

Comparative Proteomic Analysis of Chicken Primary Hepatocytes with Folic Acid Free or Supplementation Culture Mediums

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ABSTRACT

Folic acid had been reported to develop much metabolic regulation function in animals and human beings due to its roles in one carbon metabolism. The current study was conducted to explore folic acid regulation function in primary chicken hepatocytes via supplement and deprivation culture models based on proteomic analysis. Results have shown that folic acid supplement significantly increased intracellular folic acid, 5-Me-THF and SAM contents when compared with folic acid free group ($P < 0.05$). Whereas, there was no difference about genome 5mC levels and DNMTs mRNA expression between these two groups. Proteomic analysis found 85 differential expressed proteins with 35 down and 50 up regulation. COG and KEGG pathway analysis revealed that amino acid metabolism, carbohydrate metabolism and antioxidant function were affected by folic acid. Posttranslational modification, protein turnover, chaperones and transcription were gathered by COG analysis in relative high proportion. PRMT7 and ARID4B which were associated with histone methylation were up-regulated in the folic acid supplement group, suggesting that folic acid was likely to take part in metabolism regulation of hepatocytes via histone methylation manner in the study. In conclusion, proteomic analysis found 85 differential expressed proteins in hepatocytes with folic acid free and supplementation medium. Folic acid might be involved in amino acid and carbohydrate metabolism and oxidation resistance by its epigenetic modifications functions. Our study also provided fundamental differential protein profiles mediated by folic acid, which can facilitate the understanding of folic acid regulation function in hepatic metabolism.

Key words: Folic acid, Histone methylation, Primary chicken hepatocytes, Proteomics

Abbreviations: MTHFR: methylenetetrahydrofolate reductase; FA: folic acid; DNMT: DNA methyltransferase; GO: Gene ontology; COG: cluster of orthologous groups of proteins; DEP: differential expressed protein; ROS: Reactive oxygen species; KEGG: Kyoto Encyclopedia of Genes and Genomes.

INTRODUCTION

Folic acid, as an essential B vitamin, had been reported to develop many metabolic regulation functions in animals and human beings. For instance, folic acid addition could reduce hypoxia-induced inflammatory response by Reactive oxygen species and JAK2/STAT3 pathway in human pro-myelomonocytic cells (Ma et al., 2018), and also could anises acetate-induced hepatotoxicity by down-regulating NF- κ B, IL-1 β production and lipid peroxidation caused by cell injury (Allah and Badary, 2017). What's more, maternal use of folic acid can prevent many neural tube defects (Molloy et al., 2017). The previous study also revealed that folic acid decreased homocysteine level and improved antioxidative capacity in atherosclerotic rats (Cui et al., 2017). In addition, folate was reported to have

prevention function in breast cancer risk (Chen et al., 2014). On the other hand, many study reported that folic acid developed function by changing DNA methylation because of its roles in one-carbon transfer reactions; Yu et al. (2014) has found that folic acid could reduce lipid accumulation of chicken adipocytes by increasing DNA methylation of C/EBP α promoter, thereby reducing FAS and PPAR γ expression. It was reported that the mouse sperm epigenome would be altered under the condition of low paternal dietary folate (histone H3 methylation or DNA methylation), which was also associated with many negative pregnancy outcomes (Lambrot et al., 2013). Therefore, it's confirmed to some extent that folic acid could have anti-inflammation and anti-oxidation effect, and also play positive roles in some diseases.

The liver is a metabolic organ owning synthesis, transportation, detoxication functions and also a major place of folic acid metabolism. Folic acid is transported inside the cell via different processes involving membrane embedded folate receptors or reduced folate carrier (Nazki, et al., 2014), then 5,10-methylenetetrahydrofolate could be distributed towards methionine pathways, which involves in remethylation of homocysteine for genomic and non-genomic methylation, catalyzed by methylenetetrahydrofolate reductase (MTHFR) through a non-reversible process (Lucock, 2000). In poultry industry, many metabolic diseases occur under the conditions of intensive breeding environment and higher improvement of growth performance by genetic breeding. It's aimed to come up with an assumption that whether folic acid could take part in hepatic metabolism regulation through DNA methylation capacity to solve the potential problems in chickens.

Hepatocytes culture *in vitro* is a suitable model to study metabolism, pharmacology and toxicology (Hou et al., 2001, Xu et al., 2012, Chen et al., 2017). And given the importance of liver organ itself in body metabolism and the metabolism site of folic acid, primary chicken hepatocytes will be used to explore our hypothesis mentioned above preliminarily in virtue of proteomics analysis technique. In addition, folic acid supplemented and folic acid deficient culture media are used to establish two cells culture models.

MATERIALS AND METHODS

Culture of chicken primary hepatocytes

Hepatocytes were isolated from male one-day-old layer chicks by collagenase digestion and filtration according to our previous description (Liu et al., 2018). We confirm that all animals' procedures used in the current study were approved by the ethical standards of the Animal Care and Use Committee of the College of Animal Science and Technology of the Northwest A&F University (Shaanxi, China). After 12 h attachment incubation, hepatocytes were washed with PBS and replaced with growth medium; when the confluence reached to about 80%, folic acid-free (0 mg/L) or folic acid supplemented medium (15 mg/L) was used to replace the normal medium (1 mg/L folic acid) for another 12 h treatment. RPMI 1640 culture medium with folic acid-free was purchased from Gibco (Life Technologies, Carlsbad, CA) and folic acid from Sigma (St. Louis, MO). There are three replicates in each group for proteomics analysis, and six replicates for other detections. The folic acid was

dissolved in the 10% ammonium hydroxide with minimal volume, then diluted to the concentration of 500 mg/L using deionized water (Yu et al., 2014), finally filtered by 0.22- μ m filters. The stock solution was diluted further in culture medium to reach the final concentrations required.

