718	37,090,954	85.8	81.9
B4	95.5	51.75	41,999,081	39,669,251	33,790,998	85.2	81.2
C1	96.1	51.29	43,403,893	40,715,372	34,500,957	84.7	80.5
C2	94.8	52.47	43,293,879	40,944,090	35,058,192	85.6	82.0
C3	95.3	52.95	44,674,684	42,218,036	33,851,561	80.2	75.7
C4	95.4	52.53	45,592,148	43,083,742	35,987,976	83.5	79.7

Units: reads, *n*; all others, %. GC, guanine-cytosine; ID, identification; Q20, Phred quality score. [a]Mapping ratio, mapped reads/all reads. [b]The proportion of cases in which the left and right reads align.

4 replicates in each group. QC is an essential step to verify the quality of RNA-Seq data. A QC summary—per base average quality scores, read count per base for different quality score ranges, guanine-cytosine content distribution, quality distribution, and base composition—is shown in Supplemental Data 5. We concluded that the quality of the RNA-Seq data was reliable and qualified. RNA-Seq generated 40,142,534–49,825,991 raw reads for each library, with an average of 45,069,122, 42,800,755, and 44,241,151 paired-end reads for the CON, LGE, and HGE groups, respectively. Low-quality reads were filtered out, and the average numbers of clean reads were 42,453,214 (94.19%), 40,355,474 (94.29%), and 41,740,310 (94.34%) for the CON, LGE, and HGE groups, respectively. The clean reads were used for all further analyses. After assembly, a total of 13,854 mRNAs was obtained from the 3 groups. An average of 83.96% of the reads in each library was uniquely mapped to the galGal4 assembly of the chicken genome, and the average mapping rates were 82.73%, 85.65%, and 83.5% for the CON, LGE and HGE groups, respectively (**Table 6**). The mapped reads of different regions of the genome are displayed in **Fig. 3**. The top-10 most abundantly expressed genes in both the LGE and HGE groups, ranked by absolute abundance, were APOA1, FTH1, GAPDH, GAL9, NME4, RPLP1, AHSG, VTN, EF1A, and RPS17L (**Table 7**).

Identification of DGEs and isoforms

We identified 894 DGEs (663 upregulated and 231 downregulated) in the LGE group compared with the CON group ($P \leq 0.05$); 359 of these were SDE (301 upregulated and 58 downregulated) with a $|\text{fold change}| \geq 1.5$ (FDR ≤ 0.10). The expression abundance and fold changes of DGEs are shown in Supplemental Data 1. Meanwhile, we identified 1021 DGEs (845 upregulated and 176 downregulated) in the HGE group compared with the CON group; 297 of these were SDE (259 upregulated and 38 downregulated).

Real-time PCR validation of differential gene expression

To confirm the accuracy of the RNA-Seq transcriptome data, we randomly selected 12 genes, including 4 significantly upregulated genes, 3 significantly downregulated genes, and 5 genes with no significant differential expression. The expression levels of the selected genes were quantified using qRT-PCR, and the results were consistent with the findings obtained by RNA-Seq (**Fig. 4**). For example, low-dose GEN treatment increased IGFBP3 mRNA expression in the embryonic liver compared with that in the CON group, whereas high-dose GEN treatment increased

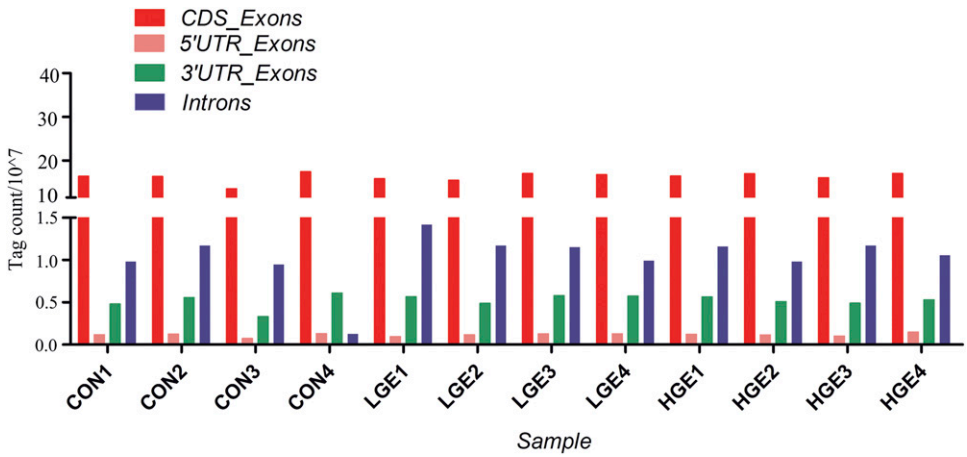

Figure 3. Numbers of exons and introns. Tag count is the number of reads mapped to the regions of exons and introns in the *G. gallus* genome. CDS, coding DNA sequence.

