Folic acid supplementation during pregnancy modulates hepatic methyl metabolism and genes expression profile of neonatal lambs of different litter sizes

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Abstract

Maternal folic acid (FA) plays an important role in the fetus development, but it is unknown the response of hepatic metabolism in the offspring from different litter sizes to maternal FA supplementation. In the present study, this was done by feeding the ewes with 0, 16 and 32 mg/(kg·DM) FA supplemented diet during pregnancy and analysing the hepatic one-carbon metabolism-related indices and gene expression in the neonatal lambs of different litter sizes (twins, TW; triplets, TR). Regardless of litter sizes, the concentrations of folate, methionine, S-adenosylmethionine and DNA methyltransferase increased significantly, but homocysteine and S-adenosylhomocysteine decreased in the liver of newborn lambs from ewes whose diet was supplemented with FA. In TW, maternal FA status has little effect on hepatic genes expression profile of newborn lambs, and no significant enriched pathway was found. However, DEG involved in cell proliferation such as *CCNA2*, *CCNB2*, *CCNE2*, *CDK1* and *BUB1* were significantly enriched when the ewes were supplemented with FA in TR groups. In addition, nucleotide synthesis-related genes such as *POLD1*, *POLD2*, *MCM4* and *MCM5* were enriched markedly in DNA replication and pyrimidine metabolism pathways in triplets when a higher FA ingestion [32 mg/(kg·DM)] was implemented in ewes. This finding demonstrated that the hepatic methyl metabolism in TW and TR newborn lambs was regulated by maternal FA status. The hepatic cell proliferation and nucleotide metabolism related genes in TR were more susceptible to maternal dietary FA supplementation during pregnancy.

Key words: Folic acid: Gestation: Gene expression: Litter size: Lambs

Fetal growth is the 'critical window' of development in early life. It is sensitive to environmental conditions, such as nutritional environment, including dietary composition and nutrient supply, which play an important role in determining the developmental plasticity of offspring during prenatal and neonatal periods[1,2]. If ontogenetic development in early life is exposed to malnutrition or undernutrition of macronutrients and micronutrients, a wide range of changes in gene expressions could occur to affect the physiological or morphological development in later life[1,3,4]. Therefore, maternal diet composition and nutrient supply, as the only nutrition source for the fetuses, should be carefully monitored and adjusted to meet the needs of the fetus.

Folic acid (FA) is one of the necessary micronutrients involved in amino acid metabolism, *de novo* purine and thymidylate synthesis and modification as well as DNA repair during cell division and growth via mediating one-carbon metabolism (the methionine and folate cycles)[5]. Due to the biological functions of FA, it is likely that requirements of FA will be increased during gestation to satisfy rapid fetal, placental and uterine cell division[6,7]. Previous studies have demonstrated that folate plays an important role in blastocyst, and FA supplementation could promote oocyte maturation and placental formation[8,9]. FA can transfer to the fetus via the placenta[10], suggesting that fetal FA status will be dependent on maternal FA availability. Moreover, on the one hand, maternal folate availability during pregnancy can affect its hepatic nutrient metabolism[11] and placental nutrient transport[12], which may regulate the metabolism of offspring; on the other hand, FA supplementation can directly affect liver metabolism and gene expression[13]. Based on these reports, we speculate that FA can not only affect fetal metabolism through the maternal effect but also affect fetal metabolism through the transport of FA into the fetus. Moreover, FA supplementation during pregnancy increased birth weight[14,15] and prevented neural tube defects of newborns in humans[16,17]. Folate status was also associated

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Abbreviations: DEG, differentially expressed genes; DNMT, DNA methyltransferase; FA, folic acid; SAH, S-adenosyl-homocysteine.

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with muscle cell development[18], immune function[19] and nutrients metabolism[20,21] with regulation of gene expression and epigenetic modification. Collectively, FA status plays a critical role in influencing fetal development and subsequently the later life of offspring.

The metabolism of FA is tightly controlled by the liver. Folate from the diet and rumen microbiota synthesis is absorbed mainly from proximal intestine and then transported to the liver via the portal vein[22]. There are three metabolic pathways for FA in liver[23,24]. First, some FA can be utilised for the self-regeneration of hepatic cell. Second, a fraction of FA is stored in the liver as 5-methyltetrahydrofolic acid and released to peripheral viscera through portal circulation when it is needed by tissues and organs. Third, FA removed by the liver is partially released into bile and then reabsorbed in intestine (the entero-hepatic recirculation), this pathway is responsible for maintaining folate concentration in plasma. In addition, dietary FA as a methyl donor could regulate the hepatic gluconeogenesis[25], lipid metabolism[26] and energy metabolism[11]. Content of FA in the fetal liver increases as gestation progresses[27] and is supplemented by maternal dietary FA throughout pregnancy[28]. Hepatic metabolism, gene expression and modification in offspring are affected by maternal FA supplementation or deficiency[29–31]. It is well known that maternal nutrition requirements increase with increasing litter size and the level of nutrients obtained by each fetus depends on maternal nutrient intake[32]. Therefore, it is important to manipulate FA supplementation precisely according to fetal number, which will be beneficial to the rational utilisation of FA and avoid waste. However, the response of hepatic metabolism in offspring from different litter sizes to maternal (*Ovis aries*) FA supplementation is unknown.

Taken together, we speculate that the effect of maternal (*O. aries*) FA supplementation on liver metabolism in offspring is associated with litter size. To study the hepatic FA metabolism is also conducive to the rational intake of FA during pregnancy. In addition, sheep was selected as the animal model because they have similar body weights, closer genetic and physiological composition with human[33], and the result may provide reference for human FA utilisation. Therefore, in the current study, methyl-metabolism and hepatic genes expression profile for newborn lambs in twins and triplets were investigated to examine the influence of maternal FA supplementation during gestation.

Material and methods

Animals and experimental design

The protocol used throughout the study was approved by the Institutional Animal Care and Use Committee of the China Agricultural University (Permit number: DK996) and in accordance with the Animal Ethics Committee of Beijing, P.R. China.

One hundred and twenty multiparous Hu sheep (all ewes had given birth twice before, 24 ± 4·2 months of age) with similar body weight (44·00 ± 0·39 kg) and showing signs of estrus were selected and randomly divided into three treatment groups after mating. Ewes in each of the three groups were fed by one of the three diets: control diet (C), control diet supplemented with 16 mg (F16) or 32 mg (F32) of rumen-protected FA per kilogram DM from mating to lambing. All animals were housed in individual pens (size: $1·5 \times 3$ m^2). The FA had a purity at 99·8 %, rumen passing rate (measured by rumen fistula method) at 92·60 % and small intestinal absorption rate (measured by small intestine fistula method) at 85·59 %. Type-B ultrasonography was used to detect whether the ewes were pregnant or not at 28 d after mating and non-pregnant ewes were removed (removed numbers in C, F16 and F32 were 13, 8 and 14, respectively). After lambing, newborn lambs were divided into six groups (TW-C, TW-F16, TW-F32; TR-C, TR-F16, TR-F32) according to the litter sizes (twins, TW; triplets, TR) and maternal FA supplementation levels (C, F16 and F32).