5mC level

Genomic DNA from hepatocytes was isolated using the TIANamp Genomic DNA Kit (Tiangen, Beijing, China) according to standard procedures. Then 100 ng of each DNA sample was used to measure global DNA methylation level using 5-mC DNA Elisa Kit (Zymo Research, Irvine, California, USA). The amount and percentage of methylated DNA (5mC) in the total DNA was calculated based on a standard curve.

Determination of folic acid, 5-Me-THF and SAM contents

Upon treatments, cells were rinsed with ice-cold PBS and trypsinized. Hepatocytes were centrifuged, washed and suspended in PBS. After ultrasonic decomposition, cells were centrifuged at 1500 g for 15 min at 4°C to remove cellular debris. The supernatant was collected to examine levels of folic acid, 5-Me-THF and SAM by Enzyme-linked Immunosorbent Assay Kits (Cloud-Clone Corp, USA). All the results were expressed as ng/10⁶ cells.

RNA isolation and gene quantification

After the removal of treatment medium, cells were washed twice with ice-cold PBS. Total RNA was extracted based on the TRIZOL reagent instruction (Invitrogen, Carlsbad, CA). Its concentration and purity were determined by the absorbance at 260 nm and A260/A280 value using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, USA). 500 ng of total RNA were used to complete cDNA synthesis by Primer Script RT Reagent Kit (TaKaRa, Dalian, China). Then the SYBR Premix Ex Taq kit (TaKaRa, Dalian, China) was used to carry out the assay for gene expression. Primers sequences were shown in table 1. Detailed procedures were operated as our previous description (Liu, et al., 2016). The $2^{-\Delta\Delta C_t}$ method was used for gene relative expression (Livak and Schmittgen, 2001).

Protein extraction

After treatment, cells were completely homogenized with a STD buffer (4% SDS, 1 mM DTT, 150 mM Tris-HCl pH 8.0, protease and phosphatase inhibitors), then the mixture was heated at 100 °C for 10 min. After centrifugation at 12000 g for 10 min when cooled to room

temperature, the supernatants were collected and protein concentration was determined using the Bicinchoninic acid (BCA) assay kit (Bio-Rad) based on its protocols.

Protein digestion and iTRAQ labelling

A total of 200 µg protein were digested following the reported methods (Du et al., 2015), and the peptide content was quantified by UV light spectral density at 280 nm. Then 80 µg peptide for each sample were used for iTRAQ labelling (Applied Biosystems). The three samples in 0 mg/L group were labelled with reagents 113, 114 and 115. The samples in 15 mg/L group were labelled with 118, 119 and 121. After labelling, all samples were pooled and dried. The mixed labeled peptides were carried out fractionating by strong cationic-exchange (SCX) chromatography separation. About 36 fractions were collected and combined, then desalted on C18 Cartridges. Each fraction was detected for liquid chromatography-tandem mass spectrometry (LC-MS/MS). Detailed procedures are on the basis of previous report (Dong, et al., 2017, Cao, et al., 2018).

Protein identification and quantification

The protein identification and iTRAQ quantification were operated using a Mascot 2.2 (Matrix Science, London, UK) and Proteome Discoverer 1.4 (Thermo Electron, San Joes, CA) as described (Wang, et al., 2013). The corresponding parameters were set as same as the description by Du et al. (2015). Database search was performed against the Gallus (Uniprot) database. For statistical analysis, student's *t* test was used to identify

significant changes between two group samples. Proteins with a statistically significant iTRAQ ratio of > 1.2 or < 0.83 ($P < 0.05$) were considered differentially abundant proteins.

Functional analysis

Gene ontology (GO), cluster of orthologous groups of proteins (COG), KEGG pathways and proteins interaction of identified differential proteins were analyzed respectively according to previously reported method (Wu, et al., 2006, Wu, et al., 2016). A schematic workflow illustrating the steps about iTRAQ process applied in this study is shown in figure 1.

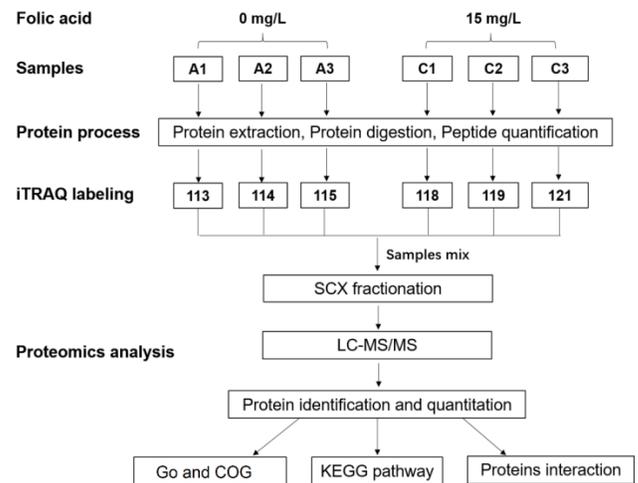


Figure 1. Experimental design and schematic diagram of proteomics analysis in the study.

