Identification of differentially expressed genes in broiler offspring under maternal folate deficiency

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1School of Life Sciences, Linyi University, Linyi, China; 2School of Agriculture and Forestry Sciences, Linyi University, Linyi, China; and 3School of Pharmacy, Linyi University, Linyi, China

Submitted 30 July 2018; accepted in final form 17 September 2018

Xing J, Jing W, Zhang Y, Liu L, Xu J, Chen X. Identification of differentially expressed genes in broiler offspring under maternal folate deficiency. Physiol Genomics 50: 1015–1025, 2018. First published September 21, 2018; doi:10.1152/physiolgenomics.00086.2018.—Folate plays an important role in DNA and RNA synthesis by donating methyl groups. To investigate the effects of maternal folate deficiency (FD) on the abdominal adipose transcriptome and on the accumulation of lipid droplets in the liver tissue of chicken offspring, differentially expressed genes (DEGs) of FD were identified with digital gene expression tag profiling. Ultramicroscopy suggested that the size of lipid droplets in hepatocytes increased with FD, while the lipid droplets population number was largely not affected. The serum parameters assay showed that the concentrations of MTHFR (476.57 vs. 395.27), DHFR (45.056 vs. 38.952), LPL (50.408 vs. 48.677), HCY (4.354 vs. 3.836), LEP (9.951 vs. 8.673), and IGF2 (1209.4 vs. 1027.7) in offspring serum of the FD group were significantly higher than those of the normal folate (NF) group (P < 0.01). The 442 DEGs between NF and FD groups were identified by digital gene expression profiling. Considering the DEGs in the FD groups vs. NF groups, 179 genes were upregulated while 263 downregulated, and in particular, 145 upregulated and 214 downregulated DEGs were successfully annotated with the nonredundant database. Gene Ontology analysis showed that FD mainly affected cellular processes, cell part and binding, cell killing, virions, and receptor regulator activity. With pathway analysis, it indicated that 123 unigenes were assigned to 115 KEGG pathways, but only five of 115 these pathways were significantly enriched with P values < 0.05. Taken together, these results provide a foundation for further studying the responses of offspring to maternal FD in breeding chickens.

INTRODUCTION

Folate, forms of which are known as folic acid (FA) and vitamin B9, is a water-soluble B vitamin that plays key roles in many biological functions, including cell differentiation, proliferation, and repair (8, 27, 32). It is a methyl donor in synthesis of S-adenosyl-L-methionine (SAM) (2, 3), and it is needed in the animal body to synthesize, repair, and methylate DNA, as well as to act as a cofactor in biological reactions involving folate (42). Consequently, folate deficiency affects gene expression through disrupting DNA methylation pattern or by inducing base substitution, DNA break, chromosomal gap, gene deletion, and gene amplification (7). These changes may lead to complex postnatal metabolic diseases, tumors, neural tube defects, and neurodegenerative or psychiatric disorders (during pregnancy) (4, 27).

Research on folate application in poultry diets has been increasing. It has been shown that folate is able to increase biochemical constituents, enhance generation of total IgG in serum of young laying hens, as well as exhibiting pleiotropic effects in inflammatory responses (30, 31). However, folate does not affect inflammatory responses in older laying hens (30, 31). Additionally, dietary FA supplementation improves production performance and decreases glucose level in the young laying hens but does not affect any performance measurements in older ones (16). These indicate that younger and older laying hens respond differently to diets with supplemental FA, suggesting that age may play a role in the development of FA response (16). In broilers, in ovo FA injection can improve the growth performance and folate metabolism as well as enhance the relationship between immune function and the epigenetic regulation of immune genes (21).

In animals, the relationship between folate status and offspring growth has been extensively investigated. It was reported that folate deficiency in pregnant ewes led to heavier and fatter adult offspring due to alteration of methylation status (35). Another study showed that maternal and peri-/postnatal folate and/or vitamin B12 restriction increased visceral adiposity and altered lipid metabolism in rat offspring, perhaps by modulating adipocyte function (18). Conversely, Lam et al. (20) demonstrated that FA supplementation had significant modulatory effects on β-adrenoceptor protein expression and lipolysis of obese/diabetic mice. Similarly, abdominal fat mass and central adiposity were reduced in the progeny of the FA-supplemented dams of 16 wk old rats (6). In vitro, folate increased the proliferation of chicken adipocytes and the expression of DNMT1 and MTHFR and methylation of C/EBPα promoter but reduced precell lipid accumulation and C/EBPα, PPARγ, and FAS expression (44). The same effects were also reported by Gouffon (12). A recent study observed that high folate levels increased DNMT1 expression in peripheral blood mononuclear cells of broilers (34). Similarly, folate could enhance differentiation and mRNA expression of myogenin and influence genome-wide DNA methylation levels in C2C12 cell lines (22). These studies suggest that FA status may be associated with animal lipid metabolism. However, the effects of folate on the metabolic pathway of animal fat are still unclear.
In the present study, a broiler breeder maternal folate deficiency model was constructed to investigate the influence of maternal folate deficiency on growth, development, and gene expression of offspring. The global gene expression profiles of the normal folate group and the folate deficiency group were identified with digital gene expression tag profiling and bioinformatics analyses. The newly identified differentially expressed genes (DEGs) can provide important information for the chicken genome and transcriptome annotation. Furthermore, they will provide new insight into the relationship between maternal folate nutrition and lipid metabolism in the offspring, which is of great theoretical and economic significance.