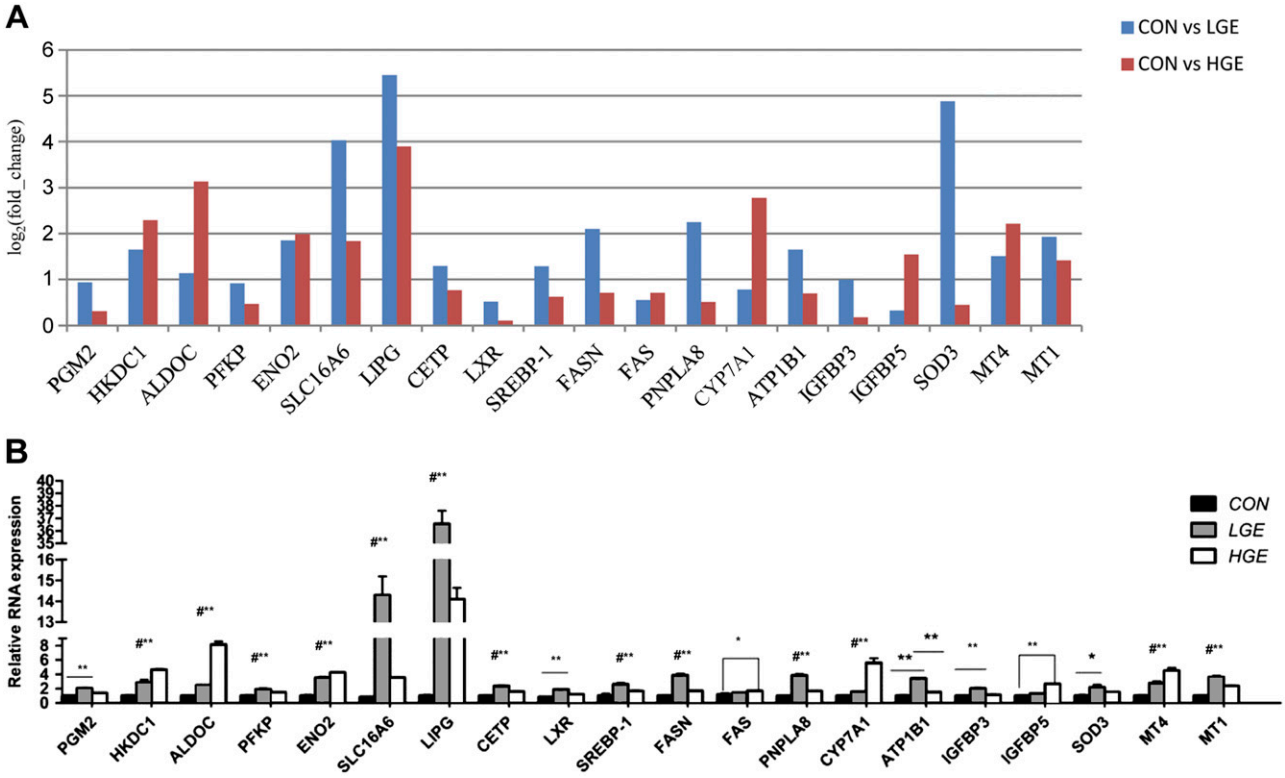

Figure 4. Validation of DGEs by qRT-PCR. *A*) Comparison [binary logarithm ($\log_2$; fold change)] of the RNA-Seq data (4 samples per group) between the experimental and CON groups. *B*) Individual variability of validated DGEs in qRT-PCR (6 samples per group) between the experimental and CON groups. Error bars represent the SE of variability among individual samples. $^*P < 0.05$, $^{**}P < 0.01$. $^{\#}$**, groups were significantly different from each other ($P < 0.01$).

IGFBP5 expression. The results suggest that RNA-Seq reliably identified differentially expressed mRNAs in the chicken liver transcriptome.

GO terms and pathway analysis using DGEs between the LGE and CON groups

To understand better the network that regulates metabolism and development in embryos from GEN-treated laying broiler breeders, we analyzed the functional distribution of DGEs in livers from the CON group compared with that in livers from the LGE and HGE groups. KEGG pathways and GO categories of Biologic Process, Cellular Component, and Molecular Function were analyzed. With the use of the genes that were differentially expressed between the CON and LGE groups, we obtained a total of 583, 28, 136, and 12 clusters based on the GO Biologic Process, Cellular Component, and Molecular Function categories and KEGG pathway analysis, respectively (**Fig. 5*A***). As shown in **Fig. 6**, an enrichment analysis of the GO term Biologic Process showed that the DGEs were significantly enriched under the terms brown fat cell differentiation, carboxylic acid transport, glucose metabolic process, monocarboxylic acid metabolic process, ovulation from ovarian follicle, dicarboxylic acid metabolic process, intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress, and positive regulation of

TABLE 7. *The gene IDs of the top-10 genes expressed in both low and high GEN-treated groups*

Gene ID	Full name	FPKM (average)
APOA1	Apolipoprotein A-I	15,957.20
FTH1	Ferritin, heavy polypeptide 1	8691.29
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	5946.14
GAL9	Avian β-defensin 9	8397.64
NME4	Nucleoside diphosphate kinase 4	4188.18
RPLP1	Ribosomal protein, large, P1	4468.14
AHSG	α-2-HS-glycoprotein	4140.23
VTN	Vitronectin	4072.26
RPS17L	Ribosomal protein S17	4075.29
EF1A	Eukaryotic translation elongation factor 1 α 1	3900.52

FPKM, fragments per kilobase of exon per million fragments mapped.