Table 1. Primers of genes for RT-PCR analysis

Gene	Accession number	Primer sequences, 5' to 3'	Product size, base pair	Reference
β-actin	L08165	Forwards: ATTGTCCACCGCAAATGCTTC Reverse: AAATAAAGCCATGCCAATCTCGTC	113	Liu et al. (2016)
DNMT1	NM206952	Forwards: ACAGCCTTCGCCGATTACA Reverse: CTCTCCACCTGCTCCACCAC	81	Liu et al. (2016)
DNMT3A	NM001024832	Forwards: CAACAACCACGACCAGGAGT Reverse: ACCATGCCACAGTGATAGAGT	84	Liu et al. (2016)
DNMT3B	NM001024828	Forwards: CCCGTTATGATCGACGCTAT Reverse: GGGCTACTCGCAGGCAAA	92	Liu et al. (2016)

Statistical analysis

Experimental data on DNMTs expression, genomic 5mC level, folic acid, 5-methyl-THF and SAM contents in chicken hepatocytes were analyzed using *t*-test in SPSS 21.0 software (SPSS Inc., Chicago, IL, USA). The identification of differential expression proteins (DEPs)

between two groups depended on the ratio of protein contents in folic acid free group to folic acid supplement group. The ratio ≥ 1.20 or ≤ 0.83 was regarded as differentially expressed proteins. In addition, a value of $P < 0.05$ was considered to be statistically significant.

Ethics Committee Approval

All the birds and experimental protocol in this study were approved by the Institution Animal Care and Use Committee of the Northwest A&F University (protocol number NWAFA1008).

RESULTS

5mC level and some metabolites content

As shown in table 2, intracellular folic acid, 5-Me-THF and SAM contents were significantly higher in folic acid group when compared with folic acid free group ($P < 0.05$). Whereas, there was no difference about genome 5mC levels between these two groups.

Table 2. Levels of genome 5mC and some metabolites in hepatocytes of layer chicks

Parameters	FA-free	FA-sup	SEM	P value
5mC (%)	1.00	0.81	0.106	0.096
FA (ng/10 ⁶ cell)	24.00	29.94*	0.430	<0.001
5-Me-THF (ng/10 ⁶ cell)	0.26	0.37*	0.024	0.002
SAM (ng/10 ⁶ cell)	1.69	1.96*	0.091	0.021

Note: The symbol * showed difference significantly in statistics between folic acid free and supplement groups ($P < 0.05$). SEM= Standard error; FA= folic acid; 5-Me-THF=5-methyl tetrahydrofolic acid; SAM= S-adenosylmethionine.

mRNA expression of DNMTs

As exhibited in figure 2, 15 mg/L folic acid supplement didn't affect genes expression about DNA methyltransferases in comparison with those in folic acid free group.

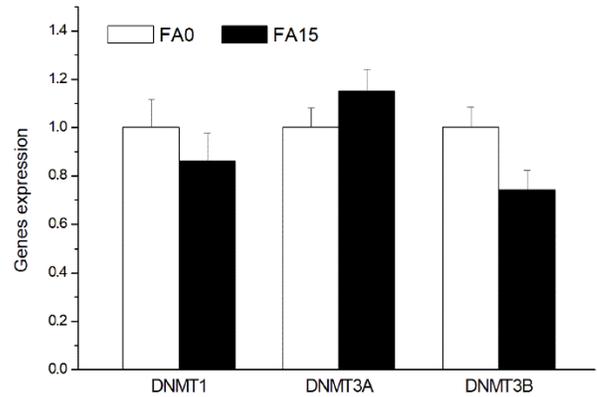


Figure 2. Gene expression of DNA Methyltransferases (DNMT1, DNMT3A, DNMT3B) in layer chicken hepatocytes between groups with folic acid free and supplement medium. Data were presented as means \pm SEM (n=6).

Protein profiling

Using the Mascot software, a total of 28725 unique peptides and 4660 proteins were identified. Among these proteins, 547 were between 0 to 20 kDa, 2393 between 20 to 60 kDa, 965 between 60 to 100 kDa and 755 over 100 kDa (Figure 3A). 1405 proteins had one unique peptide, 670 had two, 667 had more than 11, and the left had 3–10 (Figure 3B). Because iTRAQ quantification indicated the amount of real fold change between groups to some extent, proteins with a fold-change > 1.2 or < 0.83 ($P < 0.05$) were regarded as differential expressed proteins (DEPs). Based on this standard, 85 DEPs (35 down-regulation and 50 up-regulation) were detected shown in table 3.

Table 3. Differential expression proteins between folic acid free and supplement groups

Accession	Gene name	Protein name	¹ Ratio Sup/free	P value
Down-regulation				
F1N804	PLXNA1	Plexin A1	0.529	0.012
F1NL76	GAK	Cyclin G associated kinase	0.641	<0.001
Q8AWB6	SLC35B1	Solute carrier family 35 member B1	0.675	<0.001
R4GF71	TMSB4X	AM-8-amino-7-oxononanoate aminotransferase	0.707	0.002
E1B2Y2	SLC7A3	Cationic amino acid transporter-3	0.709	0.006
B5AIG4	PNPLA2	Adipose triglyceride lipase	0.711	0.007
A0A1L1S044	LOC420368	Predicted GTPase	0.711	0.038
F1P4D1	SLC30A7	Zinc transporter 7	0.746	0.031
A0A165FX80	CATH1	Cathelicidin-1	0.754	0.046
P12276	FASN	Fatty acid synthase	0.759	0.010
F1P3G3	CHN1	Chimerin 1	0.760	0.003
A0A2K6TZL8	TSNAXIP1	Translin associated factor X interacting protein 1	0.768	0.009
F1NDN6	KRT12	Keratin 12	0.768	0.001
H9L107	KRT4	Myosin heavy chain	0.771	0.003
Q5ZJ43	EXOC8	Exocyst complex component 8	0.774	0.018
F1NGI6	SGSH	N-sulfoglucosamine sulfohydrolase	0.779	0.011
E1C483	ACBD6	Acyl-CoA-binding protein	0.779	0.016