This experiment was conducted at Jiangsu Qianbao Animal Husbandry Co. Ltd, Yancheng, Jiangsu, China. The dietary components and nutrition levels were provided following the recommended requirements of small ruminants by National Research Council[34]. The formulas of total mixed ration based on DM were 50 % peanut vines, 45 % whole corn silage and 5 % concentrate in the early gestation period (from mating to 90 d) and 27 % peanut vines, 28 % whole corn silage and 45 % concentrate in the late gestation period (from 91 d after mating to lambing). Composition of concentrate and nutrient levels of total mixed ration are shown in Supplementary Table S1. FA was added into total mixed ration, which was given in equal amounts at 08.00 and 18.00 daily to each ewe. Ewes had free access to clean water throughout the trail.

Sample collection and measurement

Dietary chemical composition. Total mixed ration samples were collected every 2 weeks during gestation period and dried in an oven at 65°C for 48 h. Then the samples were ground to pass through a sieve with 1 mm mesh for chemical analyses. DM content was determined by drying the samples in an oven at 105°C for 2 h. The macro-Kjeldahl method was used to assess crude protein content by multiplying 6·25 with nitrogen content[35]. A reflux system (ANKOM XT15, Ankom Technology, Macedon, NY, USA) with petroleum ether was run at 90°C for 1 h to detect dietary ether extract content. Ash content was measured by burning the samples in a muffle furnace at 550°C until the samples reached a constant weight. Neutral- and acid-detergent fibre content was determined using the method described by Van Soest *et al.*[36]. Calcium content was determined using the atomic absorption spectrometer (Czerny-Turner AAS8000, Skyray Instruments), while phosphorus content was detected by the molybdenum blue colorimetric method[35].

Hepatic sample collection. After lambing, 80 newborn lambs (TW-C, *n* 16; TW-F16, *n* 13; TW-F32, *n* 10; TR-C, *n* 8; TR-F16, *n* 19 and TW-F32, *n* 14) with near-average birth weight [TW-C, 3·80 ± 0·07 kg; TW-F16, 3·93 ± 0·11 kg; TW-F32, 3·96 ± 0·08 kg; TR-C, 3·11 ± 0·10 kg; TR-F16, 3·50 ± 0·07 kg and TW-F32, 3·49 ± 0·08 kg][37] were euthanised by carbon dioxide inhalation followed by exsanguination[38]. The liver was separated and cleaned with saline, and then around 20 g of liver were

collected and refrigerated at –80°C for later methyl metabolism-related parameters determination. Another around 5 g of liver was sampled into RNase-free tubes and stored in liquid nitrogen for RNA extraction.

Hepatic methyl metabolism indices. The contents of folate, methionine (Met), homocysteine (Hcy), S-adenosyl-methionine (SAM), S-adenosyl-homocysteine (SAH) and DNA methyltransferase (DNMT) in newborn lamb liver was measured according to the manufacturer's instructions of sheep ELISA kits (Dogesce Biological Technology Development Co., Ltd.). Six samples were determined for each treatment.

Hepatic Transcriptome analysis

RNA Isolation. Total RNA was isolated from all the liver samples (n 6 for each group) using Trizol Reagent (Tiangen Biochemical Technology Co., Ltd.). The purity, concentration and RNA integrity number (RIN) of the extracted RNA were evaluated using NP80 NanoPhotometer (IMPLEN Inc.), Qubit RNA Assay Kit with a Qubit 2·0 Fluorometer (ThermoFisher Scientific) and RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies), respectively. Only the samples with RIN greater than 7·0 were used for RNA sequencing (RNA-seq).

RNA Sequencing and Data Analysis. A total of 2 μg RNA per sample was used for library preparation. Sequencing libraries were performed using NEBNext UltraTM RNA Library Prep Kit for Illumina (NEB), following the manufacturer's recommendations. Index codes were added to attribute sequences to each sample. Quality of the library was assessed with Agilent Bioanalyzer 2100 system. Then, according to the manufacturer's instructions, TruSeq PE Cluster Kit v4-cBot-HS (Illumia) was used for the sequence clustering according to the samples index codes by using cBot Cluster Generation System. After cluster generation, Illumina Hiseq 4000 platform was used for the library sequencing to generate paired-ended 150 bp reads. Raw RNA-seq data presented in this paper were submitted to the NCBI Short Read Archive (accession number: PRJNA650226).

The raw reads were transformed to clean reads after quality control processing (including removal of reads containing adapters, reads containing ploy-N and low-quality reads). All the following analyses were based on the clean reads. Clean reads were then mapped to the reference genome of *Ovis aries* (Oar_v4·0, https://www.ncbi.nlm.nih.gov/genome/?term=txid9940[orgn]) using Tophat2 tools[39,40]. Only the reads with a perfect match or one mismatch were kept for further analyses. The quantification of gene expression level was estimated by calculating the number of cDNA fragments per kilobase of transcripts per million fragments mapped (FPKM)[41]. Differentially expressed genes (DEG) in each pair of the three groups were determined using the model based on the negative binomial distribution analysis of DESeq R package[42]. The P values of DEG were corrected using the method of Benjamini and Hochberg for controlling the false discovery rate[43]. Genes with a corrected P-value <0·05 were assigned as DEG.

Quantitative real-time PCR (qRT-PCR) verification. Twelve candidate genes were selected for qRT-PCR using SYBR Premix Ex Taq kit (Tiangen Biochemical Technology Co., Ltd.) in the iQ5 system (Bio-Rad) to validate their RNA-seq results. Six samples from each group and each sample with four replicates were done by qRT-PCR. Primers for the candidate genes were designed using Primer 5 software and synthesised by Sangon Biotechnology (Sangon Biotechnology Co., Ltd.) (online Supplementary Table S2). The glyceraldehyde 3-phosphate dehydrogenase gene was used as the internal control. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

The results of hepatic methyl metabolism indices were analysed by using the generalised linear model (GLM) of statistical package SPSS version 22.0 (SPSS, IBM, Inc.) to assess the effects of litter sizes (TW and TR) and dietary FA levels (C, F16 and F32). Polynomial analysis was conducted to test the linear or quadratic response to dietary FA supplementation levels. $P < 0·05$ was considered as statistically significant.

DEG in each pair of the three groups of different FA supplementation levels and of the two litter sizes were functionally annotated by Gene Ontology (GO) analysis[44]. Physiological metabolism events and signal pathways of the DEG were assessed using KOBAS software to test the statistical enrichments of the DEG in KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways[45]. Results of DEG enrichment performed by GO and KEGG analyses with corrected P values less than <0·05 were considered to be significantly different.

Correlation between the selected genes expression and biochemical indexes of liver was performed by using Pearson analysis of statistical package SPSS version 22.0 (SPSS, IBM, Inc.), and $P < 0·05$ was considered as significant correlation.

Results

Effects of the litter sizes and folic acid levels on liver weight and hepatic methyl metabolism of lambs

Liver weight in TW group was significantly higher than that of TR group ($P < 0·05$), while it was not affected by dietary folic acid supplementation. The percentage of liver weight to birth weight was not affected by litter sizes and dietary FA levels ($P > 0·05$).