MATERIALS AND METHODS

Ethics statement. Experiments were conducted with the Arbor Acres strain broiler breeder in cages and were approved by Linyi University Institutional Animal Care and Use Committee. All animal work was performed according to the regulations and guidelines established by the Ministry of Science and Technology of the People’s Republic of China (approval number: 2006-398). Efforts were made to minimize suffering of birds.

Experimental design. At 30 wk of age, 120 Arbor Acres female broiler breeders were randomly allotted into two dietary groups: the normal folate group (NF, control group) with 2.0 mg/kg supplemental folate in basal diet and the folate deficiency group (FD, treatment group) without supplemental folate in basal diet. Each group had five replicates for a total of 12 birds. The broiler breeders were housed in raised wire cages in an open-sided house with two chickens per cage. A standard basal diet containing corn and soybean meal was fed as a mash, and the composition of the basal diet (Table 1) was formulated to meet or exceed the nutrition requirements for female broiler breeders established by the National Research Council (NRC, 1994). Meanwhile, all male broiler breeders were fed the same standard diet (NRC, 1994). The female broiler breeders were fed twice a day at 8:00 and 18:00 and fed 140 g per bird. The male broiler breeders were fed once a day at 8:00 and fed 100 g per bird. Water was provided ad libitum, and the light program was 16 h light-8 h dark with conventional ventilation and huddles clean and sanitary. In the final 2 wk, semen samples were collected for artificial insemination at 16:00, once every 2 days. After 12 wk, a total of 120 hatching eggs were collected from each group at three time points and then hatched for offspring. Eighty chicks from each group (groups were the same as the above NF and FD) were selected for feeding. Each group consisted of five pen replicates of 16 chicks. Two groups were provided with the same commercial diet (satisfied or exceeded nutrient requirements of broilers according to NRC, 1994), supplied by Liuhe Group, Shandong province, China) for 1–21 days and 22–42 days, respectively. The chicks were kept in floor pens and were given free access to feed and water with conventional ventilation and natural lighting at all times.

Sample harvest. When offspring reached 42 days old, 14 (7 male and 7 female) chickens per group were randomly selected for sacrifice by jugular vein bleeding. Blood samples were collected for serum isolation. All sera were harvested by centrifugation, and samples were stored at −20°C for subsequent ELISA analysis. Meanwhile, abdominal adipose samples were immediately isolated and briefly washed with PBS before being snap-frozen in liquid nitrogen and stored at −80°C for total RNA extraction. Tissues were also collected from different sites of each lobe of the liver for subsequent transmission electron microscopy.

Transmission electron microscopy. Collected liver tissue samples were immediately immersed in 2% glutaraldehyde solution (in 0.05 M phosphate buffer, pH 6.8) for 4 h at room temperature for primary fixation. Secondary fixation was completed in 1% osmium tetroxide solution (in the same phosphate buffer) for 4 h in dark at room temperature. Dehydration was carried out in a 10% graded ethanol series. Following dehydration, acetone was used to replace ethanol inside tissue. After the replacement of ethanol, tissue was embedded in Embed-812 resin, and 70 nm thick sections were obtained with a Leica ultramicrotome. Sections were then stained with 5% uranyl acetate, followed by lead citrate solution, and then photographed under a Jeol 1220 transmission electron microscope.

ELISA assay. Commercially available chicken ELISA Kit (Shanghai Yaping Biotechnology, Shanghai, China) was used for assaying of lipoprotein lipase (LPL), hormone-sensitive lipase (HSL), 5,10-methylenetetrahydrofolate reductase (MTHFR), dihydrofolate reductase (DHFR), insulin-like growth factor 2 (IGF2), leptin (LEP), and homocysteine (HCY) concentrations in chicken serum following manufacturer’s protocol.

RNA extraction, library construction, and sequencing. Total RNA was isolated from six female samples (marked as NFI, NF2, NF3, FD1, FD2, and FD3, respectively, with three duplicates for each group) with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions and then treated with DNase I (Takara, Dalian, China) to remove DNA contamination. The total RNA was measured quantitatively and qualitatively by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and 2100 Bioanalyzer (Agilent Technologies) according to the manufacturer’s instructions.

Table 1. Content and nutrition level of diet for broiler breeders

<table>
<thead>
<tr>
<th>Ingredients, %</th>
<th>NF Group</th>
<th>FD Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>64.5</td>
<td>64.5</td>
</tr>
<tr>
<td>Soybean meal</td>
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<td>Soybean oil</td>
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<td>0.17</td>
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<tr>
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<td>0.02</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
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<td>0.15</td>
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<td>Mineral premix*</td>
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<tr>
<td>Vitamin, including</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>11,000</td>
</tr>
<tr>
<td>Vitamin D₃, IU/kg</td>
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<td>3,500</td>
</tr>
<tr>
<td>Vitamin E, IU/kg</td>
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<td>100</td>
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<td>Vitamin B₉, mg/kg</td>
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<td>12.00</td>
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<tr>
<td>Vitamin K, mg/kg</td>
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<td>4.40</td>
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<tr>
<td>Niacin, mg/kg</td>
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<td>50.00</td>
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<tr>
<td>Pantothenic acid, mg/kg</td>
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<tr>
<td>Vitamin B₆, mg/kg</td>
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<td>4.40</td>
</tr>
<tr>
<td>Biotin, μg/kg</td>
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</tr>
<tr>
<td>Folic acid, mg/kg</td>
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<tr>
<td>Choline, mg/kg</td>
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<td>1,210.0</td>
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<tr>
<td>Metabolizable energy, MJ/kg</td>
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<td>12</td>
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<tr>
<td>Crude protein</td>
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<tr>
<td>Ca</td>
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<td>3.10</td>
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<tr>
<td>P, total</td>
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<tr>
<td>Met</td>
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<td>0.36</td>
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<td>Lys</td>
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<td>0.72</td>
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<tr>
<td>Ile</td>
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<td>0.56</td>
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<tr>
<td>Arg</td>
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<tr>
<td>Met+Cys</td>
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<tr>
<td>Val</td>
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<tr>
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<td>Vitamin B₉, mg/kg</td>
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<td>Vitamin K, mg/kg</td>
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<tr>
<td>Lys</td>
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<td>Ile</td>
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<tr>
<td>Arg</td>
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<td>Met+Cys</td>
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<tr>
<td>Val</td>
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<td>0.62</td>
</tr>
<tr>
<td>Thr</td>
<td>0.52</td>
<td>0.52</td>
</tr>
<tr>
<td>Trp</td>
<td>0.17</td>
<td>0.17</td>
</tr>
</tbody>
</table>