E1BS86	AIG1	Androgen induced 1	0.780	0.043
F1NQ90	C11H19ORF12	Mu-like prophage protein	0.781	0.005
F1NNN3	TCERG1L	Transcription elongation regulator 1 like	0.783	0.022
F1NKU2	MELK	Non-specific serine/threonine protein kinase	0.783	0.017
P08286	P08286	Histone H1.10	0.787	0.011
E1BSR9	RBX1	Ring-box 1	0.787	0.049
F1NRK3	RPP38	Ribonuclease P/MRP subunit p38	0.792	0.013
F1NZ92	DNAH3	Dynein, heavy chain	0.798	0.017
F1P4C2	RIPK1	Receptor interacting serine/threonine kinase 1	0.805	0.018
F1P2M3	MTIF3	Translation initiation factor 3	0.810	0.001
E1C4V2	Gga.15193	Zn-finger	0.810	0.003
E1BSI3	ENSGALG00000006435	Ubiquitin-protein ligase	0.814	0.036
F1NW64	TPX2	Microtubule nucleation factor	0.817	0.012
E1C8Q1	CEP164	Centrosomal protein 164	0.821	0.005
E1BV18	CAPSL	Calcyposine like	0.824	<0.001
A0A0A0MQ61	ENSGALG00000016325	Glutathione S-transferase	0.825	0.017
F1NWX7	SEC61B	Transcription factor about chromatin remodeling	0.826	0.023
R4GLJ6	VHL	Phosphotransferase	0.829	0.002
Up-regulation				
F1NF85	PEMT	Phosphatidylethanolamine N-methyltransferase	1.200	0.019
E1C3M0	USP45	Ubiquitin specific peptidase 45	1.213	0.006
F1NPJ3	CCDC127	Translation initiation factor 2	1.215	0.036
E1BVP5	ASPA	Aspartoacylase	1.216	0.036
F1P2G6	PIGT	Phosphatidylinositol glycan anchor biosynthesis	1.220	0.036
R4GGH1	ENSGALG00000028833	NAD-dependent aldehyde dehydrogenases	1.223	0.029
E1C0T3	PDZRN3	PDZ domain containing ring finger 3	1.225	0.013
F1NST0	DHX58	ERCC4-like helicases	1.228	0.027
E6N1V0	LAO	Amine oxidase	1.229	0.008
P07031	ACYP2	Acylphosphatase-2	1.235	0.006
Q5ZLB2	ARL6IP1	Phosphoribosylaminoimidazole carboxylase	1.240	0.018
E1C8Q2	ETNPPL	4-aminobutyrate aminotransferase	1.243	0.001
F1ND79	ZNF644	Zn-finger	1.254	0.013
Q5F366	IDUA	Iduronidase	1.259	0.020
F1NS64	MFSD4B	Major facilitator superfamily domain containing 4B	1.270	0.034
F1NK39	ARID4B	histone trimethylation	1.271	0.007
F1P3K7	PCBD2	Pterin-4-alpha-carbinolamine dehydratase 2	1.275	0.027
F1N8L2	TECR	Very-long-chain enoyl-CoA reductase	1.285	0.034
F1NJK5	RRP7A	Ribosomal RNA processing 7 homolog A	1.295	0.006
Q5ZJC0	RCJMB04	Uncharacterized protein	1.303	0.022
E1BUS8	CRYZL1	Quinone oxidoreductase-like protein 1	1.317	0.011
Q9W7G2	SALL3	Spalt protein	1.355	0.017
F1P054	CYP1A5	Cytochrome P450	1.363	0.001
E1C7X0	VRK2	Serine/threonine protein kinase	1.367	0.018
F1NB56	PDE4D	Phosphodiesterase	1.368	0.019
B8XA33	ADAM23	Disintegrin and metalloprotease 23	1.374	0.016
F1P3X6	YTHDF2	Membrane proteins	1.377	0.001
R4GG24	AKR1B1L	Aldo/keto reductases	1.380	0.041
E1BZD6	RIPK4	Ankyrin repeat	1.382	0.013
F1NWX7	MRPL42	Mitochondrial ribosomal protein L42	1.391	0.010
Q5ZL23	APBB1IP	Protein-binding family interacting protein	1.395	0.014
Q5ZJ36	PLK1	Serine/threonine-protein kinase PLK	1.413	0.008
F1P586	SFSWAP	Splicing factor SWAP	1.428	0.002
O73884	PHOSPHO1	Phosphoethanolamine/phosphocholine phosphatase	1.429	0.042
Q5ZLE1	PRPF4B	Permeases	1.438	0.045
G4XJS0	TLR1LB	Toll-like receptor 1 type 2	1.447	0.004
Q5ZHK9	LLPH	Protein LLP homolog	1.456	0.015
F1NW34	KDM8	Lysine demethylase 8	1.459	0.024
E1C4M9	SLC43A2	Solute carrier family 43 member 2	1.473	0.027
F1P5K8	APTX	Aprataxin	1.575	0.009
F1NII4	TXLNG	Taxilin gamma	1.594	0.011
E1C6E5	TSPAN3	Tetraspanin	1.630	0.036
E1C6D5	KDM4A	PHD zinc finger-containing protein	1.910	0.028
P28568	SLC2A3	Solute carrier family 2	2.119	0.020
Q5ZIB9	PRMT7	Protein arginine N-methyltransferase 7	2.169	0.020
R4GJY5	FAM108A1	Protein ABHD17B	2.179	0.049
Q5F4A8	AK6	Adenylate kinase isoenzyme 6	2.246	0.041
G8HUH5	BACT	Beta-actin (Fragment)	2.331	0.006
Q5ZK96	BTBD9	BTB domain containing 9	2.518	0.045
Q5F3Q0	NUP205	Asp-tRNAAsn/Glu-tRNA ^{Gln} amidotransferase	3.167	0.014

[†] Ratio sup/free = Protein expression in folic acid supplement group / that in folic acid free group.