No significant interaction effect between the litter sizes and FA levels was found on the contents of folate, Met, Hcy, SAM, SAH and DNMT and the ratio of SAM/SAH in the liver ($P > 0·05$) (Table 1). The concentrations of folate, Met, SAM and DNMT in the liver of lamb increased linearly ($P < 0·05$) with dietary FA supplementation, but Hcy and SAH decreased linearly ($P < 0·05$) in response to dietary FA supplementation (Table 1). The ratio of SAM/SAH increased ($P < 0·05$) with FA supplemented in the diet ($P < 0·05$) (Table 1).

Effects of the litter sizes on hepatic transcriptome profile of the lambs

In the present study, 38 913 780 to 54 401 634 clean reads in 150 bp were generated for individual samples (online Supplementary Table S3). The estimates of Q20 and Q30 values were larger than 97 % and 92 %, respectively (online

Table 1. Liver weight and methyl metabolism-related parameters affected by litter sizes and folic acid (FA) supplementation
(Mean values and standard errors of the mean, n 6)

Items	Litter Sizes		FA			SEM	P-values				
	TW	TR	C	F16	F32		Litter Sizes	FA	I×	L	Q
Liver weight/g	85·91	69·92	73·88	80·61	79·26	1·86	< 0·001	0·261	0·714	0·956	0·312
Liver weight/birth weight	2·16	2·12	2·15	2·17	2·10	0·038	0·651	0·764	0·598	0·603	0·467
Folate, nmol/g	16·88	18·32	13·67	17·70	21·42	0·75	0·198	< 0·000	0·699	< 0·000	0·892
Met, nmol/g	18·41	17·63	15·80	18·30	19·96	0·54	0·428	0·006	0·758	0·001	0·678
Hcy, nmol/g	19·33	20·51	23·69	19·18	16·89	0·68	0·248	< 0·000	0·490	< 0·000	0·302
SAM, nmol/g	21·41	21·33	16·66	22·61	24·83	0·78	0·947	< 0·000	0·807	< 0·000	0·111
SAH, nmol/g	28·07	29·67	35·94	26·61	24·06	1·20	0·365	< 0·000	0·489	< 0·000	0·071
SAM/SAH	0·82	0·78	0·48	0·87	1·04	0·05	0·550	< 0·000	0·639	< 0·000	0·049
DNMT, ng/g	57·56	56·73	46·71	58·45	66·28	1·75	0·731	< 0·000	0·988	< 0·000	0·425

TW, twins. TR, triplets. C, F16 and F32, lambs form ewes fed 0, 16 or 32 mg/(kg·DM) FA in the basal diet, respectively. Met, methionine; Hcy, homocysteine; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; DNMT, DNA methyltransferase. I×, P value of interaction effects between litter size and folic acid supplementation. L, P value of linear effect to folic acid levels. Q, P value of quadratic effect response to folic acid supplementation. S.E.M., standard error of mean. The unit of folate, Met, Hcy, SAM and SAH are nmol/g. The unit of DNMT is ng/g.
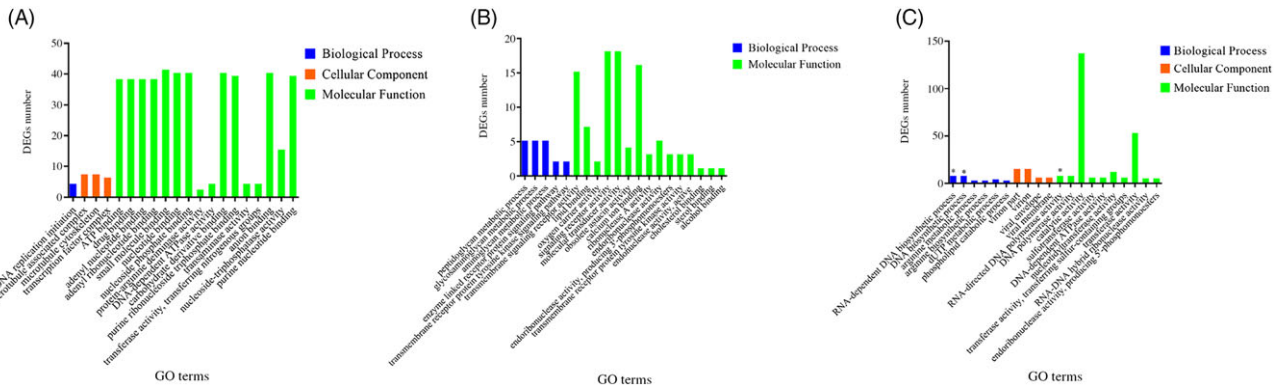

Fig. 1. The most enriched GO terms (biological process, cellular component and molecular function) affected by litter sizes (A, TR-C *v.* TW-C; B, TR-F16 *v.* TW-F16; C, TR-F32 *v.* TW-F32). DEG, differentially expressed genes. TW-C, TW-F16 and TW-F32 mean newborn twin lambs from ewes fed control diet supplemented with 0, 16 and 32 mg/(kg·DM) FA, respectively. TR-C, TR-F16 and TR-F32 indicate newborn triplet lambs from ewes fed with 0, 16 and 32 mg/(kg·DM) folic acid (FA) supplemented in control diet, respectively. '*' means enriched significantly. n 5 in TW-C group and n 6 in other groups.

Supplementary Table S3). Around 84·51–87·97 % of the reads were mapped to the Oar_v4·0 reference genome, while approximately 80 % of the reads from each sample were uniquely mapped to the reference genome (online Supplementary Table S4). The Person correlation R^2 values of $\log_{10}$ (FPKM + 1) between any two samples were larger than 0·90, supporting the high reliability and repeatability of our results (online Supplementary Fig. S1).

The numbers of totally expressed genes in liver samples ranged from 17 927 to 19 222, and the average numbers of expressed genes in TW-C, TW-F16, TW-F32, TR-C, TR-F16 and TR-F32 groups were 18 597, 18 660, 18 663, 18 590, 18 617 and 18 551, respectively.

Results of Venn analysis indicated the overlapped DEG number between the comparison of TR-C *v.* TW-C and TR-F16 *v.* TW-F16, TR-C *v.* TW-C and TR-F32 *v.* TW-F32, TR-F16 *v.* TW-F16 and TR-F32 *v.* TW-F32 were 14, 15 and 23, respectively (online Supplementary Fig. S2I).

Between twin- and triplet-born lambs, 316 DEG (198 up- and 118 down-regulated) were found when comparing TR-C with TW-C (online Supplementary Fig. S3A). Totally, 471 DEG (217 up- and 254 down-regulated) were identified between

TR-F16 and TW-F16 (online Supplementary Fig. S3B). For the comparison of TR-F32 and TW-F32, 597 DEG were detected, among them 296 up-regulated and 301 down-regulated (online Supplementary Fig. S3C).