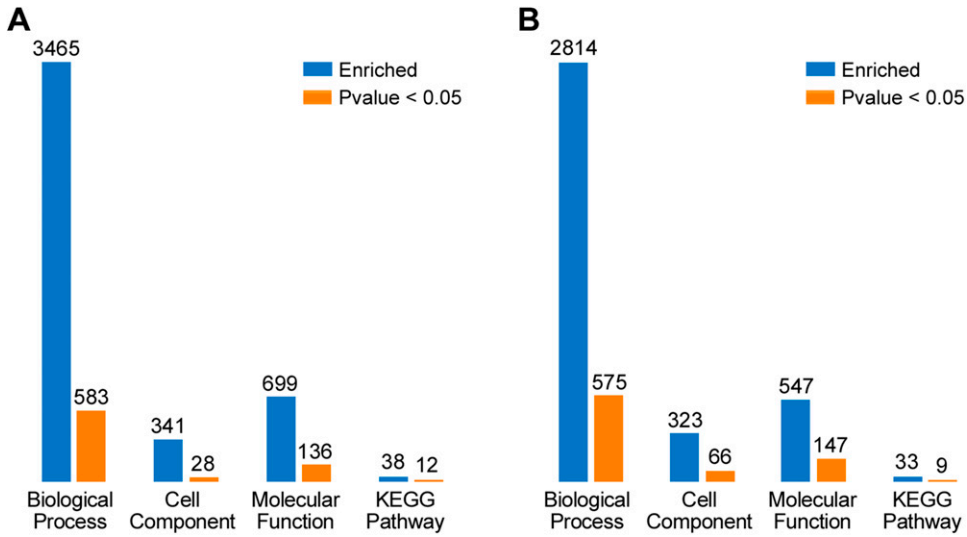

Figure 5. Biological process, cellular component, molecular function and KEGG pathway are the 4 enrichment categories that we used for the functional analysis of DGEs from the CON *vs.* LGE groups (*A*) and the CON *vs.* HGE groups (*B*), respectively. Counts for each category represent the total number of terms in the database associated with the query gene/protein list. Values of $P < 0.05$ are statistically significant.

myoblast differentiation. The variables pathway activation strength (PAS) value and *P* value_adjusted of the top 10-enriched Biologic Process terms are shown in Supplemental Data 2, indicating that the processes were enhanced in the breeders by 40 mg/kg GEN treatment. The top enriched terms under Molecular Function included organic anion transmembrane transporter activity, protein serine/threonine kinase activity, transcription regulatory region DNA binding, PKA catalytic subunit, MAPK binding, IL-12 receptor activity, inositol tetrakisphosphate 3-kinase activity, fatty acid elongase activity, major histocompatibility complex (MHC) class I protein complex, IL-12 receptor complex, autophagosome, IL-23 receptor complex, endocytic vesicle, protein kinase complex, and very low density lipoprotein (VLDL) particle. The top enriched terms under Cellular Component included MHC class I protein complex, IL-12 receptor complex, autophagosome, IL-23 receptor complex, and VLDL particle. Therefore, the GO term enrichment analysis showed that the DGEs between the CON and LGE groups were significantly enriched in metabolism, development, and immunity. The enriched KEGG pathways included biosynthesis of amino acids [enolase 2 (ENO2)|fold change = 3.60; GLUL|3.42], *Salmonella* infection/TLR signaling pathway (FOS|3.17; JUN|3.87), GnRH signaling pathway (CACNA1D|2.76; JUN|3.87), and synthesis and degradation of ketone bodies (HMGS1|12.07). Additionally, oxidoreductase activity acting on paired donors [CYP1A2|2.06; CYP1A4|1.99; CYP2H1|2.96; ENSGALG00000004050|2.01; ENSGALG00000005469|5.75 (Pvalue_adjusted <0.05)], fatty acid synthase (FASN) activity [elongation of very long-chain fatty acid (ELOVL)4|4.12; ELOVL5|1.92; FASN|4.29 (Pvalue_adjusted <0.10)], lipid transporter activity [ATP-binding cassette transporter G1 (ABCG1)|3.55; ATP10B|4.45; cholesteryl ester transfer protein (CETP)|2.45; COL4A3BP|2.02; solute carrier (SLC) 51B|2.63; HNRPK|0.29; lipase G|43.68; OSBPL6|2.76; patatin-like phospholipase A8 (PNPLA8)|4.75; SIRT1|1.93; SLCO2A1|2.18; StAR-related lipid transfer protein 5 (STARD5)|2.27 (Pvalue_adjusted <0.05)], carboxylic acid transmembrane transporter activity [SLC16A10|2.52; SLC16A3|11.59; SLC16A6|16.32; SLC25A48|2.33; SLC26A4|6.62; SLC38A2|3.49; SLC6A14|7.25; SLC6A17|8.63; SLCO2A1|2.18 (Pvalue_adjusted <0.10)], and cholesterol transporter activity [ABCG1|3.55; CETP|2.45; STARD5|2.27 (Pvalue_adjusted <0.10)] was enhanced.

GO terms and pathway analysis using DGEs between LGE and HGE groups

With the use of the DGEs between the CON and HGE groups, we obtained a total of 575, 66, 147, and 9 clusters based on the GO Biologic Process, Cellular Component, and Molecular Function annotations and KEGG pathway analysis, respectively (Fig. 5*B*). As shown in Fig. 6*B*, among the Biologic Process terms enriched for DGEs between the CON and HGE groups, we found that 400 mg/kg GEN treatment inhibited the monocarboxylic acid metabolic process and enhanced the transmembrane receptor protein tyrosine kinase signaling pathway, the apoptotic signaling pathway, and activation of lymphocyte proliferation. Furthermore, 400 mg/kg GEN treatment increased the activity of hexokinases (HKDCs; fructokinase activity, mannokinase activity, and glucokinase activity), IL-12 receptor activity, and lipoprotein lipase (LPL) activity in the category, Molecular Function. The categories MHC class I protein complex, IL-12 receptor complex, IL-23 receptor complex, and autophagosome, under Cellular Component, were enhanced, whereas the VLDL particle was inhibited (Supplemental Data 3). The DGEs between the 400 mg/kg GEN-treated group and the CON group were clustered into the annotations JAK-STAT signaling pathway, adipocytokine signaling pathway, and insulin resistance (**Table 8**).

