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# 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-κB, IL-1β 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/EBPα promoter, thereby reducing FAS and PPARγ 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 methylenetetra-hydrofolate 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 the10% 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-μm 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$^{o}$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/$10^6$ 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 $2^{-\Delta\Delta\ Ct}$ 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 ℃ 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.

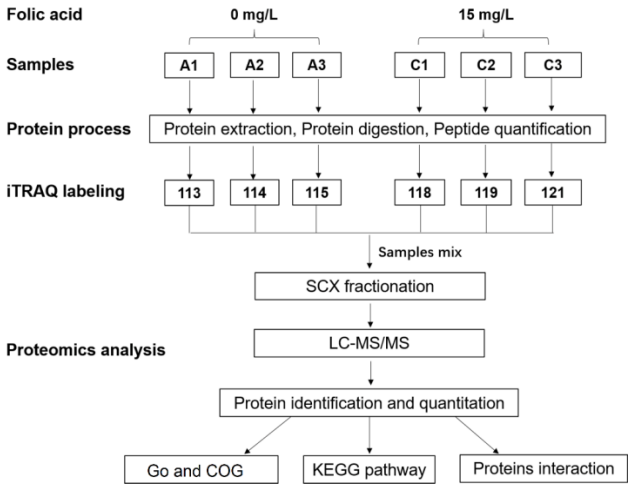


**Figure 1.** Experimental design and schematic diagram of proteomics analysis in the study.

**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<br>Reverse: AAATAAAGCCATGCCAATCTCGTC | 113 | Liu et al. (2016) |
| DNMT1 | NM206952 | Forwards: ACAGCCTTCGCCGATTACA<br>Reverse: CTCTCCACCTGCTCCACCAC | 81 | Liu et al. (2016) |
| DNMT3A | NM001024832 | Forwards: CAACAACCACGACCAGGAGT<br>Reverse: ACCATGCCCACAGTGATAGAGT | 84 | Liu et al. (2016) |
| DNMT3B | NM001024828 | Forwards: CCCGTTATGATCGACGCTAT<br>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 $\geqslant 1.20$ or $\leqslant 0.83$ 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 NWAFAC1008).

## 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.

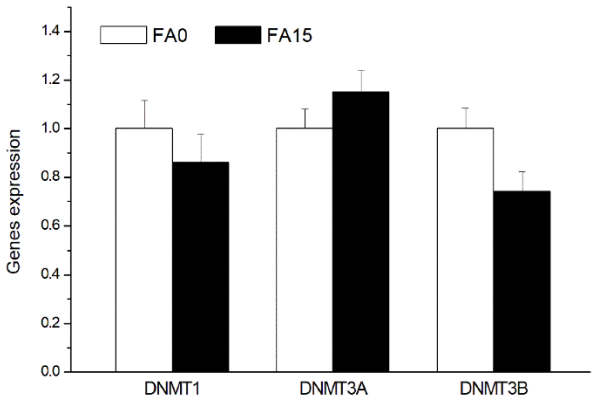


**Figure 2.** Gene expression of DNA Methyltransferases (DNMT1, DNMT3A, DNMT3B) in layer chicken hepatocytes between groups with folic acid free and supplement medium. Data were presented as means ± SEM (n=6).

**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 | Calcyphosine 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 |
| F1NWY7 | 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 |
| F1NI14 | 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-tRNAGln amidotransferase | 3.167 | 0.014 |

[1] 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 hided. 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).