The DEG between the groups of different FA supplementation levels were categorised into biological process (BP), cellular component (CC) and molecular function (MF). GO analysis of DEG form different litter sizes indicated DEG between TR-C and TW-C enriched in nucleic acid-related terms as DNA replication initiation, adenyl nucleotide/ribonucleotide binding, nucleotide/nucleotide phosphate binding and purine nucleotide binding (Fig. 1(a)). DEG between TR-F16 and TW-F16 were involved in MF such as receptors activity (transmembrane signalling receptor activity and signalling receptor activity) and enzymes activity (ribonuclease A/endoribonuclease/activity and transmembrane receptor protein tyrosine kinase activity) (Fig. 1(b)). DEG between TR-F32 and TW-F32 also found involved in nucleic acid-related (RNA-dependent DNA biosynthetic process and DNA biosynthetic process) and enzymes activity (RNA-directed DNA polymerase activity, DNA polymerase activity, catalytic activity and transferase activity) terms (Fig. 1(c)).
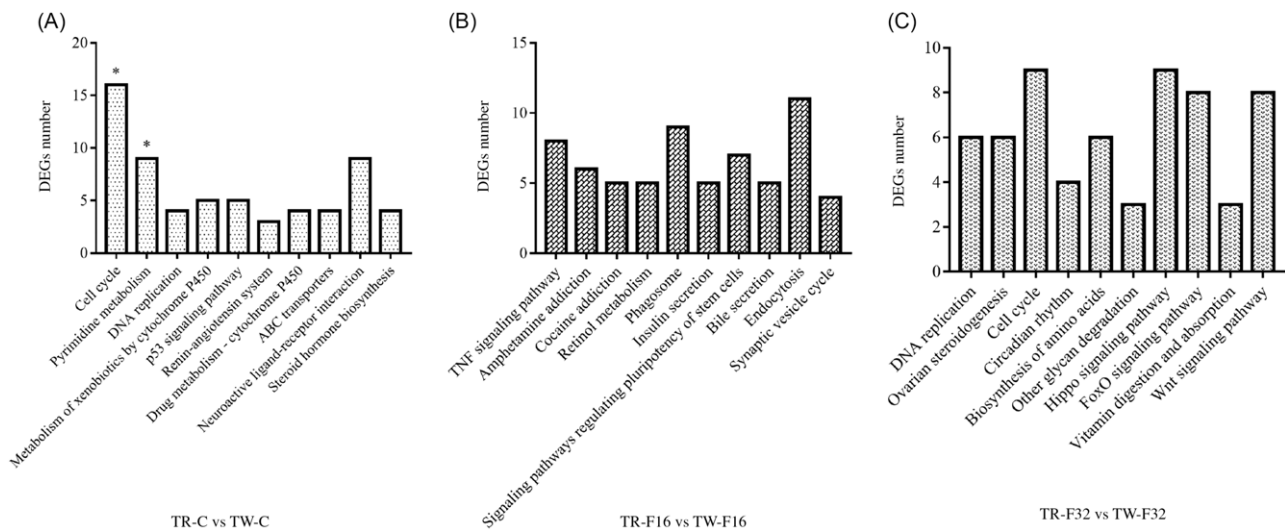
Fig. 2. The most enriched pathways of differentially expressed genes (DEG) between twin- and triplet-born lambs' liver by KEGG analysis. TW-C, TW-F16 and TW-F32 mean newborn twin lambs from ewes fed control diet supplemented with 0, 16 and 32 mg/(kg·DM) folic acid (FA), respectively. TR-C, TR-F16 and TR-F32 indicate newborn triplet lambs from ewes fed with 0, 16 and 32 mg/(kg·DM) FA supplemented in control diet, respectively. '*' means enriched significantly. n 5 in TW-C group and n 6 in other groups.

Results of KEGG pathway analysis revealed that the cell cycle and pyrimidine metabolism pathways were enriched significantly by DEG from TR-C $v.$ TW-C (Fig. 2(a)). More specifically, DEG, such as *CCNB2*, *CCNE2*, *CDC6*, *CDC20*, *CCNA2*, *E2F2*, *MCM4*, *BUB1* and *TTK*, were involved in the cell cycle pathway, and genes such as *POLE*, *TK1*, *TXNRD3*, *RRM2* and *DCK* were enriched in the pyrimidine metabolism (online Supplementary Table S5). While no significant and valuable pathway was found between TR-F16 and TW-F16 groups (Fig. 2(b)). With FA level increased (TR-F32 $v.$ TW-F32), down-regulated DEG as *DNA2*, *MCM3*, *MCM6* and *POLD2* were enriched in DNA replication pathway and *MCM3*, *MCM6*, *SMAD3* and *CHEK1* enriched in cell cycle pathway (Fig. 2(c)) (online Supplementary Table S5).

Effects of the folic acid on hepatic transcriptome profile in twins and triplets

In the twin born groups, the overlapped DEG between the comparison of TW-F16 $v.$ TW-C and TW-F32 $v.$ TW-C, TW-F16 $v.$ TW-C and TW-F32 $v.$ TW-F16, TW-F32 $v.$ TW-C and TW-F32 $v.$ TW-F16 were 53, 281 and 137, respectively (online Supplementary Fig. S2II). In the triplet born groups, the number of overlapped DEG between the comparison of TR-F16 $v.$ TR-C and TR-F32 $v.$ TR-C, TR-F16 $v.$ TR-C and TR-F32 $v.$ TR-F16, TR-F32 $v.$ TR-C and TR-F32 $v.$ TR-F16 were 89, 86 and 42, respectively (online Supplementary Fig. S2III).

In the twin born lambs, a total of 576 DEG (227 up- but 349 down-regulated) were found when compared TW-F16 with TW-C (online Supplementary Fig. S4A), while 386 DEG (185 up- but 201 down-regulated) were detected between TW-F32 and TW-C (online Supplementary Fig. S4B). Moreover, 1284 DEG were identified between TW-F16 and TW-F32, including 668 to be up- but 616 down-regulated (online Supplementary Fig. S4C). For triplet born lambs, compared with TR-C, 701 DEG (186 up- but 515 down-regulated) were found in TR-F16

(online Supplementary Fig. S4D), whereas 309 DEG (127 up- but 182 down-regulated) were detected in TR-F32 (online Supplementary Fig. S4E). In addition, 333 DEG were found between TR-F16 and TR-F32, of which 208 were up-regulated but 125 down-regulated (online Supplementary Fig. S4F).

In twin born lambs from ewes fed diet with FA supplementation or not, most of the enriched DEG from TW-F16 $v.$ TW-C comparison were involved in glycolmetabolism processes as glucose/hexose/monosaccharide metabolic process, gluconeogenesis and catalytic activities (protein methyltransferase activity, transferring one-carbon groups, methyltransferase activity and S-adenosylmethionine-dependent methyltransferase activity) (Fig. 3(a)). DEG between TW-F32 and TW-C were enriched in BP also involved in glycometabolism-related processes such as galactose/hexose/monosaccharide metabolic process. In addition, MF enrichment indicated that enzyme activity (S-adenosylmethionine-dependent methyltransferase activity, adenylyltransferase activity and enzyme activator/regulator activity) and transporter activity (transmembrane and nucleoside transmembrane transporter activity) terms were involved (Fig. 3(b)). Most of the DEG between liver samples from the TW-F32 and TW-F16 groups were involved in biological processes related to organic/carboxylic acid metabolic/biosynthetic process, small molecule metabolic process, glutamine family amino acid metabolic/biosynthetic process and enzyme regulator activity (Fig. 3(c)).