To compare further the effects of high- and low-dose GEN treatments on the livers of broiler chick embryos, we further searched for DGEs between the LGE *vs.* CON groups and the HGE *vs.* CON groups. Eighty-three common DGEs were found for the LGE *vs.* CON groups and the HGE *vs.* CON groups, and 214 genes were differentially expressed only in the HGE *vs.* CON groups,

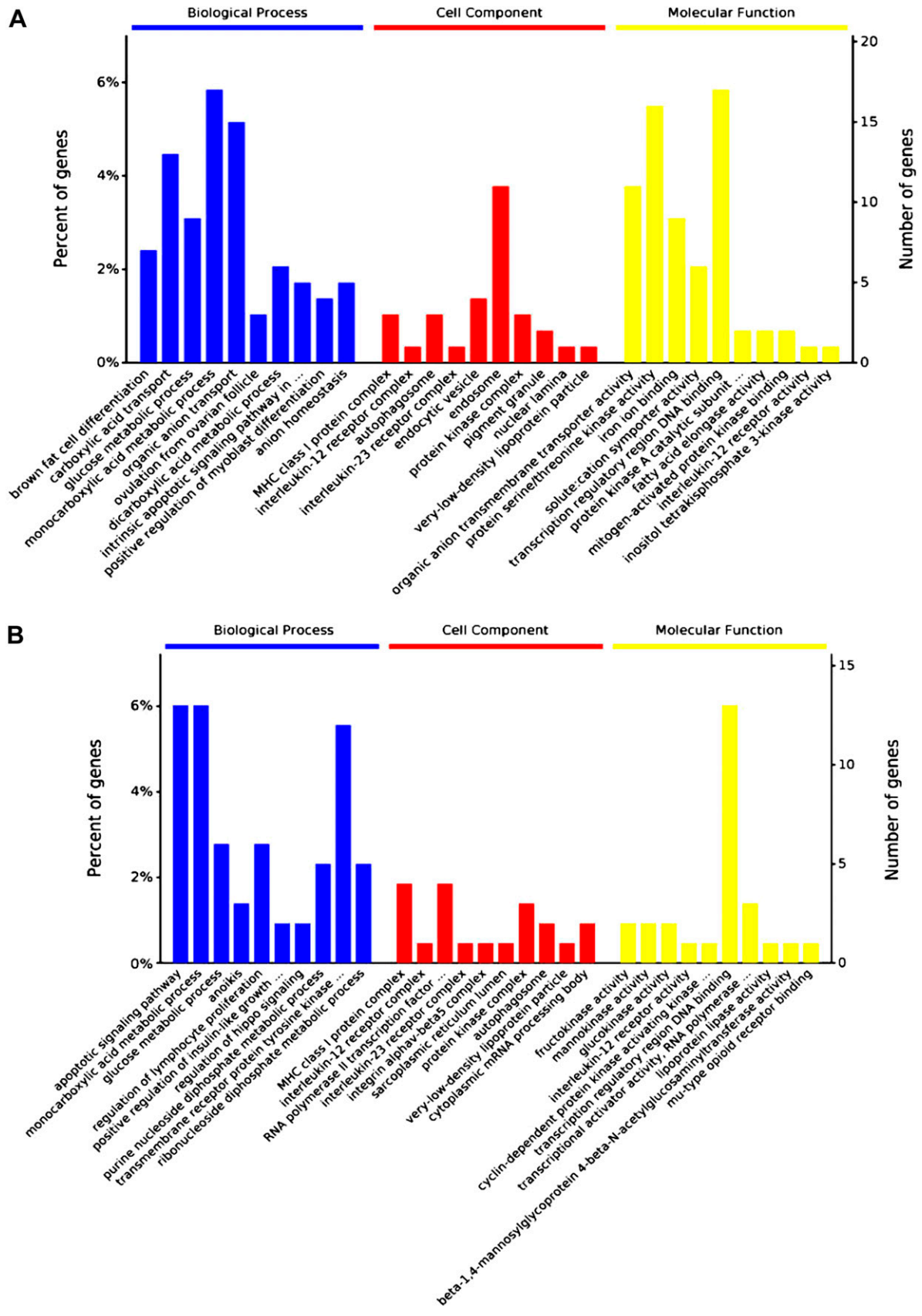

Figure 6. The top 10-enriched terms of DGEs in each main category (Biologic Process, Cell Component, and Molecular Function) of the GO database. *A*) Enriched terms based on DGEs between the LGE and CON groups. *B*) Enriched terms based on DGEs between the HGE and CON groups.