Classification of DEPs

Of the 85 DEPs, 79 DEPs could be assigned to 21 categories using the COG database. As shown in figure 4, the largest group was general function prediction only (26.6%) followed by amino acid transport and metabolism (10.1%), replication, recombination and repair (10.1%), carbohydrate transport and metabolism (7.6%), transcription (7.6%), posttranslational modification, protein turnover, chaperones (6.3%), and signal transduction mechanisms (6.3%). Further, GO classification analysis of DEPs was performed. The number for significant enriched biological process, cell component and molecular function is 162, 29 and 75 respectively (data not shown). In terms of GO term distributions in the second level as presented in figure 5, for biological processes, more than 60% of the notable proteins were respectively related to regulation of cellular process, single-organism process, and metabolic process; for cell component, about 68%, 57% and 35% were correlated with cell, organelle and membrane respectively; for molecular function, about 59% and 50% were respectively associated with binding and catalytic activity.

To characterize the functional consequences of DEPs associated with folic acid intervention in chicken primary hepatocytes, KEGG pathway mapping based on DEPs were also carried out and demonstrated in figure 6. Results indicated that folic acid could significantly affect metabolism of xenobiotics by cytochrome P450, drug metabolism- cytochrome P450, retinol metabolism, steroid hormone biosynthesis, pyruvate metabolism, tryptophan metabolism and glutathione metabolism. It was worth mentioning that some proteins such as ENSGALG00000016325, CYP1A5 and ACYP2 were involved in these pathways. ENSGALG00000016325 could code glutathione S-transferase which was down-regulated in 15 mg/L folic acid group, while CYP1A5 and ACYP2 were up-regulated when compared with the no folic acid group which coded cytochrome P450 and acylphosphatase proteins respectively.

Proteins interaction analysis

The protein-protein interaction networks were performed by the web-tool STRING 10.5 (<https://string-db.org/cgi/input.pl>). The DEPs interactions were shown in figure 7, in which the stronger associations are represented by thicker lines. The results showed that some functional modules were clustered in the network and formed tight connections with DEPs in chicken primary hepatocytes between folic acid free and supplement groups.

Disconnected nodes in the network were hidden. The functional modules were mainly involved in cell cycle (SKP1, RBX1, SKP2, CDC27, CDC20, MAD2L1, CUL1, BUB1B, PLK1, BUB1 and CCNB2), ubiquitin mediated proteolysis (CUL4A, CUL2, TCEB1, RBX1, SKP1, FBXW7, SKP2, CDC27, VHL, CUL1 and CDC20), protein export (SEC63, SEC61A1, SEC61B, SEC61G and SEC61A2), protein processing in endoplasmic reticulum (SEC63, SEC61A1, SEC61B, SEC61G, SEC61A2, SKP1, RBX1 and CUL1), phagosome (SEC61A1, SEC61B, SEC61G, SEC61A2, and ACTB), lysosome (IDUA, GALNS, CLTC and CLTCL1), ribosome biogenesis in eukaryotes (LOC425215, RRP7A and RPP38), TGF beta signaling pathway (SKP1, RBX1 and CUL1) and fatty acid biosynthesis (FASN and ENSGALG00000005439).

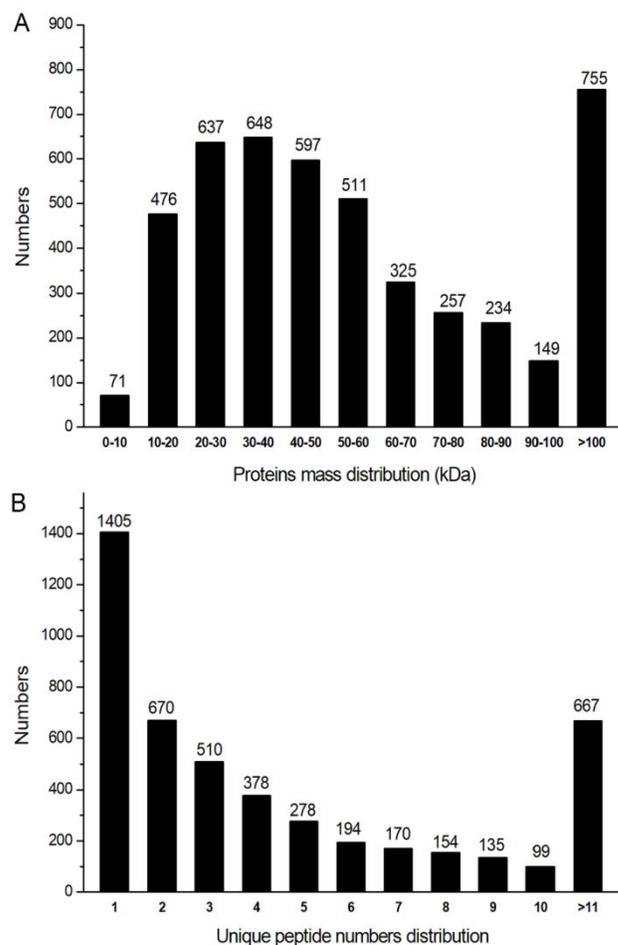


Figure 3. Basic information of iTRAQ identification. **A:** Different molecular weights distribution of proteins identified among samples. **B:** The number of unique peptides that identified proteins in the current study.