In triplet born lambs with or without FA supplementation in maternal diets, DEG from the comparison of TR-F16 $v.$ TR-C were mostly enriched in the nucleic acid metabolism (nucleoside diphosphate metabolic process, deoxyribonucleotide metabolic process, deoxyribose phosphate metabolic process and nucleoside phosphate metabolic process) and energy metabolism (carbohydrate catabolic process, glycolytic process and ATP generation from ADP) terms (Fig. 3(d)). The most enriched GO category terms between TR-F32 and TR-C groups were

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associated with DNA metabolic process and binding (drug binding, ATP binding, adenyl nucleotide/ribonucleotide binding and purine nucleotide/ribonucleotide binding) (Fig. 3(e)). DEG from the results of TR-F32 *v.* TR-F16 were involved in processes such as stimulus response (acute inflammatory response, acute-phase response and inflammatory response) and enzyme activity-related terms (hydro-lyase activity, carbon-oxygen lyase activity and lyase activity) (Fig. 3(f)).

Though no significant enriched pathway of DEG was found between TW-F16 and TW-C groups (Fig. 4(a)) and between TW-F32 and TW-C groups (Fig. 4(b)), some functional DEG regulated by FA supplementation were involved in cell growth-related pathways, for example, the p53 signalling pathway (e.g., *IGF1, GADD45B, THBS1, IGFBP3* and *CCNG1*, online Supplementary Table S6), apoptosis (*LMNB1, TUBA3E, TUBA1C* and *TUBA8*, online Supplementary Table S6), AMPK and FoxO
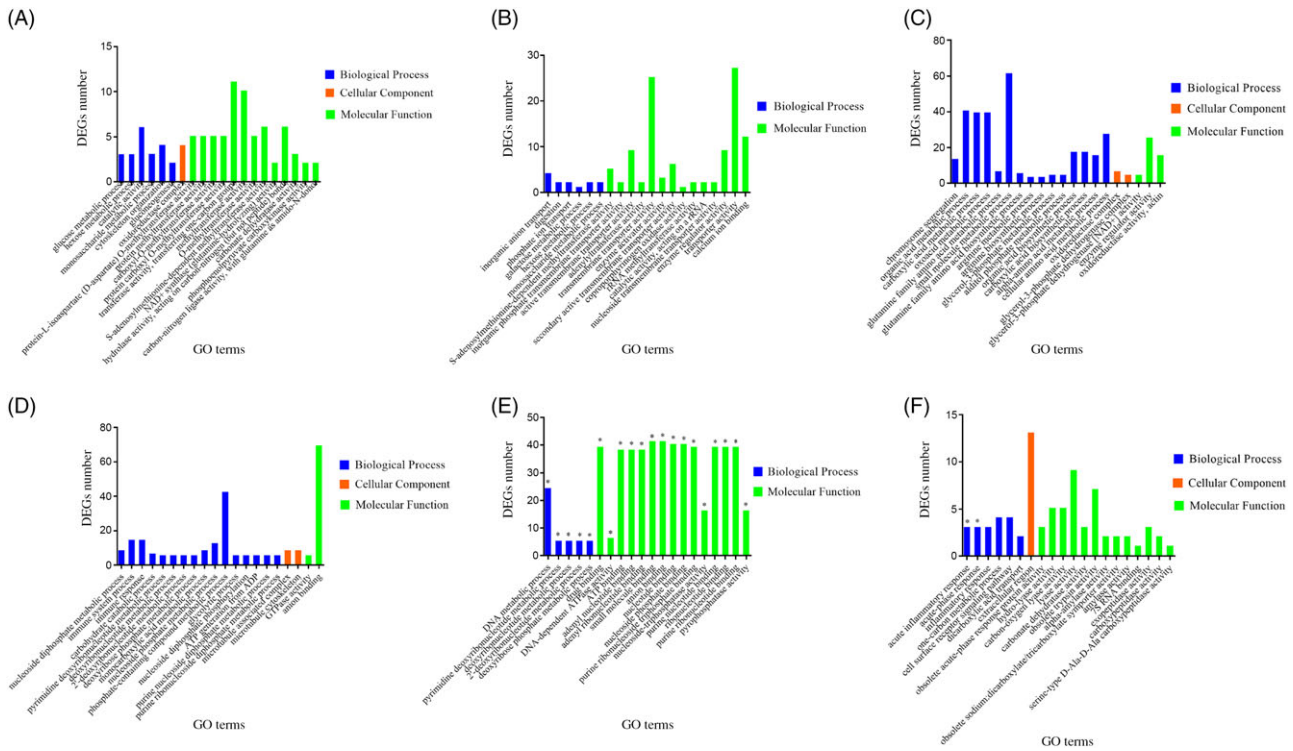

Fig. 3. The most enriched GO terms (biological process, cellular component and molecular function) affected by dietary folic acid supplementation in the liver of twin born (A, TW-F16 *v.* TW-C; B, TW-F32 *v.* TW-C; C, TW-F32 *v.* TW-F16) and triplet born lambs (D, TR-F16 *v.* TR-C; E, TR-F32 *v.* TR-C; F, TR-F32 *v.* TR-F16). DEG, differentially expressed genes. TW-C, TW-F16 and TW-F32 mean newborn twin lambs from ewes fed control diet supplemented with 0, 16 and 32 mg/(kg·DM) folic acid (FA), respectively. TR-C, TR-F16 and TR-F32 indicate newborn triplet lambs from ewes fed with 0, 16 and 32 mg/(kg·DM) FA supplemented in control diet, respectively. '*' means enriched significantly. n 5 in TW-C group and n 6 in other groups.
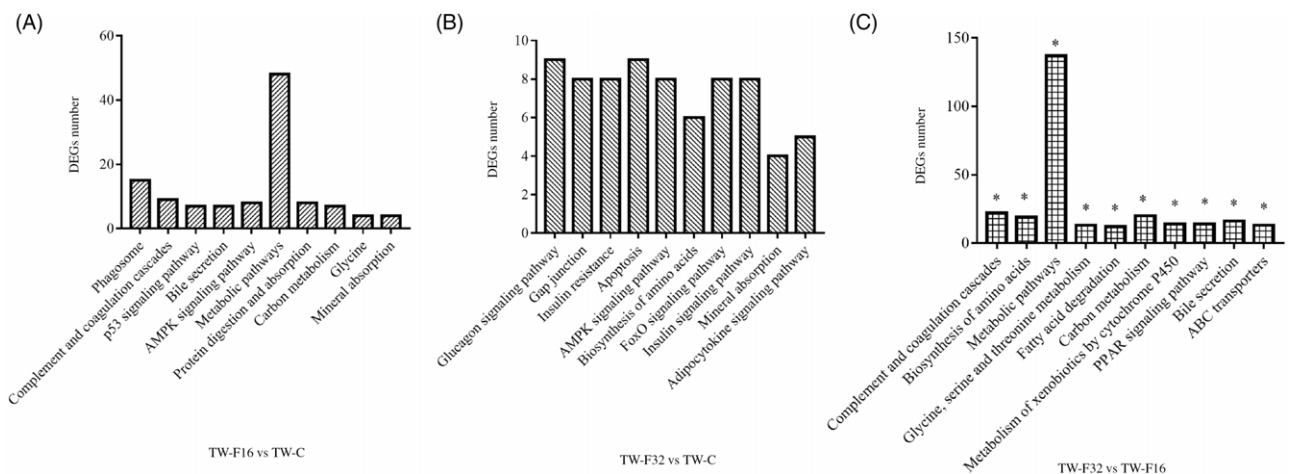