TABLE 8. *Cluster analysis (CON vs. LGE)*

KEGG_PATHWAY	Gene count	*P*
Metabolic pathways	31	0.031
Protein digestion and absorption	5	0.064
Biosynthesis of amino acids	9	0.000
Glycine, serine, and threonine metabolism	4	0.032
Tryptophan metabolism	5	0.006
Fructose and mannose metabolism	5	0.002
Glycolysis/gluconeogenesis	5	0.026
Glycerolipid metabolism	4	0.083
Glyoxylate and dicarboxylate metabolism	4	0.015
Butanoate metabolism	3	0.074
Synthesis and degradation of ketone bodies	3	0.011
MAPK signaling pathway	13	0.002
TNF signaling pathway	6	0.038
AMPK signaling pathway	6	0.068
TLR signaling pathway	5	0.094

indicating that the dose of maternal GEN treatment influenced the embryonic liver transcriptome (**Fig. 7**). The GO and KEGG analysis showed that the 214 DGEs were related to the categories cell adhesion, endodermal cell differentiation, apoptotic process and B cell lymphoma 2 family protein complex, VEGF receptor signaling pathway, insulin receptor signaling pathway, PI3K-Akt signaling pathway, adipocytokine signaling pathway, hypoxia-inducible factor 1 signaling pathway, and type II diabetes mellitus (**Table 9**).

Protein–protein interaction analysis

Cytoscape bioinformatics analysis of potential protein interactions for all DGEs was performed (**Fig. 8**). The major biologic functions revealed by the software OmicsBean were as follows. The DGEs between the GEN-treated groups and the CON group were significantly related to the categories GnRH, TLR signaling pathway, biosynthesis of amino acids, herpes simplex infection, and *Salmonella* infection. Maternal GEN treatment significantly upregulated JUN, FOSL2, MAPK14, HKDC, ENO2, and other genes in the embryonic liver. Among them, JUN and MAPK14 are associated with the largest numbers of genes. DGEs between the HGE group and the CON group were also clustered in the categories adipocytokine signaling pathway and glycolysis/gluconeogenesis signaling pathway.

DISCUSSION

The chick embryo is a common model system for research on the embryonic development of vertebrates. Egg quality can reportedly regulate the individual morphology and production performance of offspring chickens (31). In the current study, the GO cluster analysis of DGEs in the embryonic liver transcriptome showed that 40 mg/kg dietary GEN treatment in hens significantly promoted protein absorption and amino acid synthesis in chick

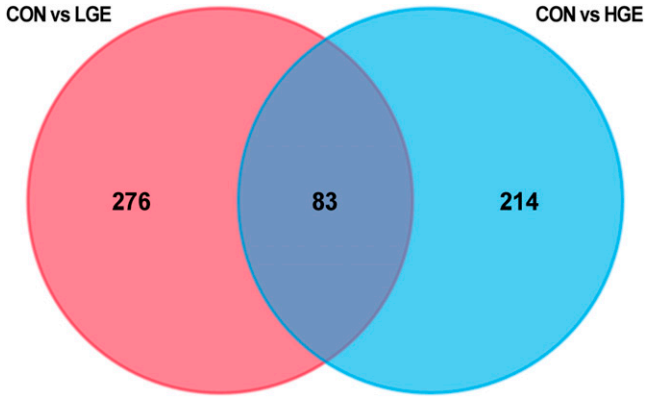

Figure 7. A Venn diagram of DGEs between the CON *vs.* LGE groups and the CON *vs.* HGE groups.

embryos, in addition to activation of myoblast differentiation. Likewise, high-dose GEN treatment promoted the development of embryonic tissue and organs. IGFBP3 can help IGF-I combine with its receptor, which promotes cell proliferation and differentiation, as well as metabolism. In the present experiment, low-dose GEN treatment significantly upregulated the transcription of IGFBP3 in the embryonic liver. IGFBP3 can also promote cell division and fetal growth directly (32). Therefore, low-dose GEN supplementation in the diets of hens increased the embryonic weight and length, as well as the liver index. Meanwhile, the width of the proliferative zone in the growth plate of the embryonic tibia was increased after low-dose GEN treatment, which is consistent with a report that GEN can increase alkaline phosphatase activity and osteocalcin secretion, promoting bone formation with increased type I collagen in osteoblasts (33). Short-term administration of GEN to gilts has been reported to increase the relative expression of IGFBP-5 and GnRHR in the anterior pituitary (34). KEGG pathway analysis indicated that high-dose GEN treatment significantly activated the insulin-like signaling pathway and the VEGF receptor signaling pathway, with upregulated IGFBP5 transcription in the embryonic liver. The different effects of low- and high-dose GEN on the expression of IGFBP-3 and IGFBP-5 may be a result of its estrogenic potency. This is the first report that maternal GEN could promote embryonic development through IGFBPs. Additionally, 400 mg/kg GEN treatment in hens significantly influenced the apoptosis and tyrosine kinase signaling pathways, which may explain why high-dose GEN treatment decreased the relative embryo weight and liver index. However, the mechanism requires further study.

Embryonic development is heavily dependent on metabolic status. The liver plays an important role in the growth and development of the chick embryo, especially in glucose and lipid metabolism, bile acid production, and hematopoietic organ formation. Dietary intake of GEN (250 mg/kg diet) can reportedly improve hyperglycemia, glucose tolerance, and blood insulin levels in obese diabetic mice (35). In this experiment, GO cluster analysis showed that the addition of GEN to the diet of hens significantly affected the metabolism of their embryos. Low-dose GEN treatment promoted the glycolysis process with

