miR-375 upregulates lipid metabolism and inhibits cell proliferation involved in chicken fatty liver formation and inheritance via targeting recombination signal binding protein for immunoglobulin kappa J region (RBPJ)

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ABSTRACT Poultry is susceptible to fatty liver which lead to decrease egg production and increase mortality. But the potential molecular mechanisms remain largely unclear. In the current study, in combination with transcriptome sequencing and miRNA sequencing data analysis from F1 generation of the normal liver and fatty liver tissues, the differentially expressed miR-375 and its target gene RBPJ were screened and verified. The expression levels of miR-375 and RBPJ gene in the liver between control and fatty liver groups of F0-F3 generation for Jingxing-Huang (JXH) chicken are different significantly (P < 0.05 or P < 0.01). And downregulated RBPJ expression can promote TG content and lipid droplets in primary hepatocytes cultured in vitro (P < 0.01). Cell proliferation-related genes, including PMP22, IGF-1, IGF-2, and IGFBP-5, increased or decreased significantly after overexpression or knockdown RBPJ (P < 0.05 or P < 0.01), respectively. This study uniquely revealed that miR-375 induced lipid synthesis and inhibited cell proliferation may partly due to regulation of RBPJ expression, thereby involving in fatty liver formation and inheritance in chicken. The results could be useful in identifying candidate genes and revealing the pathogenesis of fatty liver that may be used for disease-resistance selective breeding in chicken.

Key words: chicken, fatty liver, miR-375, RBPJ, lipid metabolism

INTRODUCTION

Unlike mammals, the liver is the main organ for lipid synthesis in poultry. Abnormal liver fatty acid oxidation, lipoprotein transport, or fat synthesis will cause excessive accumulation of fat in the liver, thereby inducing fatty liver (Burdge and Lillycrop, 2014). Fatty liver is a metabolic disease (Lewis and Mohanty, 2010), which is more common in laying hens(Zhang et al., 2018). Although the fatality rate induced by fatty liver is low (Clark and Diehl, 2003), but it affects the chicken’s performance such as egg production, fertilization rate, and hatchability rate (Zhang et al., 2018).

Non-coding RNAs including miRNA are important transcription regulators in physiological and pathological processes (Sayed and Abdellatif, 2011; Hulsmans et al., 2011; Huang et al., 2014). More and more researches have been focused on the role of miRNA in the development of related diseases. Recent study demonstrates that miRNA has an important effect on liver lipid metabolism in poultry (Marques-Rocha et al., 2015). Some studies have shown that in the development of nonalcoholic fatty liver, some tissue specific miRNA may play a key role in regulating the post transcriptional regulation of the key process of liver injury related to this disease (Panera et al., 2014). A study of 47 patients with nonalcoholic fatty liver showed that the expression levels of miR-122, miR-192, and miR-375 are significantly upregulated, and the upregulation levels are related to the severity of the disease (Pirola et al., 2015). Auguet et al. (2016) found that the expression of miR-33 in liver tissue and blood of nonalcoholic fatty liver disease (NAFLD) patients is also upregulated, and upregulated miR-33 can prevent obesity and liver steatosis in mice (Price et al., 2018). The expression of miR-34a is related to their susceptibility to NAFLD based on the study for mice fed a high-fat diet (Pogribny et al., 2010). Inhibiting the expression of miR-34a in obese...
mice can change the expression of related metabolic genes and metabolic results (Fu et al., 2012). Studies for the NAFLD patients found that the expression of miR-34a in the serum is upregulated (Cermelli et al., 2011; Yamada et al., 2013; Liu et al., 2016; Salvoza et al., 2016), and the upregulated level is related to the severity of the disease (Castro et al., 2013). In short, miRNAs could affect liver lipid metabolism and the occurrence of nonalcoholic fatty liver disease by regulating target genes in related pathways (Butler, 1976; Burdge and Lillycrop, 2014; Ferreira et al., 2014; Huang and Hu, 2015; Rozenboim et al., 2016; Ansen-Wilson and Lipin- ski, 2017; McCarty, 2017).

In the present study, the F1 generation from the chicken fatty liver model induced by high-fat and low-protein diets was used (Zhang et al., 2018). In combination with transcriptome and miRNA sequencing data analysis for F1 generation of the control and fatty liver groups, the differential expression miRNAs and targeted genes were screened, and verified between control and fatty liver group of F0–F3 generation for Jingxing-Huang (JXH) chicken. At the same time, the regulation to lipid metabolism for the differential expression miRNA and its targeted gene was also studied in primary hepatocytes cultured in vitro. This study will provide a valuable resource to reveal the formation and inheritance of fatty liver. The results could be useful in identifying candidate genes for fatty liver that could be used for disease-resistance selective breeding in chicken.