Fig. 4. The most enriched pathways in liver of newborn twin lambs from ewes fed with different levels of folic acid (FA) during gestation period. TW-C, TW-F16 and TW-F32 mean newborn twin lambs form ewes fed 0, 16 or 32 mg/(kg·DM) FA in the basal diet, respectively. '*' means enriched significantly. n 5 in TW-C group and n 6 in TW-F16 and TW-F32 groups.
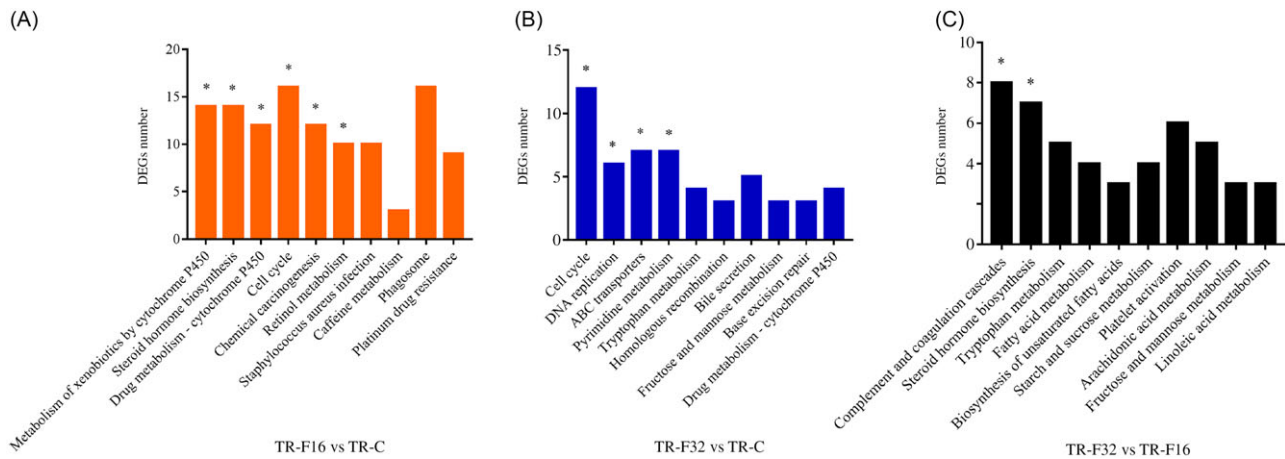
Fig. 5. The most enriched pathways in liver of newborn triplet lambs from ewes supplemented different levels of folic acid (FA) in the diet during gestation period. TR-C, TR-F16 and TR-F32, newborn triplet lambs form ewes fed 0, 16 or 32 mg/(kg·DM) FA in the basal diet, respectively. '*' means enriched significantly. n 6 of each group.

signalling pathways (*PRKAG2, PRKAG3, PCK1, INSR* and *G6PC*, online Supplementary Table S6). In addition, genes including *SLC7A9, SLC16A10, SDS* and *SDSL* (online Supplementary Table S6) were amino acid metabolism-related and involved in protein digestion and absorption, glycine, serine and threonine metabolism and biosynthesis of amino acids. Interestingly, the RNA-seq data of DEG between TW-F32 and TW-F16 groups (Fig. 4(c)) implied that the down-regulated genes in TW-F32 (e.g., *SDS, SDSL, CTH, SARDH, GLDC* and *GNMT*, online Supplementary Table S6) were found to be significantly enriched in the biosynthesis of amino acids, glycine and serine as well as the threonine and carbon metabolisms. Other DEG between TW-F16 and TW-F32 groups were markedly involved in the metabolism, fatty acid degradation and PPAR signalling pathway (Fig. 4(c)).

KEGG analysis of DEG between TR-F16 and TR-C groups indicated that the steroid hormone biosynthesis pathway was enriched as the ewes were supplemented with FA. Genes such as *HSD17B2, HSD17B6, AKR1D1, CYP1A2, CYP1A1* and *UGT2A3* (online Supplementary Table S7) were down-regulated in TR-F16 (Fig. 5(a)). The significantly enriched cell cycle pathway of the DEG from TR-F16 v. TR-C involved in down-regulated genes associated with cell proliferation in TR-F16 (e.g., *CCNA2, CCNB2, CCNE2, CDK1, CDKN2C, CDC20, TTK, BUB1* and *PKMYT1*, online Supplementary Table S7). The significantly enriched cell cycle pathway of the DEG between TR-F32 and TR-C groups (Fig. 5(b)) and identified down-regulated genes such as *CCNA2, CCNE2, CDC6, TTK, BUB1, PKMYT1, MCM4* and *MCM5* (online Supplementary Table S7) in TR-F32. In addition, the down-regulated genes in TR-F32 (*POLD1, POLD2, MCM4* and *MCM5*, online Supplementary Table S7) were also significantly enriched in DNA replication pathway. On top of *POLD1* and *POLD2*, the nucleotide metabolism-related genes of *TYMS, TK1* and *DCK* (online Supplementary Table S7) were significantly involved in the pyrimidine metabolism pathway.

qRT-PCR validation of RNA-seq results

Six genes (*IGF1, PRKAG2, PRKAG3, PCK1, SDS* and *SDSL*) were selected for qRT-PCR to validate their RNA-seq results from TW

groups while another six genes (*CCNE2, CCNA2, CDC20, BUB1, POLD1* and *POLD2*) were selected from TR groups. As shown in Fig. 6, all the selected genes displayed the same expression patterns in both qRT-PCR and RNA-seq results from the TW and TR groups, indicating that the RNA-seq data were reliable.

Pearson correlation analysis between gene expression and biochemical indexes in liver

The correlation result between gene expression and biochemical parameters in the liver of twin born lambs was shown in Supplementary Table S8. Hepatic folate concentration was significantly correlated with the expression of *IGF1, PRKAG2, PRKAG3* and *SDS* ($P < 0.05$). Met concentration had a significant correlation with *IGF1, PRKAG2* and *SDS* ($P < 0.05$). Content of SAM and DNMT was significantly negatively correlated with *PRKAG2* and *PCK1* ($P < 0.05$). In triplet born lambs (online Supplementary Table S9), significant correlations were found between the liver folate content and the expression of *CCNE2, CCNA2, CDC20, POLD1* and *POLD2* in liver ($P < 0.05$). Content of Met, SAM, DNMT and the ratio of SAM/SAH were significantly correlated with the expression of *CCNE2, CCNA2, BUB1, POLD1* and *POLD2* ($P < 0.05$). In addition, the content of Hcy and SAH had a positive correlation with the expression of *CCNE2* and *CCNA2* ($P < 0.05$).