TABLE 9. *Cluster analysis (LGE vs. HGE)*

Analysis	Gene count	*P*
GO term_BP		
Cell adhesion	18	<0.001
Endodermal cell differentiation	4	0.001
Apoptotic process	12	0.002
Cell migration	7	0.004
Hepatocyte apoptotic process	3	0.005
Apoptotic mitochondrial changes	3	0.012
Negative regulation of intrinsic apoptotic signaling pathway	3	0.015
VEGF receptor signaling pathway	4	0.024
Bcl-2 family protein complex	2	0.025
RNA polymerase II transcription factor complex	3	0.032
KEGG_PATHWAY		
Pathways in cancer	13	<0.001
PI3K-Akt signaling pathway	12	<0.001
Focal adhesion	8	0.004
Adipocytokine signaling pathway	5	0.005
ECM-receptor interaction	5	0.011
Insulin signaling pathway	6	0.012
Insulin resistance	5	0.022
Bile secretion	4	0.031
Arrhythmogenic right ventricular cardiomyopathy	4	0.034
Proteoglycans in cancer	6	0.049
HIF-1 signaling pathway	4	0.074
Type II diabetes mellitus	3	0.083
Transcriptional misregulation in cancer	5	0.086

KEGG_PATHWAY analysis was performed using OmicsBean software. BP, biological process; Bcl-2, B cell lymphoma 2; ECM, extracellular matrix; HIF-1, hypoxia-inducible factor 1.

upregulated mRNA expression of phosphoglucomutase-2; this enzyme catalyzes the conversion between glucose-1-phosphate and glucose-6-phosphate. In glycolysis, fructose-1,6-bisphosphate can be catabolized into glyceraldehyde-3-phosphate and dihydroxyacetone phosphate by fructose-biphosphate C aldolase (ALDOC). HKDC1 catalyzes the conversion of glucose into the active state. ENO2, another key enzyme in glycolysis, catalyzes the dehydration of 2-phosphoglycerate to form phosphoenolpyruvate. Low-dose GEN treatment significantly upregulated the expression of HKDC1, ALDOC, phosphofructokinase, platelet (PFKP), and ENO2, clearly demonstrating enhancement of the glycolysis process in the embryonic liver. Likewise, a study suggested that GEN could significantly increase the activity of glycolytic enzymes—HKDC, PFK, phosphoenolpyruvate carboxykinase, and lactate dehydrogenase (36). KEGG pathway analysis showed that low-dose GEN treatment activated the insulin signaling pathway in the chick embryo liver and alleviated insulin resistance, which is consistent with a report that GEN can improve insulin sensitivity in fructose-treated mice (37). Additionally, we found that the clustered PI3K-Akt signaling pathway and insulin signaling pathway contained more DGEs between the HGE and CON groups, indicating that feeding high-dose GEN to hens may have a more obvious promoting effect on glucose metabolism in the chick embryo. The above content was the first report to clarify that maternal GEN could enhance glycolysis in the embryonic liver.

The development and lipid metabolism of chick embryos during the late hatching period are vigorous. To provide a sufficient energy source for development from hatching to feed intake, embryos must have sufficient fat deposition. Chick embryos must absorb fatty acids (mainly C18:ln-9 and C16:0) from the yolk and then convert them into C18:0 and C20:4n-6AA in the embryonic liver for use (38). AA can increase the expression of C-fos and early growth response protein 1, which induce cell growth and promote organ development (39). We found that the addition of GEN to the diet of laying broiler breeder hens significantly increased the content of AA in the chick embryonic liver. DGEs associated with GEN treatment were clustered in the process of unsaturated fatty acid metabolism; for example, an increase in the transcriptional level of PNPLA2 was observed in the embryonic liver. PLA2 is the rate-limiting enzyme in AA anabolism. Therefore, GEN treatment in hens may increase AA anabolism in the embryonic liver by upregulating PNPLA expression. Furthermore, the gene PNPLA8 is also involved in signal transduction, cell growth, gene expression, innate immunity, and the inflammatory response (40). GEN is different from estrogen in promoting the expression of fat synthesis-related genes (including PPAR-γ, adipose triglyceride lipase, LPL, and hormone-sensitive lipase) (41). A mixture of GEN and di(2-ethylhexyl)phthalate was reported to increase significantly the mRNA expression of cholesterol (3-hydroxy-3-methyl-glutaryl-coenzyme A) and phospholipid [sterol regulatory element-binding protein 1c (Srebp1c), FASN] mediators by activating upstream liver X receptor (LXR)-α (42). In this study, quantitative PCR analysis showed that the expression of LXR and SREBP-1 in the embryonic liver was increased significantly by low-dose GEN treatment. Additionally, the feeding of GEN to laying broiler breeder hens significantly upregulated the transcriptional levels of fatty acid elongation factors (ELOVL4 and ELOVL5) and FASN, which promote fatty acid synthesis, which conflicts with reports that GEN inhibits fatty acid synthesis in the livers of mice fed with sucrose. This difference may be a result of the metabolic and physiologic status of the chick embryo. Furthermore, low-dose GEN treatment upregulated the expression of CYP7A1 in the chick embryonic liver, which may promote the conversion of cholesterol into bile acids. The clustering results showed that high-dose GEN treatment in hens promoted bile acid secretion in chick embryos compared with low-dose GEN treatment. Therefore, maternal GEN could increase the expression of LXR and SREBP-1, as well as downstream genes, and increase AA anabolism and bile acid secretion in the embryo.