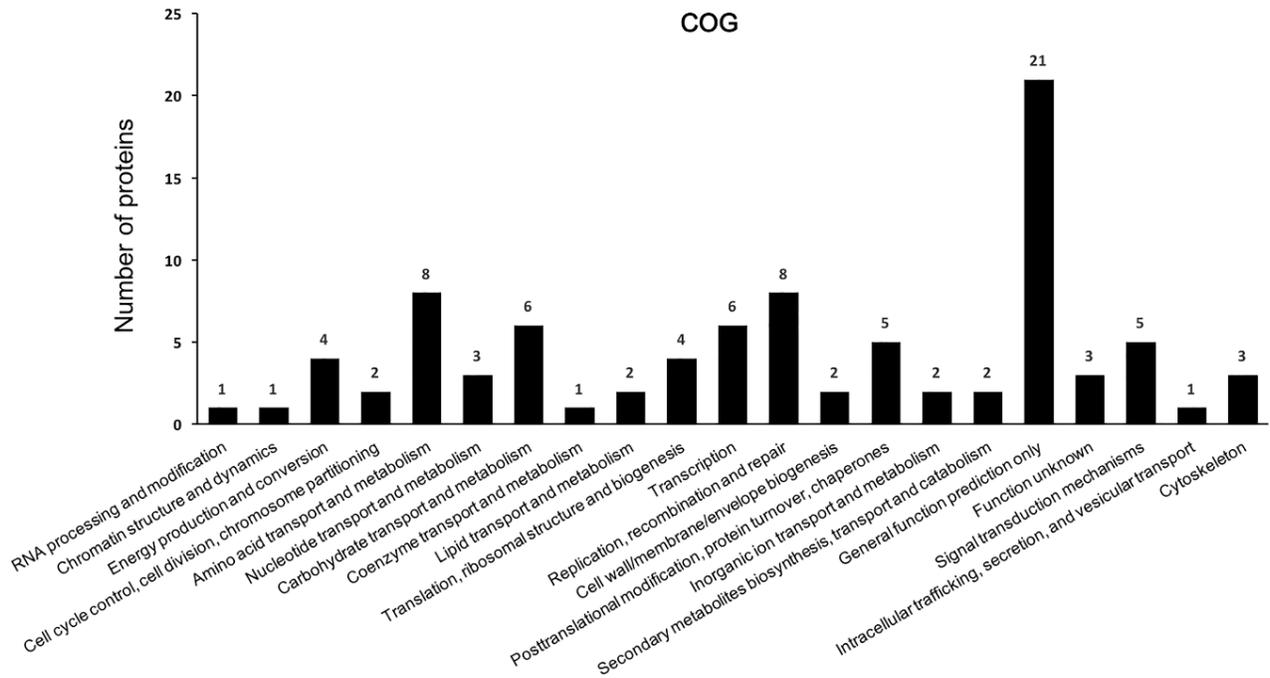


Figure 4. Clusters of Orthologous Groups (COG) of proteins classification of DEPs between folic acid free and supplement groups. The Y-axis is the numbers of DEPs annotated to the category.

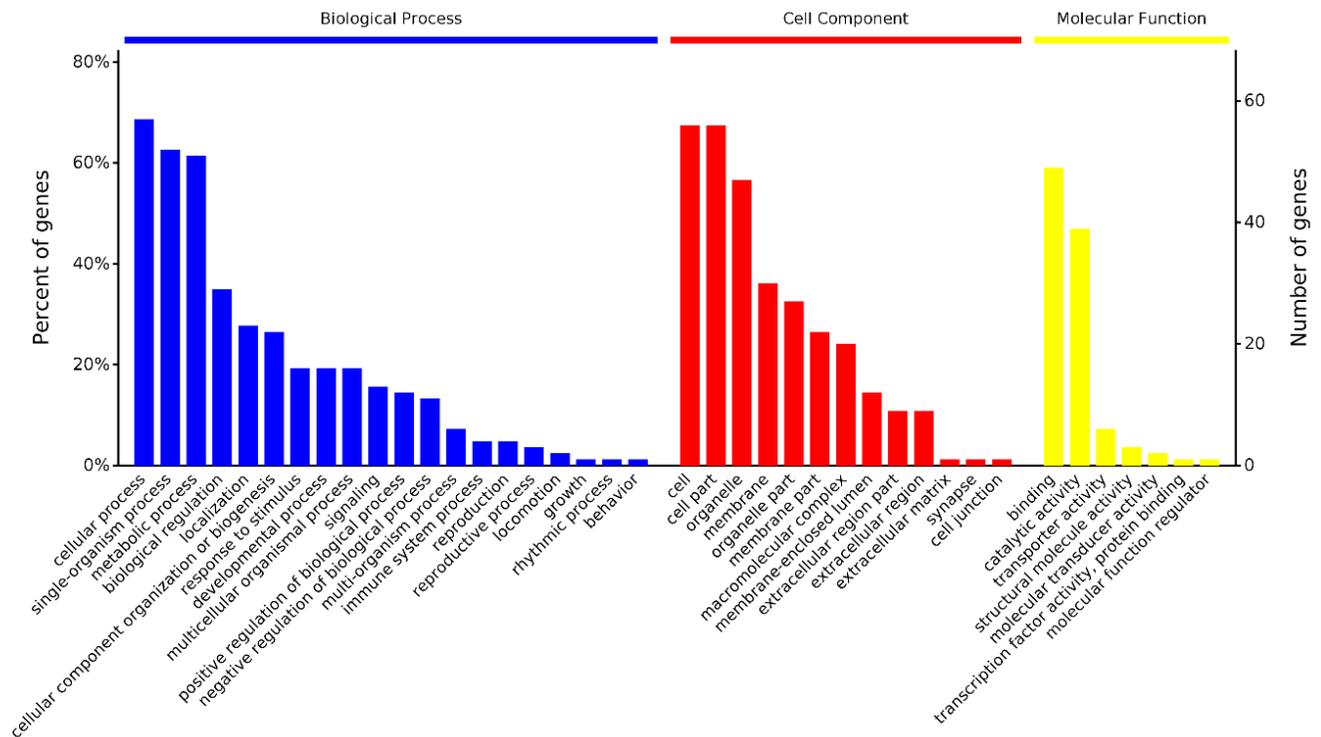


Figure 5. Functional classification of differential proteins by Gene Ontology analysis including biological process, cellular component, and molecular function. All data are presented based on GO second-level terms. The Y-axis is on behalf of the numbers of DEPs annotated to the corresponding category.