Discussion

Folate, as an essential nutrient and involved in one carbon metabolism, is critical for many metabolic processes. Processes including nucleotide synthesis and modification and amino acid and vitamin metabolism, especially during gestation period, because of the growth and development of fetus. FA is important for cell division and homoeostasis during organ growth and metabolic renewal of tissues[46,47]. The adverse consequence of FA deficiency and the benefit of FA supplementation during the early development period to metabolic problems and health of the offspring were well reported previously[48–50]. Maternal FA requirement during pregnancy
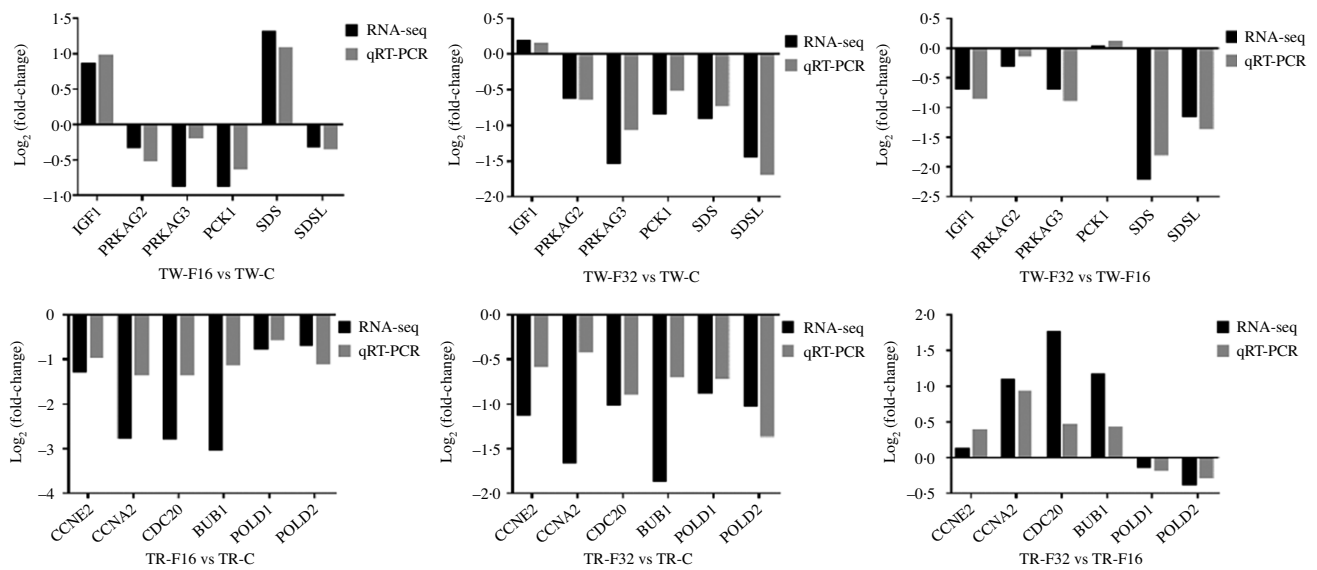
Fig. 6. Comparison of candidate genes expression between RNA-seq and qRT-PCR in TW and TR groups. TW-C, TW-F16 and TW-F32 indicate newborn twin lambs from ewes fed control diet supplemented with 0, 16 and 32 mg/(kg·DM) folic acid (FA), respectively. TR-C, TR-F16 and TR-F32 indicate newborn triplet lambs from ewes fed with 0, 16 and 32 mg/(kg·DM) FA supplemented in control diet, respectively. *n* 6 of each group.

is 5–10 times higher than that during non-pregnancy due to uterine, placental and fetal growth[51]. Therefore, FA supply during pregnancy plays an important role in regulating the development and metabolism of offspring. Our previous study found that the maternal blood metabolism and the growth performance (birth weight increased with dietary FA supplementation) of newborn lambs were affected by dietary FA supplementation[37,52]. However, the response of offspring's hepatic metabolism from different litter size to maternal FA supplementation was still unknown, which was investigated in the present study.

Serum folate concentration is an indicator of body folate status and its concentration increases with dietary FA addition[10,53,54]. During pregnancy, the folate transported by the placenta is dependent on maternal plasma folate concentration as demonstrated by the positive relationship between maternal plasma and placental folate concentration[55]. Therefore, maternal dietary FA supplementation might improve the folate transportation from mother to fetuses and increase its accumulation in fetuses. In the current study, hepatic folate content increased linearly in the newborn lambs from maternal diet with FA supplementation, and the corresponding ewes and lambs (lineally increased in both twin- and triplet-newborn lambs) plasma folate concentrations were increased as previously reported[37,52]. The result is consistent with the report that folate content increases significantly in the liver of offspring that come from a dam supplemented with FA in the diet throughout pregnancy[28]. According to the one-carbon metabolism (methionine and folate cycle)[56], the elevated folate in the liver of neonatal lambs might induce the decrease of Hcy and the increase of Met by improving the conversion of Hcy to Met. Previous research in rats[57], humans[58] and cows[54] reported similar results that higher folate concentration is accompanied with lower Hcy. As Met is the precursor of SAM, increased folate could contribute to the generation of SAM by the DNMT

catalysed reaction[59,60]. In the FA supplemented groups of the current study, the increased SAM (a universal methyl donor for DNA, histone, protein and lipid), decreased SAH (a product inhibitor of methyltransferase) and the higher SAM/SAH ratio suggested maternal FA supplementation during pregnancy might modulate the hepatic metabolism of the offspring through an epigenetic mechanism[61,62]. Surprisingly, the methyl metabolism-related indices in the liver from different litter sizes were identical. This might be explained that though the placental efficiency decreased with increased litter size and resulted in the restriction of nutrient transport from mother to fetuses, maternal folate is preferentially being distributed to the fetuses to protect the fetuses' development[20,63,64].

Litter size, as a very important life-long trait, indicated a permanent influence on offspring metabolic phenotype[65,66]. In the present study, the cyclin-related genes such as *CCNB2*, *CCNE2*, *CDC6*, *CDC20*, *CCNA2* and the cyclin-dependent kinases downstream target gene *E2F2* were up-regulated in the liver lambs from larger litter size (triplets), which suggested that the increased litter size has a positive regulation on cell cycle progression. Previous studies demonstrated that the initiation of DNA replication is coordinated with the cell cycle[67,68]. This is consistent with the results that the DNA replication and the nucleotide metabolism-related pathway-pyrimidine metabolism were enriched by the up-regulated DEG. However, when compared the TR-F16 with TW-F16 and TR-F32 with TW-F32, we only found part of down-regulated DEG involved in the cell cycle or DNA replication pathway with no significant enrichment. Based on this, we speculated that hepatic genes expression profile might be affected by the interaction between litter sizes and maternal diet FA supplementation levels. Therefore, we discussed the hepatic genes profile of offspring in response to maternal FA supplementation separately according to twin-born and triplet-born lambs.