The energy source for early embryonic development is glucose from albumen, whereas later embryonic stages rely on lipids from the egg yolk. In fact, 80% of yolk lipids are depleted during the last 7 d of incubation (43). GO clustering analysis showed that the addition of GEN to the diet of broiler breeder hens affected carboxylic acid metabolism in the embryonic livers. Low-dose GEN treatment enhanced carboxylic acid transport and increased

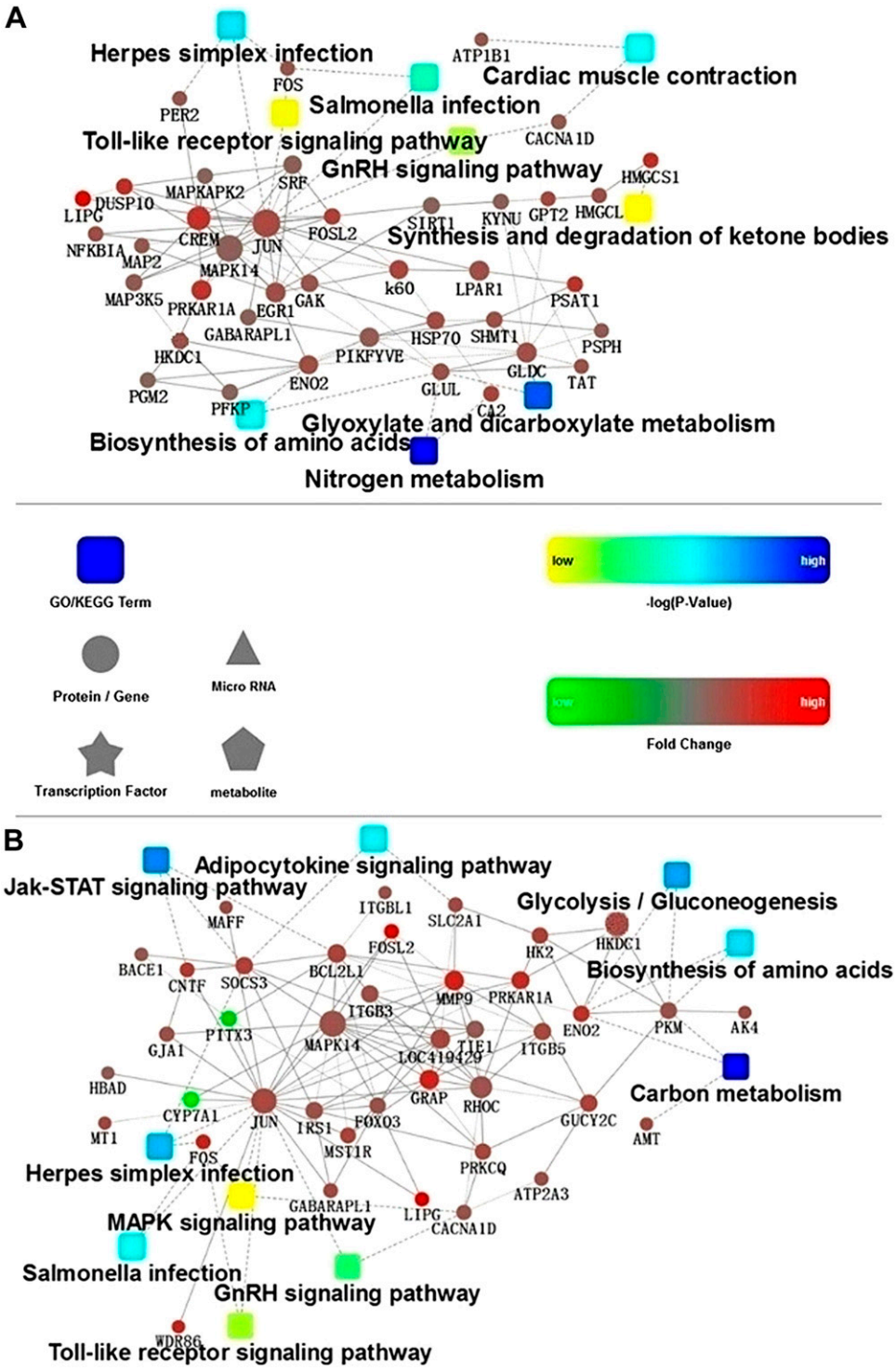

Figure 8. Protein–protein interaction analysis. Circular nodes represent genes/proteins; rectangles represent KEGG pathways or GO Biologic Process terms. The pathways are colored with a gradient from yellow to blue, in which yellow indicates a smaller *P* value, and blue indicates a larger *P* value. GO biologic processes are colored red. In the fold-change analysis, genes/proteins are colored red for upregulation or green for downregulation. *A*, *B*) Protein–protein interaction analyses using DGEs of the CON *vs.* LGE groups and the CON *vs.* HGE groups, respectively.

the mRNA expression of SLC family genes (SLC51B, SLC13A3, SLC16A10, SLC16A3, SLC16A6, and SLC1). SLC1 can promote phosphatidic acid synthesis using the fatty acids 18:1 and 14:0 as substrates and especially enhances the acylation of short-chain saturated fatty acids (44). In the present experiment, GO cluster analysis showed that GEN treatment significantly improved lipid transporter activity in the chick liver. Low-dose GEN treatment significantly increased the expression of lipase G and genes related to cholesterol transporter activity (ABCG1, CETP, and STARD5). ABCG1 can promote the transmembrane transport of sterols and phospholipids (45), and CETP mainly mediates the selective uptake of HDL-cholesterol by the liver (46). In poultry, VLDL transports triglycerides in the liver to the surrounding tissues through the blood circulation. Then, triglycerides are hydrolyzed into fatty acids and glycerol by LPL. High-dose GEN treatment in laying broiler breeders increased LPL activity, which was consistent with the increase in LPL expression and the reduction of serum lipid levels in mice that were fed GEN (47). Therefore, maternal GEN could promote fatty acid transport in chick embryos.