NF, normal folate; FD, folate deficiency. *The amount of minerals provided per kilogram of diet: Mn 150 mg; Zn 100 mg; Fe 70 mg; Cu 18 mg; I 1.1 mg; Se 0.25 mg.
Six rounds of cDNA library sequencing were conducted with the Digital Gene Expression Tag Profile kit for Illumina (NEBNext Ultra RNA Library Prep Kit; NEB, Beijing, China), according to the manufacturer’s instructions. Briefly, the mRNA was purified from 5 μg RNA using oligo (dT) magnetic beads. The mRNA was fragmented with a fragmentation buffer. With the short fragments as templates, a random hexamer primer was used to synthesize first-strand cDNA. Second-strand cDNA was synthesized with DNA polymerase I, RNase H, dNTPs, and buffer. Short fragments were purified with a QiAquick PCR Extraction Kit (Qiagen, Beijing, China) and eluted in EB buffer for end repair with the addition of poly (A). These short fragments were ligated with sequencing adapters. After suitable fragments were selected and amplified by PCR, the cDNA library was successfully constructed. Finally, the libraries were sequenced on the Illumina HiSeqTM 2500 platform of Beijing Biomarker Technologies (Beijing, China).

Sequencing data analysis. The collected data were MIAME compliant. Raw reads were evaluated and filtered to remove low-quality tags, adapters, and RNA. All cleaned sequences were aligned to the reference sequences (Gallus gallus genome/ESTs, http://genome.ucsc.edu/index.html) using TopHat (38) with mismatches no more than one base.

Gene abundances were estimated, and DEGs were tested with Cufflinks/RSEM (40) according to the results of the alignment. The reads per kilobase per million (RPKM) method was used for a relative assessment of unigene abundance, the length of each gene, and mapped reads (39). The DEGs between the NF group and FD group were identified by the DESeq package for comparative gene expression analysis. cDNA synthesis was conducted according to the manufacturer’s instructions, and the primer sequences for these reactions are shown in Table 2. The qPCR was carried out with Brilliant SYBR Green qPCR Master Mix (Agilent, Santa Clara, CA, USA) following the manufacturer’s instructions. Briefly, the mRNA was purified from 5 μg RNA using oligo (dT) magnetic beads. The mRNA was fragmented with a fragmentation buffer. With the short fragments as templates, a random hexamer primer was used to synthesize first-strand cDNA. Second-strand cDNA was synthesized with DNA polymerase I, RNase H, dNTPs, and buffer. Short fragments were purified with a QiAquick PCR Extraction Kit (Qiagen, Beijing, China) and eluted in EB buffer for end repair with the addition of poly (A). These short fragments were ligated with sequencing adapters. After suitable fragments were selected and amplified by PCR, the cDNA library was successfully constructed. Finally, the libraries were sequenced on the Illumina HiSeqTM 2500 platform of Beijing Biomarker Technologies (Beijing, China).

Sequencing data analysis. The collected data were MIAME compliant. Raw reads were evaluated and filtered to remove low-quality tags, adapters, and RNA. All cleaned sequences were aligned to the reference sequences (Gallus gallus genome/ESTs, http://genome.ucsc.edu/index.html) using TopHat (38) with mismatches no more than one base.

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Hierarchical clustering analysis of DEGs between the NF group and FD group was performed with Cluster 3.0 software. Gene ontology (GO) enrichment analyses were conducted with the Goseq R package for comparative gene ontology categories, including molecular function (MF), biological process (BP), and cellular component (CC). The Clusters of Orthologous Groups of proteins (COGs) database was used to predict and classify possible protein functions. The DEGs were also imported into the online software Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.genome.jp/kegg/pathway.html) for biological pathway mapping.

Quantitative real-time PCR. Quantitative (q)PCR was employed to validate the DEGs from Illumina sequencing. We selected 14 genes based on the magnitude of changes in gene expression and their relevance to folate metabolism, methylation, or lipid transportation and metabolism. In addition, the expression levels of LPL, MTHFR, IGF2, and LEP genes involved in lipid and FA metabolism were also analyzed by qPCR to confirm the ELISA results. The RNA samples prepared for DEG library construction were also used for qPCR validation. cDNA synthesis was conducted according to the manufacturer’s instructions, and the primer sequences for these reactions are shown in Table 2. The qPCR was carried out with Brilliant SYBR

Table 2. Primers used for qPCR

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<tr>
<th>Gene</th>
<th>Sequence (5′→3′)</th>
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<th>Annealing Temperature, °C</th>
<th>GenBank Accession Number</th>
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</table>

qPCR, quantitative PCR.
Green qPCR Master Mix (Stratagene, La Jolla, CA). All PCR reactions were performed in triplicate with negative controls. Using the 18S-actin gene for normalization, we analyzed the relative expression levels of the DEGs by the \(2^{-\Delta\Delta CT}\) method (26).