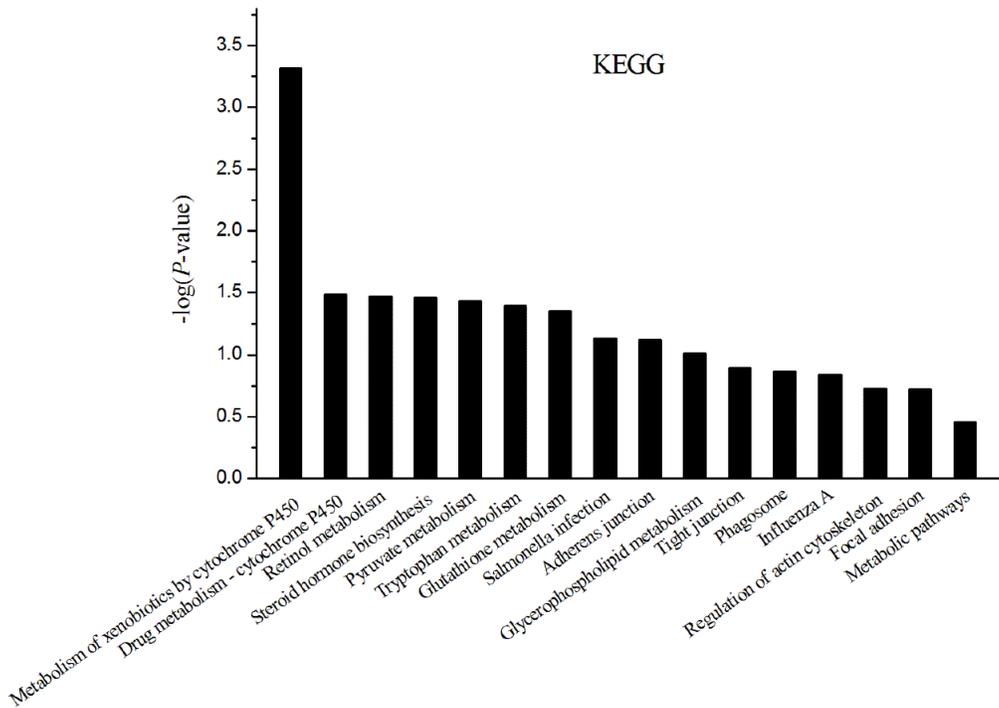


Figure 6. Distribution of enriched KEGG pathway according to DEPs between folic acid free and supplement groups.

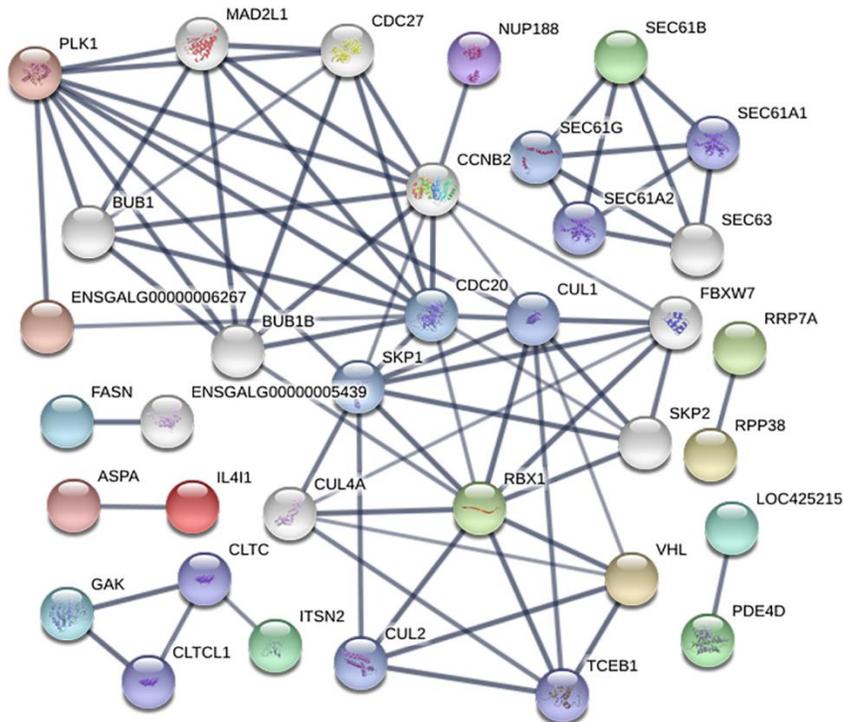


Figure 7. Interaction network analysis of DEPs using STRING software (<http://string-db.org>). In this network, nodes are proteins; lines represent functional associations between proteins. The resulting networks were constructed with confidence scores higher than 0.7. The gray lines between nodes represent functional associations between proteins and the thickness of the lines represents the level of confidence in association reported.