Furthermore, GEN treatment significantly increased VLDL particles in the chick embryo. A strong correlation exists between VLDL and fat deposition, which can be used as an indicator of carcass fatness in turkeys (48). Therefore, we further confirmed that maternal GEN intake enhanced the transport and deposition of fatty acids in the embryonic liver, which may improve the growth performance of offspring.

The energy source for early embryonic development is glucose from albumen, whereas later embryonic stages rely on lipids from the egg yolk. In fact, 80% of yolk lipids are depleted during the last 7 d of incubation (43). β-Oxidation in the chick embryo is vigorous, along with abundant mitochondria and high carnitine acyltransferase activity (49). PPARα and PPARδ are involved in energy metabolism, inflammation, cell proliferation and differentiation, apoptosis, and immune regulation (50, 51). High- and low-dose GEN treatment upregulated the expression of PPARα and PPARδ, respectively, in the embryonic liver. Therefore, maternal GEN could also enhance β-oxidation in the chick embryo. Phytoestrogens from soybean can reportedly activate the transduction of AMPK cascade signaling and enhance energy consumption (52). GO clustering analysis showed that the feeding of GEN to broiler breeders promoted brown adipocyte differentiation in chicken embryos, which can convert ATP into heat energy. Further research showed that low-dose GEN treatment increased the transcription levels of ATP1B1, ATP10B, and ATP5A1 in the livers of chick embryos, whereas high-dose GEN treatment increased the transcription levels of ATP2A3 and ATP1B1, which is consistent with the finding that GEN treatment significantly increased the activity of Na^+/K^+-ATPase in the livers of chick embryos. Therefore, maternal GEN could enhance energy metabolism in the chick embryo through the AMPK and PPARs signaling pathways, which may promote development.

GEN can reportedly upregulate the expression of glutathione peroxidase and SOD mRNAs, reduced by ethanol treatment in mouse fetuses (53). In the experiment, GO cluster analysis of DGEs in the embryonic liver transcriptome showed that GEN supplementation in the diets of laying broiler breeder hens significantly increased the oxidoreductase activity of embryos. GEN treatment in hens also significantly increased the activity and mRNA expression of Mn-SOD in the embryonic liver. SOD can reduce reactive oxygen species with catalase and glutathione peroxidase, thus inhibiting lipid peroxidation (54). Furthermore, 40 mg/kg GEN treatment increased the activity of hepatic CuZn-SOD in the embryo. In addition, we found that the expression of MT in embryonic livers was increased, with upregulated transcription of MT1 and MT4 after GEN treatment. MT has ~10,000 times the reactive oxygen species scavenging capacity of SOD and mainly serves to maintain the redox balance in the cell (55, 56). Therefore, maternal GEN increased the T-AOC of chick embryos. In addition to the AOC, dietary GEN has been shown to improve the humoral and mucosal immunity of growing broilers by increasing the abundance of intestinal intraepithelial lymphocytes (57). In the present experiment, cluster analysis showed that the addition of GEN to the diet of laying broiler breeder hens significantly activated the MHC class I protein complex, the IL-12 receptor complex, and the IL-23 receptor complex. MHC I protein complexes can recognize antigens for $CD8^+$ T cells and activate B cells to produce antibodies. The transcription levels of MHC-like class I Y-coding gene (LOC768350) and MHC-like class I F10 α-chain coding gene (LOC430600) in the embryonic livers were significantly upregulated after GEN treatment. IL-12 can promote T cell proliferation and induce the secretion of cytokines, such as IFN-γ, TNF-α, and GM-CSF in cytotoxic T cells and NK cells. Accordingly, dietary GEN treatment upregulated the transcription levels of genes involved in lymphocyte proliferation (CDKN1A, IL12RB1, Sox11, PRKAR1A, PRKCQ, and TCF3). Therefore, the feeding of laying broiler breeder hens a diet with GEN can enhance the AOC and immunity of their embryos, which are main factors influencing embryonic development.

In summary, our results provide a novel inside-out molecular mechanism of maternal GEN in regulating the development and metabolism of chick embryos. The feeding of GEN to laying broiler breeder hens could promote the development and cell proliferation of chick embryos through IGFBPs (IGFBP3 and IGFBP5). However, high-dose GEN treatment activated the apoptosis and protein tyrosine kinase signaling pathways, decreasing the growth parameters of embryos compared with the low-dose GEN treatment. Our work first demonstrated that maternal GEN could promote the glycolysis process through the insulin signaling pathway and enhance the transport of carboxylic acids and cholesterol, as well as the synthesis of fatty acids in the embryonic liver through upregulated LXR and SREBP-1. Additionally, GEN treatment increased β-oxidation in the embryonic liver through activated PPARs (PPARα and PPARδ) and the AMPK signaling pathway, which could provide energy for embryonic development. We also found that maternal GEN could improve the immune function and AOC of the embryonic chick. These findings provide a basis for the use of bioactive compounds to treat developmental malfunctions and metabolic syndrome. FJ

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AUTHOR CONTRIBUTIONS

Z. Lv and Y. Guo performed most of the experiments, designed the study, and wrote the manuscript; Z. Lv, H. Fan, and K. Xing performed the animal experiments; Z. Lv and C. Ning performed most of the bioinformatics studies; Z. Lv, B. Zhang, and H. Fan performed quantitative PCR and analyzed the data.

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