Statistical analysis. The two-sample \(t\)-tests and Wilcoxon rank tests were employed to compare concentrations of LPL, HSL, MTHFR, DHFR, IGF2, LEP, and HCY in serum and the qPCR data between NF and FD groups. A significance level of 0.05 was chosen for testing the difference between NF and FD groups.

RESULTS

Ultramicroscopy of the accumulation of lipid droplets. Lipid droplets in hepatocytes from liver tissues were examined by microscopy. A large number of lipid droplets accumulated in the hepatocytes of both the FD and NF groups. There was no significant difference between the two groups regarding to the their lipid droplets population, but droplet sizes were significantly different. Lipid droplets were up to 5 \(\mu\)m in dimension in FD group (Fig. 1A). In contrast, the lipid droplets in the hepatocytes of the NF group were dramatically smaller, with the biggest droplet measuring only 2.5 \(\mu\)m (Fig. 1A’). Other organelles in both groups, including endoplasmic reticula (ER), are known to be responsible for protein synthesis and sorting and the production of lipid droplets. Mitochondria, which are thought to be responsible for energy conversion, were also compared, and no significant differences were found regarding to their number, structure, or behavior (Fig. 1, B and B’). ER were often observed to surround mitochondria in both FD (Fig. 1C) and NF groups (Fig. 1C’). Folate-deficient food remarkably increased the size of the lipid droplets, while its number was not significantly affected. Moreover, FD did not produce significant changes to ER or mitochondria.

ELISA analysis. The concentrations of MTHFR, DHFR, HCY, LPL, LEP, and IGF2 in the sera collected from chicken in NF and FD groups were detected to analyze the effect of the FD on enzyme activities. As shown in Table 3, the concentrations of MTHFR (476.57 vs. 395.27), DHFR (45.056 vs. 38.952), LPL (50.408 vs. 48.677), HCY (4.354 vs. 3.836), LEP (9.951 vs. 8.673), and IGF2 (1,209.4 vs. 1027.7) in offspring serum from the FD group were significantly higher than those of the NF group (\(P < 0.01\)), while the concentrations of HSL in both groups had no significant difference (\(P > 0.05\)).
Table 3. Some serum parameters of enzyme, adipose metabolism, and lipogenesis

<table>
<thead>
<tr>
<th>Item</th>
<th>NF (n = 14)</th>
<th>FD (n = 14)</th>
<th>P</th>
</tr>
</thead>
<tbody>
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<td>MTHFR, U/l</td>
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<td>476.57±2.916</td>
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<td>DHFR, U/l</td>
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<td>45.056±0.3455</td>
<td>0.0001</td>
</tr>
<tr>
<td>HSL, U/l</td>
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<td>104.33±0.928</td>
<td>0.6078</td>
</tr>
<tr>
<td>LPL, U/l</td>
<td>48.677±1.296</td>
<td>50.408±1.489</td>
<td>0.0021</td>
</tr>
<tr>
<td>HCY, μmol/l</td>
<td>3.836±0.049</td>
<td>4.354±0.038</td>
<td>0.0001</td>
</tr>
<tr>
<td>LEP, ng/l</td>
<td>8.673±0.085</td>
<td>9.951±0.161</td>
<td>0.0001</td>
</tr>
<tr>
<td>IGF2, ng/l</td>
<td>1027.7±6.080</td>
<td>1209.4±14.564</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

*The different uppercase superscripted letters in the same row indicate differences (P < 0.05). No superscripts in the same row means no significant difference (P > 0.05).

Apparently, FD enhanced the levels of enzymatic activities of MTHFR, DHFR, HCY, LPL, LEP, and IGF2, while the level of HSL was not affected.

Analysis of DEG libraries. To identify genes that were differentially expressed by FD in broiler breeder offspring, six DEG libraries (three libraries from each group) were constructed and sequenced with the Illumina sequencing platform. The major characteristics of these six libraries are summarized in Table 4. A total of 58,268,510 raw reads were generated by the Illumina HiSeqTM 2500 platform (Table 4). After filtering adaptors, empty tags, and low-quality reads, we obtained a total of 46,558,157 clean reads and 4,655,514,214 bases (Table 4). The base Q30 was more than 95.27%, and around 75% of clean reads were matched to the chicken reference genome in six samples with TopHat software. In these clean reads, more than 97% were uniquely matched, and only around 2.00% of clean reads was mapped to multiple sites (Table 4), indicating that the sequence data were of good quality. The relative number of reads mapped to exons was ~64%, while around 25 and 11% of reads were mapped to intergenic and intron regions, respectively. This may have occurred because of the incompleteness of sequence annotation libraries and background noise. Analysis of the distribution of reads across the reference genes indicated that the reads were evenly distributed at every position, showing that the randomness of the sequence data from every sample was reliable. Sequencing data saturation was analyzed to estimate whether the number of detected genes increased in proportion to the amount of sequencing data. The results indicate that the number of identified genes approached ~2.0 million.