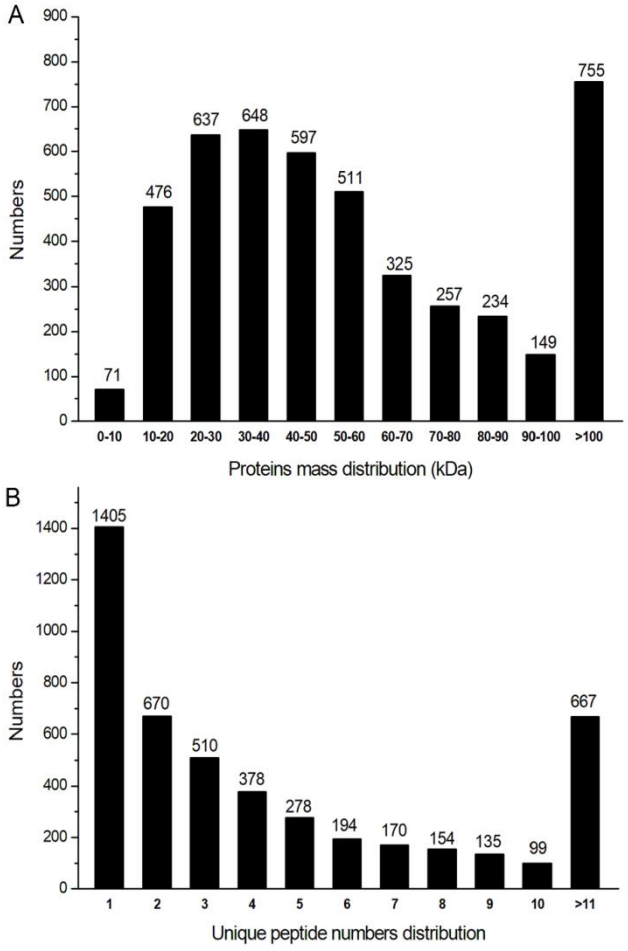


**Figure 3.** Basic information of iTRAQ identification. **A:** Different molecular weights distribution of proteins identified among samples. **B:** The number of unique peptides that identified proteins in the current study.

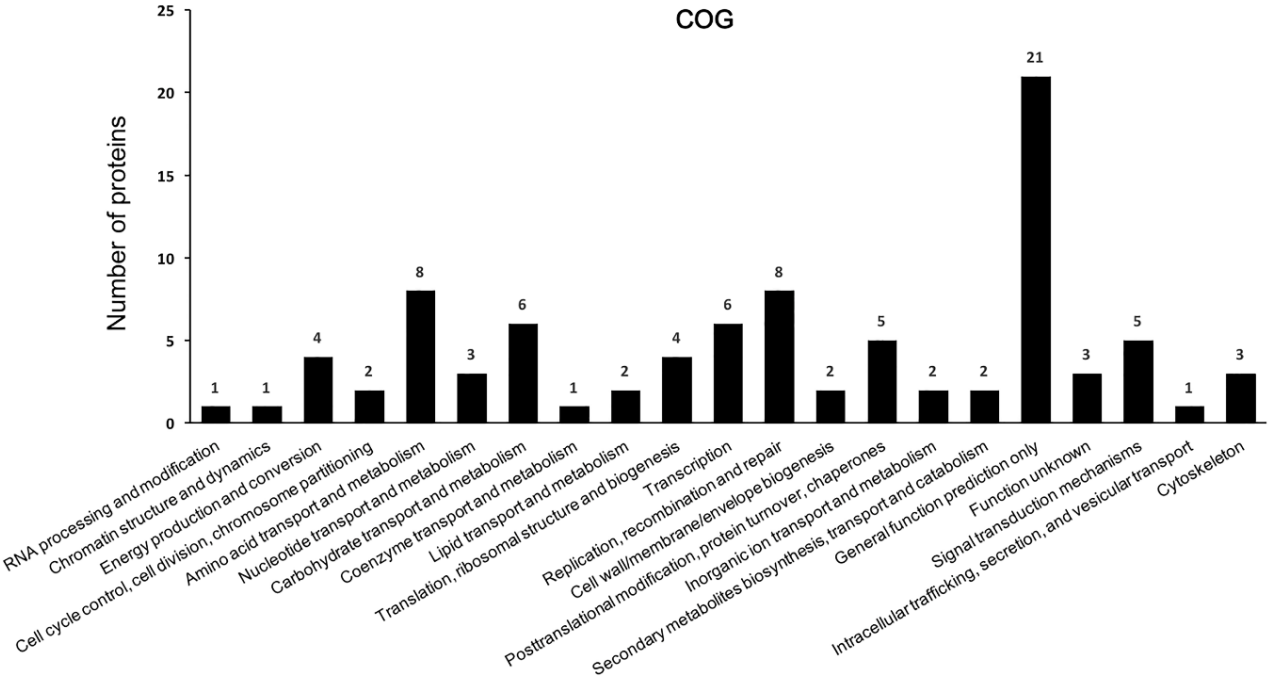


**Figure 4.** Clusters of Orthologous Groups (COG) of proteins classification of DEPs between folic acid free and supplement groups. The Y-axis is the numbers of DEPs annotated to the category.