DISCUSSION

In the current study, chicken primary hepatocytes are used as the model to explore folic acid metabolism regulation function through deprivation and supplementation ways. As we all known, folic acid was commonly recognized due to its significance for the development of neurological systems in newborns. Many study have stated that there existed negative correlations between dietary or plasma folic acid and the occurrence rate of some diseases (Sie et al., 2011, Chen et al., 2014, Molloy et al., 2017). But the causal mechanisms that define the role of folic acid in these complex diseases are not established. It's generally accepted that folic acid-mediated 1-carbon metabolism could affect genes expression by DNA methylation and chromatin structure, thereby disturbing metabolic pathways about pathologies (Stover, 2009). Previous study pointed out that folic acid could slow down the aggressiveness of glioma by increasing methylation levels of DNA repeats element and genes related to apoptosis and proliferation (Hervouet et al., 2009). It was reported that low folate intake could result in genomic DNA hypomethylation and improve the risk of colorectal neoplasia, and daily supplementation with 400 mg/day folic acid for 10 weeks resulted in a marginal increase in leucocyte DNA methylation and rectal mucosa DNA methylation in patients with colorectal adenoma (Pufulete et al., 2005).

Considering the role of folic acid in DNA methylation and the fact that DNA methylation is critical to normal genome regulation and development (Crider et al., 2012), we examined genomic 5-methylcytosine (5mC) contents in hepatocytes with folic acid free and supplementation medium. Surprisingly, folic acid didn't increase DNA methylation level in the folic acid addition group. DNA methylation is catalyzed by DNA methyltransferases (DNMTs). DNMT1 is a maintenance methyltransferase and responsible for restoring the methylated status of newly synthesized daughter strands; DNMT3a and DNMT3b are de novo methyltransferases (Li et al., 2016). Consistently, these DNMTs expression were also not affected by folic acid supplementation in the current study. However, intracellular folic acid, 5-Me-THF and SAM concentrations were higher in culture medium with folic acid supplemented when compared with folic acid free group. These results may be illogicality taken together, but the relationship between folic acid and DNA methylation is complex. DNA methylation also involved in the participation of other substances such as choline,

betaine and other B vitamins (Niculescu and Zeisel, 2002). On the other hand, SAM could inhibit MTHFR activity, which provides 5-Me-THF by catalyzing a unidirectional reaction (Smith et al., 2013). But other review also suggested that there was no correlation between global DNA methylation and folate status (Crider et al., 2012).

In addition, there was no difference about cell viability, albumin and lactic dehydrogenase concentration in culture medium between folic acid free and addition groups (data not shown), which suggested that the dosage of folic acid used in the study was reasonable and non-toxic for cells growth. Hence, proteomic analysis was further employed to assess folic acid metabolism regulation function in primary chicken hepatocytes. We found folic acid changed some metabolic pathways enriched by 85 DEPs including cytochrome P450 metabolism, retinol metabolism, steroid hormone biosynthesis, pyruvate metabolism, tryptophan metabolism and glutathione metabolism. Cytochrome P450 was reported to be involved in oxidation-reduction reactions (Meunier, et al., 2004), and up-regulated in the current study indicating that folic acid improved antioxidant ability. ENSGALG00000016325 which coded glutathione S-transferase (GSTs) was also contained in the pathway of cytochrome P450 metabolism, and was down-regulated in folic acid addition group. GSTs are the ubiquitous enzymes that play a key role in cellular detoxification (Jain et al., 2010), and its lower protein abundance suggested that folic acid seemed to be protective for hepatocytes. Folic acid, as an antioxidant (Gliszczynskaswiglo, 2007), has good therapeutic effects on hypoxia-induced inflammatory response by decreasing ROS activity (Ma et al., 2018).

Besides, retinol metabolism, steroid hormone biosynthesis, pyruvate metabolism, and tryptophan metabolism were also enriched. These could be contained amino acid and carbohydrate metabolism as COG analysis that amino acid or carbohydrate transport and metabolism were clustered in relative high proportion. However, how does folic acid affect these metabolism change? It is interesting to note that arginine N-methyltransferase 7 (PRMT7) and ARID4B were up-regulated proteins by folic acid addition based on proteomics though no evidence was found about DNA methylation. PRMT7 has been implicated in roles of transcriptional regulation, DNA damage repair, RNA splicing, cell differentiation, metastasis and epigenetic regulation by transferring methyl groups to arginine residues on protein substrates (Feng et al., 2013). Biological process analysis of GO has

suggested that ARID4B was associated with histone H3K9 and H4K20 trimethylation which were all related to nucleosome and chromatin structure (Xu et al., 2008, Hahn et al., 2011). These results indicated that folic acid might take part in metabolism regulation by histone methylation which contributed to transcription and post-transcriptional modification. And posttranslational modification, protein turnover, chaperones and transcription were gathered by COG analysis based on DEPs. Li et al. (2016a) has reported that folic acid increased H3K9 methylation of IL-6 promoter. Therefore, we speculated that folic acid might regulate hepatocellular metabolism via the histone methylation manner rather than DNA methylation in the present study.

CONCLUSION

In conclusion, the present proteomic analysis found 85 differential expressed proteins in primary chicken hepatocytes with folic acid free and supplementation medium. The pathways of those altered proteins are related to amino acid and carbohydrate metabolism, and oxidation resistance. Folic acid regulated these metabolisms more likely by histone methylation rather than DNA methylation. These results indicated that proteomics with bioinformatics analysis is a good starting point for understanding regulation function of some substances. A deep and broad understanding of the DEPs identified is ongoing to make clear their specific role. Our findings might provide comprehensive protein expression information that can facilitate the understanding of folic acid regulation function in hepatic metabolism.

DECLARATIONS

Competing interests

The authors declare that they have no competing interests.

Author's contributions

XJY and YLL designed the research; JFZ, FYW, JHZ and YLL performed the research and analysed the data; YLL wrote the manuscript; XY and XJY have taken part in the revision of the manuscript. All authors read and approved the final version of the manuscript.

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