Analysis of DEGs between NF and FD groups. Gene expression abundance was calculated and normalized by the RPKM method. RPKM density distribution showed that there were few different genes in these two groups. In all six libraries, 17,934 known reference genes were expressed in each abdominal adipose sample of the NF and FD groups. After quantile normalization, 442 DEGs were identified with a fold change of ≥2 and FDR ≤ 0.01 between the NF and FD groups (Fig. 2). Of these DEGs, 179 genes were upregulated and 263 genes were downregulated in the NF vs. FD groups (Fig. 2). Among these DEGs, 359 of these 442 DEGs were successfully annotated in the nonredundant database, including 145 upregulated and 214 downregulated genes due to folate deficiency. The top 10 up- and downregulated DEGs that were regulated by FD in offspring between the NF and FD groups are listed in Table 5. According to the annotation, the most upregulated DEG by FD encodes substance-P receptor. The most downregulated DEG by FD encodes fibroblast growth factor 10 precursor.

Cluster analysis of DEGs. Hierarchical cluster analysis was performed with Cluster 3.0, and genes with the same or similar functions were clustered. The results indicate that the expres-

Table 4. Statistics of high-quality sequences of six samples

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>NF1</th>
<th>NF2</th>
<th>NF3</th>
<th>FD1</th>
<th>FD2</th>
<th>FD3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw reads</td>
<td>9,685,250</td>
<td>9,797,955</td>
<td>9,378,754</td>
<td>9,292,286</td>
<td>10,410,115</td>
<td>9,704,150</td>
</tr>
<tr>
<td>Clean reads</td>
<td>7,688,027</td>
<td>7,759,385</td>
<td>7,423,904</td>
<td>7,428,389</td>
<td>8,336,707</td>
<td>7,921,745</td>
</tr>
<tr>
<td>Total bases</td>
<td>768,757,016</td>
<td>775,887,878</td>
<td>742,339,772</td>
<td>742,794,716</td>
<td>833,616,463</td>
<td>792,118,369</td>
</tr>
<tr>
<td>GC content, %</td>
<td>51.09</td>
<td>51.68</td>
<td>51.52</td>
<td>50.59</td>
<td>51.66</td>
<td>51.81</td>
</tr>
<tr>
<td>Q30, %</td>
<td>95.61</td>
<td>95.37</td>
<td>95.39</td>
<td>95.74</td>
<td>95.27</td>
<td>95.29</td>
</tr>
<tr>
<td>Mapped reads</td>
<td>5,775,745 (75.13%)</td>
<td>5,802,872 (74.79%)</td>
<td>5,569,853 (75.03%)</td>
<td>5,619,663 (75.65%)</td>
<td>6,269,866 (75.21%)</td>
<td>5,916,630 (74.69%)</td>
</tr>
<tr>
<td>Unique mapped reads</td>
<td>5,658,070 (97.96%)</td>
<td>5,677,343 (97.84%)</td>
<td>5,455,278 (97.94%)</td>
<td>5,500,929 (97.89%)</td>
<td>6,132,412 (97.81%)</td>
<td>5,796,993 (97.98%)</td>
</tr>
<tr>
<td>Multiple mapped reads</td>
<td>117,675 (2.04%)</td>
<td>125,529 (2.16%)</td>
<td>114,575 (2.06%)</td>
<td>118,734 (2.11%)</td>
<td>137,454 (2.19%)</td>
<td>119,637 (2.02%)</td>
</tr>
</tbody>
</table>

NF1–NF3 and FD1–FD3 represent three libraries for each group.

Fig. 2. Scatter plots of differentially expressed genes (DEGs) in the abdominal adipose of NF and FD groups. RPKM, reads per kilobase per million.
Table 5. Top 10 DEGs according to log2FC value between NF and FD groups

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>log2FC</th>
<th>P</th>
<th>Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENSGALG000000013853</td>
<td>2.57611</td>
<td>0.02519</td>
<td>substance-P receptor</td>
</tr>
<tr>
<td>ENSGALG00000004510</td>
<td>2.26501</td>
<td>0.04544</td>
<td>uncharacterized protein LOC101747664</td>
</tr>
<tr>
<td>ENSGALG00000013327</td>
<td>2.20746</td>
<td>0.01852</td>
<td>C-C motif chemokine 17</td>
</tr>
<tr>
<td>ENSGALG00000024466</td>
<td>2.14190</td>
<td>0.02010</td>
<td>B box and SPRY domain-containing protein isoform X2</td>
</tr>
<tr>
<td>ENSGALG00000008861</td>
<td>2.04604</td>
<td>0.02957</td>
<td>soluble carrier family 15 member 1</td>
</tr>
<tr>
<td>ENSGALG00000000921</td>
<td>1.91888</td>
<td>0.01553</td>
<td>transmembrane protein 132B isoform X4</td>
</tr>
<tr>
<td>ENSGALG00000010881</td>
<td>1.53576</td>
<td>0.03216</td>
<td>ficolin-2 isoform X12</td>
</tr>
<tr>
<td>ENSGALG00000023460</td>
<td>1.38743</td>
<td>0.03999</td>
<td>ankyrin repeat and SOCS box protein 2 isoform X3</td>
</tr>
<tr>
<td>ENSGALG00000013953</td>
<td>1.25946</td>
<td>0.03982</td>
<td>zinc finger protein GLIS1, partial (Anas platyrhynchos)</td>
</tr>
<tr>
<td>ENSGALG00000009069</td>
<td>1.21192</td>
<td>0.01781</td>
<td>ETS-related transcription factor Elf-3</td>
</tr>
</tbody>
</table>