In the twin lambs, even though no significant pathways were enriched when comparing the hepatic DEG from maternal FA supplementation groups (F16 and F32) with the control group, genes involved in cell growth regulation were enriched in the apoptosis, p53 signalling pathway, AMPK signalling pathway and FoxO signalling pathway. The DEG were also significantly correlated with the liver methyl metabolism indexes, such as folate, Met, SAM and DNMT. In human folate-deficient HepG2 cells, cell growth and viability decreased, and an increased apoptotic propensity associated with cell cycle-specific mechanism was observed[69]. Apoptosis as a stress factor could activate the p53 pathway that plays a critical role in the cell division, cell cycle and cellular homoeostatic regulation[70]. Crott et al. reported that folate status affects cell growth by regulating the genes and their products of which involved in the p53 pathway[71]. The IUGR (intrauterine growth retarded) model of pig also revealed that maternal FA supplementation reversed IUGR that altered apoptosis-related gene expression in newborn piglets[72]. Moreover, the DEG such as PCK1, LEPR, PRKAG2, PRKAG3, INSR, GYS2 and G6PC were glucose metabolism-related genes and involved in AMPK pathway. A previous report demonstrated that nutrition deprivation as one of stress signals could trigger the p53 pathway via the mediator of AMPK pathway[70], which acted as a master coordinators of cell growth, metabolism and ultimately cell fate[73]. The identified FoxOs signalling pathway, which contributes to cell survival, growth and proliferation[74], was influenced by maternal FA supplementation in the twin born lambs. The result was in accordance with a previous study in chickens[75]. In addition, DEG, such as SLC7A9, SLC16A10, SDS and SDSL, were amino acid metabolism-related genes and involved in the serine metabolism, which may affect the hepatic one-carbon metabolism. Pathways related to amino acids and lipid metabolism such as biosynthesis of amino acids, glycine, serine and threonine metabolism pathway, carbon metabolism, fatty acid degradation, PPAR signalling pathway were significantly enriched in the liver of lambs from mothers diet supplemented with 16 and 32 mg/(kg·DM) FA groups. According to the methionine-folate cycle, amino acids are an important part of this cycle, and folate status is significantly associated with amino acid metabolism[5]. The folate deficiency during pregnancy had widespread changes on methyl metabolism and amino acids in the rat fetus[20]. In addition, previous reports have demonstrated that folate intake and folate status are associated with changes in the expression of genes involved in lipid metabolism[26,76], but the underlying mechanism needs to be further studied. Therefore, FA supplementation during gestation period may affect the hepatic metabolism to some extent by regulating the cell growth, amino acids and lipid metabolism in the twin lambs.

In the triplet lambs, the cell cycle pathway was enriched in both 16 and 32 mg/(kg·DM) FA supplementation groups when compared with the control group. The correlation analysis also found that there was a significant relationship between liver biochemical indicators (such as folate, Met, SAM and DNMT) and related-genes expression. Cell cycle progression is accomplished through the DNA replication (S phase) and mitosis (M phase), which are separated temporally by G1 and G2 phases. Folate-mediated one-carbon metabolism plays a vital role in cell cycle by comprising an interconnected network of folate-dependent metabolic pathways and responsible for the de novo purine synthesis and de novo thymidylate synthesis[5]. Folate is necessary for maintain the normal cell cycle, and the folate deprivation-induced cell cycle arrest at G0/G1 phase and apoptosis in the cell experiments[77,78]. Cyclin family contains a series of protein and function as key regulator of cell cycle. Lin et al. reported that the content of cell cycle-related protein cyclin A, D1, D3 and E, CDK2 and CDK4 were not affected by FA concentrations (0, 0·1, 1 and 10 μmol/l) in the human umbilical venous endothelial cells[79]. While Kuo et al. used the same FA concentrations to culture LoVo colon cancer cell lines found FA concentration-dependently decreased the levels of CDK2 protein, increased CDKN1A, CDKN1B and TP53 protein and no significant effect on the levels of cyclin A, D1, D3 and E and CDK4 protein[80]. In the present study, we found a series of cyclin like CCNA2, CCNB2, CCNE2 and other cell cycle-related genes as CDK1, CDKN2C, CDC20, TTK, BUB1 and PKMYT1 were down-regulated with higher FA levels in the liver of neonatal lambs. These might be explained by the suggestion that the effects of folate on the cell cycle-related key genes expression in a cell-specific manner[81] and might be related to the environmental difference between in vitro and in vivo experiments. In addition, we also found that the steroid hormone biosynthesis pathway was co-enriched with lower FA (16 mg/(kg·DM)) supplementation in the liver of triplet born lambs, we speculated it may because FA could regulate the lipid metabolism[26,76]. As we all know, IUGR is more likely to occur with the increase of litter size, which was consistent with our previous finding that birth weight of triplet is lower than that of twins. The lipid metabolism and expression of genes related to the process were changed by IUGR, but maternal FA supplementation was an effective way to prevent the changes[82]. Furthermore, the down-regulated genes like POLD2 and POLD1, which play a crucial role in DNA replication and repair, were significant involved in the DNA replication and pyrimidine metabolism pathways with higher FA (32 mg/(kg·DM)) supplementation, which is consistent with the genes enriched in cell cycle. The result was supported by the reports that folate plays a critical role in DNA replication and the synthesis of nucleotides and indicates a correlation with the cell cycle[5,83]. Moreover, the up-regulated genes were enriched in cell cycle, pyrimidine metabolism and DNA replication between triplet- and twin-born lambs. While genes enriched in the same pathways were down-regulated in the triplet born lambs with maternal FA supplementation, which also suggested maternal FA supplementation is critical for neonatal lambs cell cycle progression. Collectively, FA supplementation during pregnancy had an influence on hepatic metabolism by regulating genes expression and pathways involved in cell cycle and nucleotides metabolism in the triplet newborn lambs.

Based on the current study, FA supplementation during pregnancy improves hepatic methyl metabolism in both twins and triplets. However, in terms of hepatic genes expression profiles, triplet lambs had significant response to maternal FA supplementation than that of twin lambs. Moreover, FA supplementation had effective influence on regulating cell cycle and nucleotide metabolism related genes expression in the liver. The result

suggested that it is important to consider the fetal number in uterus for FA supplementation during pregnancy, especially when the mother is pregnant with large number of fetuses.

Conclusion

In conclusion, the hepatic methyl metabolism of newborn lambs was improved by supplementing FA in their mothers' diet during gestation regardless of the litter size, while the triplet born lambs were more sensitive to maternal FA supplementation than the twin born lambs by analysing genes expression profile. In the twin lambs, maternal FA status indicated a gentle influence on the hepatic genes expression involved in cell growth, amino acid and lipid metabolism. However, in the triplet lambs, genes were significantly enriched in the cell cycle, DNA and nucleotides synthesis-related pathways in response to the maternal FA supplementation. The underlying reason of the different responses between twins and triplets to maternal FA addition may depend on the requirements of FA in different litter size during the fetal development period. More research is needed to elucidate the relationship and difference between maternal FA supply and requirement of different litter sizes during gestation period, to avoid the potential impairment of FA deficiency and excess for the metabolism of offspring.

Acknowledgments

Supplementary material

For supplementary material/s referred to in this article, please visit https://doi.org/10.1017/S0007114521002841

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