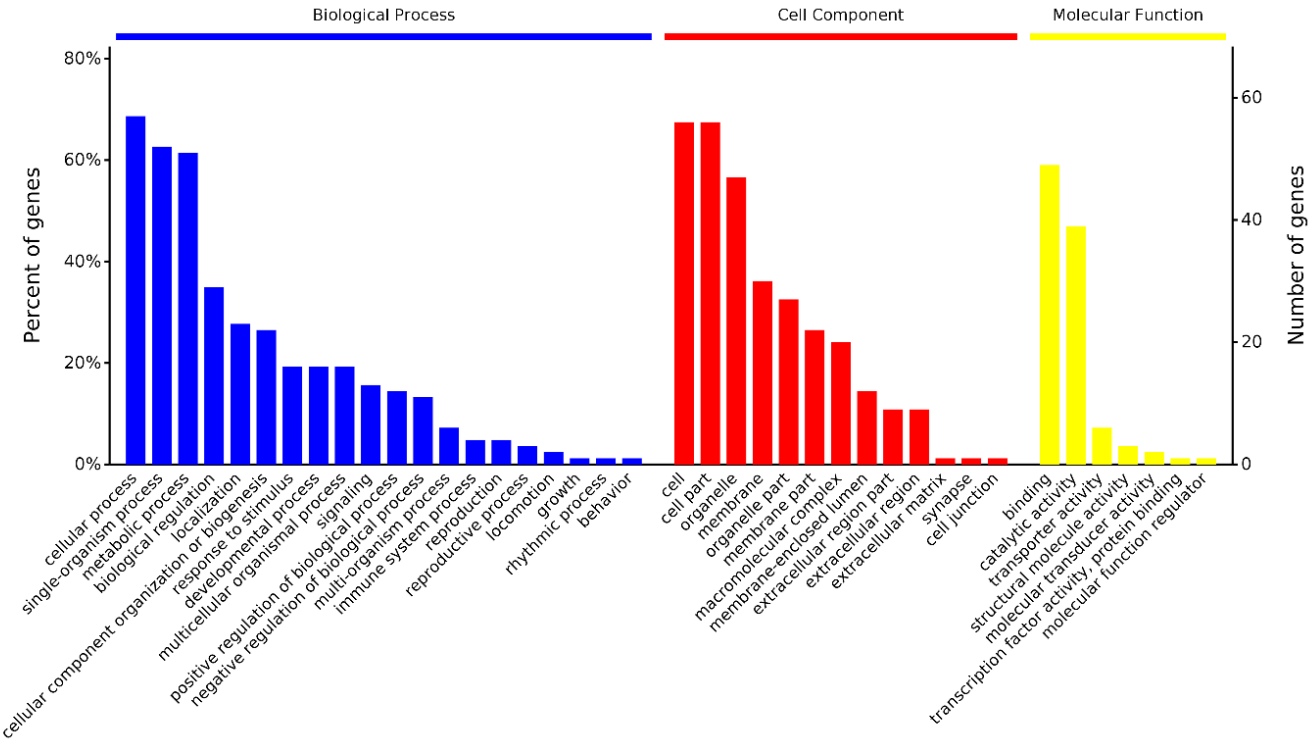


**Figure 5.** Functional classification of differential proteins by Gene Ontology analysis including biological process, cellular component, and molecular function. All data are presented based on GO second-level terms. The Y-axis is on behalf of the numbers of DEPs annotated to the corresponding category.