Downregulated

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>log2FC</th>
<th>P</th>
<th>Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENSGALG00000014872</td>
<td>−2.96957</td>
<td>0.01324</td>
<td>fibroblast growth factor 10 precursor</td>
</tr>
<tr>
<td>ENSGALG00000023460</td>
<td>−2.48350</td>
<td>0.04022</td>
<td>hypothetical protein Anapl_12714</td>
</tr>
<tr>
<td>ENSGALG00000024286</td>
<td>−2.48205</td>
<td>0.03962</td>
<td>lymphoid-restricted membrane protein-like isoform X7</td>
</tr>
<tr>
<td>ENSGALG00000009069</td>
<td>−2.47312</td>
<td>0.03590</td>
<td>low quality protein: calpain-14</td>
</tr>
<tr>
<td>ENSGALG00000010721</td>
<td>−2.37360</td>
<td>0.04135</td>
<td>zinc finger protein GLIS1, partial (Anas platyrhynchos)</td>
</tr>
<tr>
<td>ENSGALG00000014844</td>
<td>−2.27710</td>
<td>0.04829</td>
<td>sodium/potassium-transporting ATPase subunit beta-1-interacting protein 2-like</td>
</tr>
<tr>
<td>ENSGALG00000023879</td>
<td>−2.17898</td>
<td>0.01871</td>
<td>uncharacterized protein C2orf72 homolog</td>
</tr>
<tr>
<td>ENSGALG00000012396</td>
<td>−1.94371</td>
<td>0.02768</td>
<td>prostaglandin E2 receptor EP2 subtype</td>
</tr>
<tr>
<td>ENSGALG00000014990</td>
<td>−1.91259</td>
<td>0.01874</td>
<td>inward rectifier potassium channel 13</td>
</tr>
<tr>
<td>ENSGALG00000007732</td>
<td>−1.82878</td>
<td>0.02977</td>
<td>reticulon-4 receptor</td>
</tr>
</tbody>
</table>

*DEG*, differentially expressed gene; *FC*, fold change.

FOLATE DEFICIENCY INFLUENCE ON GENE EXPRESSION

GO enrichment analysis and COG annotation. To understand the role of these DEGs between NF and FD groups, we performed GO term enrichment analysis with Blast2GO software on the GO database. The results show that 442 DEGs were categorized into 55 subcategories, including 23 (41.8%) BP, 17 (30.9%) CC, and 15 (27.3%) MF (Fig. 4). Most genes in the BP class were related to cellular processes, and cell part and binding were represented in the CC and MF groups. Conversely, only a few genes contributed to “cell killing,” “virus,” and “receptor regulator activity” in BP, CC, and MF groups, respectively.

The COGs database comprises a framework for functional and evolutionary genome analysis (37), and the database is constructed based on system evolution relationships of bacteria, algae, and eukaryotes. Orthologous gene products are classified with the COGs database. Based on sequence homology, 205 DEG unigenes were found in the COGs database between NF and FD groups and were classified into 23 categories. The largest group was the “general function prediction only” (55, 26.8%) group, and “defense mechanisms” and “cell motility” were the two smallest groups (1 each group, 0.5% each group) among 23 categories.

KEGG pathway analysis. To further characterize the functions of these genes, we performed a pathway analysis of the DEGs based on the KEGG database with two-sided Fisher's exact tests. Consequently, 123 unigenes were assigned to 115 KEGG pathways. The pathways with the highest representation of unique sequences were ubiquitin-mediated proteolysis (ko04120), neuroactive ligand-receptor interaction (ko04080), MAPK signaling pathway (ko04010), endocytosis (ko04144), and spliceosome (ko03040). However, only five of the 115 pathways were significantly enriched with *P* values ≤ 0.05. These enriched pathways include steroid biosynthesis (ko00100), synthesis and degradation of ketone bodies (ko00072), histidine metabolism (ko00340), lysine degradation (ko00310), and ubiquitin-mediated proteolysis (ko04120) (Table 6). Moreover, two unigenes involved in one carbon pool for the folate (ko00670) and three unigenes related to FA metabolism (ko00071) pathways were identified with KEGG analysis. KEGG analysis also provided lipid metabolism pathway of broilers, including glycerophospholipid metabolism (ko00564), sphingolipid metabolism (ko00600), glycerolipid metabolism (ko00561), biosynthesis-ganglio series (ko00604) and glycosphingolipid biosynthesis-globo series (ko00603), fatty acid metabolism (ko00071), and fatty acid elongation in mitochondria (ko00062). However, these pathways were not significantly enriched between NF and FD groups (*P* > 0.05).

Validation of differentially expressed genes by qPCR. To validate the results of our gene expression analysis, we selected 14 DEGs identified by digital gene expression tag profiling analysis for validation by qPCR. The selected genes included six upregulated genes (*SIN3B*, *GPD1L*, *DHCR7*, *MSMO1*, *METTL5*, and *MTHFD2L*) and eight downregulated genes (*LOC101749600*, *CNRI*, *ATP10D*, *IDH1*, *ZNF521*, *GPR56*, *HIF*, and *PRPF19*), which are related to FA metabolism, methylation, or lipid transportation and metabolism. Collectively, the fold change in the mRNA expression levels of the 14 genes between the NF and FD groups, as determined by qPCR, were in concordance with the DEGs results (Fig. 5). According to the qPCR results, five (*SIN3B*, *GPD1L*, *DHCR7*, *METTL5*, and *MTHFD2L*) of the six DEGs upregulated were significantly upregulated, and seven (*LOC101749600*, *ATP10D*, *IDH1*, *ZNF521*, *GPR56*, *HIF*, and *PRPF19*) of the eight DEGs downregulated genes were significant (*P* < 0.05). Only two genes (*MSMO1* and *CNRI*) did not show significant differences between NF and FD groups (*P* > 0.05). Meanwhile, the magnitude of expression of *MSMO1* and *CNRI* genes showed discrepancies between qPCR and DEGs data sets. However, the direction of expression was consistent between the two methods, suggesting that sequencing is a reliable method for
genome-wide analysis of expression profiles in the NF and FD groups.

**Effect of FD on expression levels of LPL, MTHFR, IGF2, and LEP.** To further investigate the effects of maternal FD on FA and lipid metabolism in offspring, expression levels of the LPL, MTHFR, IGF2, and LEP genes were examined in abdominal adipose tissues by qPCR. The results show that the expression levels of LPL and IGF2 in the FD group were significantly higher than those of the NF group \((P < 0.05)\), and the expression levels of MTHFR and LEP were also enhanced by FD, but no significant difference was found in FD group compared with NF group \((P > 0.05)\) (Fig. 6). However, the expression tendency of MTHFR and LEP was consistent with concentrations of MTHFR and LEP in serum.

**DISCUSSION**

Currently, FA, as an important functional trace element, is widely used in poultry feeding. Our study found that genes responding to folate deficiency were involved in many biological functions, including metabolic processes, catalytic activities, cell proliferation, etc. This suggests that maternal folate deficiency indeed affects adipose gene transcription and expression of offspring. In addition, size differences in liver tissue lipid droplets and some key markers of enzymes in serum, genes of adipose metabolism, and lipogenesis were also observed between FD and NF groups, respectively. At present, the effect of folate reducing lipid accumulation in vitro has been reported by Gouffon (12) and Yu et al. (44). However,
little is known of the role of folate in adipose metabolism in broilers. Hence, this work provides the first evidence linking maternal FD with adipose deposition in the offspring, which contributes to a better understanding of the molecular mechanism of folate metabolism.

The liver is the primary organ for lipid metabolism in chickens. Hepatic lipid homeostasis in the chicken is regulated by many factors, including heredity, feed energy level, nutrient elements, hormones, and others. Our transmission electron microscopy results indicate that the morphology and structure of hepatocytes such as lipid droplet size (Fig. 1) were changed by folate deficiency, implying that this maternal folate deficiency can alter hepatic lipid deposition of offspring.

There is an FA transport system in the intestine of chickens (36). FA is absorbed in the small intestine and then enters the liver through the portal vein. Folate metabolism involves at least 30 different enzymes in the liver (24). In our study, concentrations of MTHFR, DHFR, HCY, IGF2, LPL, and LEP in chicken serum increased due to folate deficiency, which may be associated with folate metabolism and adipose deposition. Previous studies indicate that folate deficiency could lead to an accumulation of HCY in serum, resulting in amyasthenia, skeletal muscle dysfunction, cardiovascular disease, and neural tube defects (17, 29). A recent study also reported that hepatic IGF2 expression was upregulated by modulation of DNA hypomethylation following the injection of 150 μg FA on embryonic day 11 of incubation of broilers (25). However, our data revealed that folate deficiency causes a rise of IGF2 in abdominal adipose tissues, as well as serum. We speculate that this difference is likely due to tissue specificity, developmental stage, and differences in assay parameters (IGF2 expression in hepatic cell and abdominal adipose tissue and IGF2 enzyme activity in serum). More recently, it was found that mRNA and protein expression levels of adipose LPL were significantly decreased in the offspring of FA-supplemented female rats compared with the offspring of controls (43). Interestingly, in the present study, FD caused expression levels of LPL in adipose tissue to increase, which is consistent with the results of LPL enzyme activities in serum. Furthermore, serum LEP at day 21 of offspring was strongly reduced in the methyl donor (FA, betaine, etc.) supplemented groups in rats as compared with the controls (11). This is in agreement with serum LPL and LEP levels that were increased by folate deficiency in our study.

Until now, the molecular response of offspring to maternal FD has not been well studied in chickens, and the underlying

Table 6. Significantly enriched pathways for DEGs between NF and FD groups

<table>
<thead>
<tr>
<th>Pathway ID</th>
<th>Pathway</th>
<th>DEGs Associated with the Pathway (%)</th>
<th>All Genes with Pathway Annotation (%)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ko00100</td>
<td>steroid biosynthesis</td>
<td>3 (2.44%)</td>
<td>17 (0.42%)</td>
<td>0.01343</td>
</tr>
<tr>
<td>ko00072</td>
<td>synthesis and degradation of ketone bodies</td>
<td>2 (1.63%)</td>
<td>8 (0.20%)</td>
<td>0.02250</td>
</tr>
<tr>
<td>ko00340</td>
<td>histidine metabolism</td>
<td>3 (2.44%)</td>
<td>21 (0.52%)</td>
<td>0.02406</td>
</tr>
<tr>
<td>ko00310</td>
<td>lysine degradation</td>
<td>4 (3.25%)</td>
<td>39 (0.96%)</td>
<td>0.02887</td>
</tr>
<tr>
<td>ko04120</td>
<td>Ubiquitin-mediated proteolysis</td>
<td>8 (6.50%)</td>
<td>124 (3.05%)</td>
<td>0.03272</td>
</tr>
</tbody>
</table>

Fig. 4. Gene Ontology (GO) analysis of DEGs between NF and FD groups.
mechanisms of the adipose-depositing effects of FD are so far poorly understood. In this study, a total of 46,558,157 clean reads were generated from digital gene expression tag profiling, and 34,221,025 unigenes were successfully mapped to the reference database (Table 4). Among these unigenes, only 442 genes (179 upregulated genes and 263 downregulated genes) exhibited differential expression between NF and FD groups, and most of these DEGs were annotated in the public databases. Fortunately, some DEGs that were involved in the FA metabolic process, adipose tissue development, lipid transportation and metabolism, and methyltransferase activity in abdominal adipose were identified, indicating that maternal folate deficiency may cause adipose deposition in the offspring.