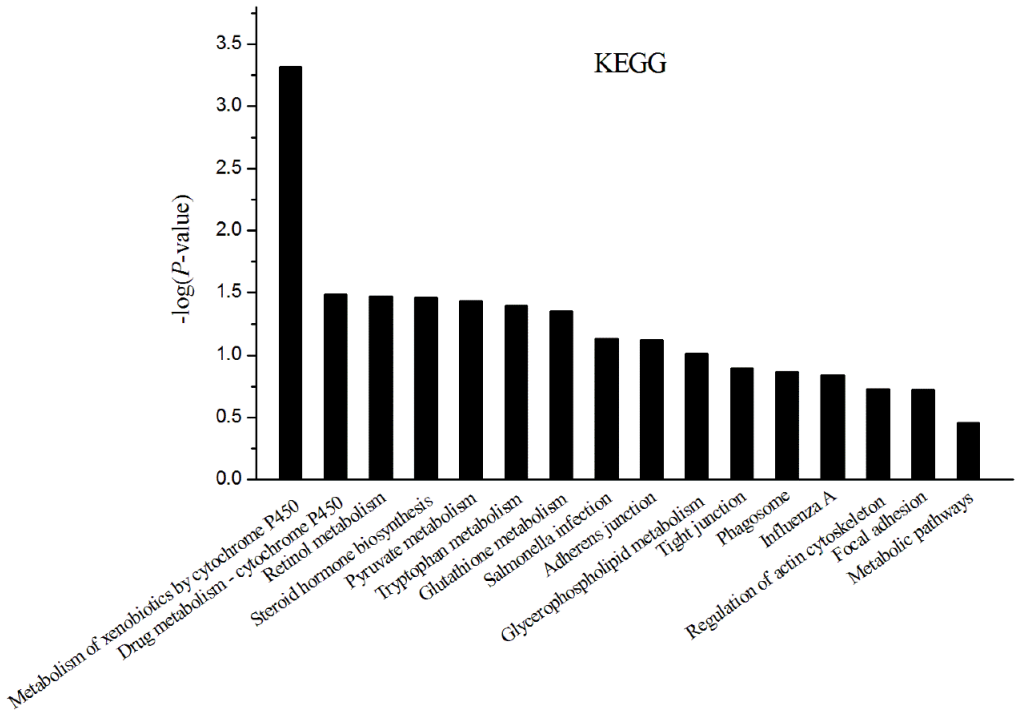


**Figure 6.** Distribution of enriched KEGG pathway according to DEPs between folic acid free and supplement groups.

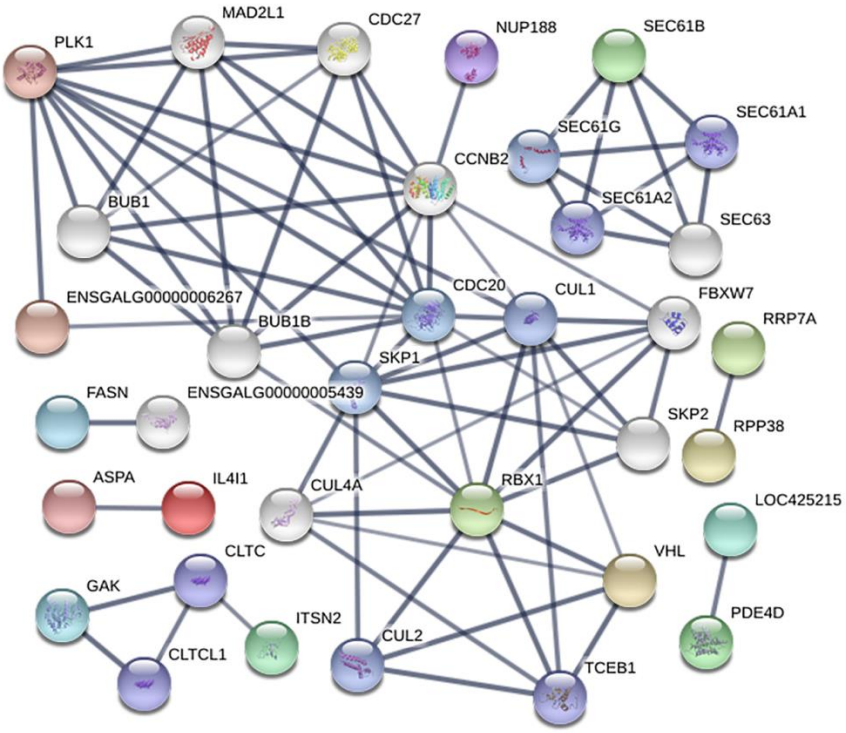


**Figure 7.** Interaction network analysis of DEPs using STRING software (http://string-db.org). In this network, nodes are proteins; lines represent functional associations between proteins. The resulting networks were constructed with confidence scores higher than 0.7. The gray lines between bodes 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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