On the basis of the DEGs results, we identified 10 unigenes with known functions in folate metabolism and methyltransfer. Among these genes, expression levels of five (MTHFD2L, METTL23, METTL5, SHMT1, and SETD7) were enhanced in the FD group. Importantly, MTHFD2L encodes a nuclear-encoded mitochondrial bifunctional enzyme that catalyzes the interconversion of 5,10-methylenTHF and 10-formylTHF (9) and is involved in FA metabolic, one-carbon metabolic, and tetrahydrofolate metabolic processes. A recent study reported that MTHFD2L protein mediates the oxidation of phospholipids to regulate amino acid metabolism in endothelial cells (15). METTL5 and METTL23 belong to methyltransferase superfamily and are related to methyltransferases and catalysis of the transfer of a methyl group to an acceptor molecule (28). Moreover, SETD7, an H3-K4-specific methyltransferase, is a group of enzymes that utilize the cofactor SAM to achieve methylation of its substrates and is able to mediate the monomethylation of histone H3 at lysine 4 (H3K4me1) in vitro (14, 41). Conversely, the expression levels of SETD2, NSD1, DIM1, CMTR2, and PHF12 in the FD group were lower than those in the NF group. Surprisingly, the expression of SETD2, which belongs to the same gene family as SETD7, was decreased by FD. This was inconsistent with the expression of SETD7. Future work is needed to investigate the functions of SETD2 and SETD7.

In addition, nine genes were found to be associated with lipid transport and metabolism. Among these genes, five (GPAT4, ACSS1, GNPAT, HEXDC, and ACA2) were significantly downregulated by FD. ACSS1, a mitochondrial isoform of acetyl-CoA short chain synthetase, plays a key role in energy expenditure and maintaining normal body temperature during fasting (5). This may have a positive effect on lipolysis. Upregulated expression of MSON1, ACADL, OXCT1, and FDFT1 indicated that FD may have a positive effect on lipid deposition. Notably, ACADL is involved in mitochondrial fatty acid beta-oxidation and in lipid metabolism (19).

As discussed above, DEGs may be involved in methyltransferase activity, rRNA and DNA methylation, protein methylation, histone lysine methylation, histone demethylase activity, methylated histone residue binding, and histone H3-K36 trimethylation. Consistently, other studies also indicate that adipose metabolism was significantly affected by folate supplementation or deficiency (18, 20, 23, 35). These results suggest that maternal folate status can influence lipid metabolism of offspring.

KEGG pathway analysis identified five significantly enriched pathways from the DEGs. These enriched pathways
were involved in steroid biosynthesis, synthesis and degradation of ketone, histidine metabolism, lysine degradation, and ubiquitin-mediated proteolysis. The steroids, for example, are components of cell membranes and can decrease membrane fluidity (33). The biosynthesis of steroids is an anabolic pathway and follows the mevalonate pathway in humans and animals (13). Another finding provided support for effects of sex steroids on plasma HCY metabolism and showed that deficiencies of folate lead to markedly increased plasma HCY level in humans (10). In addition, one pathway of one carbon pool for folate (ko00670) was regulated by FD, although there was no significant difference between the NF and FD groups. Several enzymes are involved in folate biosynthesis, including 7,8-dihydrofolate, 5,6,7,8-tetrahydrofolate, 10-formyl-THF, 5,10-methenyl-THF, 5-formyl-THF, 5,10-methylene-THF, 5-formiminothf, and 5-methyl-THF. Hence, these finding will provide valuable theoretical basis for a better understanding of relationship between maternal folate deficiency and offspring fat metabolism.

In summary, this work revealed the gene expression responses to maternal folate deficiency in offspring. A total of 442 potential genes were found to be significantly differentially expressed under FD by digital gene expression profiling. These genes are involved in complex biologic processes including folate and lipid metabolic processes, methylation, and methyltransferase activity, suggesting that maternal folate deficiency can influence the gene expression and lipid metabolism of offspring.

ACKNOWLEDGMENTS
The authors gratefully thank Drs. Mingxiao Feng and Shuying Li for valuable suggestions and revisions of the manuscript.

GRANTS
This work was financially supported by the Nature Science Foundation of Shandong Province of China (ZR2017LC018, ZR2017LC012, ZR2017LC001) and the National Natural Science Foundation of China (31372333).

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
J. Xing conceived and designed research; J. Xing, W.J., Y.Z., J. Xu, and X.C. analyzed data; J. Xing, W.J., and Y.Z. interpreted results of experiments; J. Xing, W.J., and Y.Z. drafted manuscript; J. Xing edited and revised manuscript; J. Xing, W.J., and Y.Z. interpreted results of experiments; J. Xing prepared figures; J. Xing performed experiments; J. Xing, W.J., Y.Z., J. Xu, and X.C. analyzed data; J. Xing, W.J., and Y.Z. interpreted results of experiments; J. Xing reviewed drafts; J. Xing edited and revised manuscript; J. Xing approved final version of manuscript.

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