MATERIALS AND METHODS

Ethics Approval

All the animal experiments were conducted in accordance with the guidelines for experimental animals established by the Ministry of Science and Technology, Beijing, China. Ethical approval on animal survival was given by the animal ethics committee of the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing, China (IASCAAS-AE-03).

Sample Collection

The JXH chickens of F0 generation for the fatty liver were induced by a high-fat diet, but the chickens for the control group were fed basal diet, as reported by Zhang (Zhang et al., 2018). The offspring of the F1, F2, and F3 generations were produced by male JXH chickens without (control group) or with (fatty liver group) fatty liver. All the F1, F2, and F3 generations in two groups were fed a basal diet. The time for liver collection from F0 to F3 generation was at the end of the 36th wk. And the liver tissues for miRNA sequencing of F1 generation including the control group and fatty liver group were the same as Xiaodong Tan et al. (2021).

miRNA Sequencing and DE miRNA Analysis

After the sample passed the test, the Small RNA Sample Pre-Kit was used to construct the library (Novogene, Beijing). Using the special structures of the 3′ and 5′ ends of small RNA (the 5′ end has a complete phosphate group and the 3′ end has a hydroxyl group), with total RNA as the starting sample, the 2 ends of small RNA were directly spliced, and then the reverse transcription was used to synthesize cDNA. After PCR amplification, the target DNA fragment was separated by PAGE gel electrophoresis, and the cDNA library was recovered by cutting gel. The libraries were constructed and sequenced on the Illumina HiSeq2000 platform (Illumina, San Diego, CA) in a single lane.

When the sample has biological duplication, the default screening condition of differential miRNA is: \( P < 0.05 \). In order to control the false positive rate, we need q-value combined with foldchange to screen. The default screening condition is: q-value < 0.01 & \( | \log_2 \text{(fold-change)} | > 1 \).

KEGG Enrichment Analysis

KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.kegg.jp/) was a database of whole genome and metabolic pathway. Each pathway in KEGG was enriched through hypergeometric test, and the P-value was corrected by multiple tests with q < 0.05 as the threshold. The pathways satisfying this condition were defined as those significantly enriched from target genes of differentially expressed miRNAs. Here, KEGG enrichment was measured by rich factor, Q-value and the number of genes enriched in this pathway. Rich factor refers to the ratio of the number for differentially expressed genes to the total number of annotated genes in the pathway entry. The larger the rich factor, the greater the degree of enrichment. Q-value is the P-value corrected by multiple hypothesis tests. The value range of Q-value is [0,1]. The closer to zero, the more significant the enrichment. And the first 30 enriched path entries were selected to display in this figure. If the number of enriched path entries is less than 30, all of them will be displayed.

GO Enrichment Analysis

GO (Gene Ontology, http://geneontology.org/) was used for function annotation of target genes of differentially expressed miRNA. According to the number of differentially expressed genes contained in each GO term, hypergeometric test was used to find out the significantly enriched GO term compared with the whole genome background. GO term with q < 0.05 was selected as the highly enriched GO term for target genes. The main biological functions of targets of differentially expressed miRNAs could be determined by GO enrichment analysis. This method is based on Wallenius non-central hyper-geometric distribution. Compared with the general Hyper-geometric distribution, the
distribution is characterized by that the probability of extracting individuals from a certain category is different from that of extracting an individual from a certain category. The difference of this probability is obtained by estimating the preference of gene length, so that the probability of GO term being enriched by candidate target gene can be calculated more accurately.

**Target Prediction and Luciferase Assay**

Base on the miRNA sequencing result, significantly differentially expressed (DE) miRNAs were selected for subsequent studies. In addition, combined with GO and KEGG analysis, we focused on the role of miR-375 in fatty liver. As well as, we chose the target genes of miR-375 via predicting software TargetScan (http://www.targetscan.org/vert_72/). Importantly, in our previous research, we found RBPJ is a key DE gene in inherited phenotype of acquired fatty liver in chicken. In addition, RBPJ may be an important gene related to chicken fatty liver (Tan et al., 2021). Therefore, we speculate that miR-375 may regulate fatty liver through RBPJ.

Then, 3’UTR fragment (wild type and mutation) of RBPJ were cloned into luciferase assay vectors, respectively. miR-375 mimic co-transfected with wild-RBPJ-3’UTR and mutate-RBPJ-3’UTR into cells. The luciferase assay refers to Dual Luciferase Reporter Gene Assay Kit (Roche, Basel, Switzerland).

**qRT-PCR**

β-actin and U6 were used as house-keeping gene for mRNA and miRNA detection, respectively, and the gene expression levels of miR-375, RBPJ, PMP22, IGF-1, IGF-2, and IGFBP-5 were detected. Primers used for qRT-PCR were designed using the Primer 5.0 software. All primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and listed in Table 1. Trizol reagent (ThermoFisher SCIENTIFIC, Shanghai) was used to extract total RNA from liver tissues and primary hepatocytes cultured in vitro. The mRNA expression levels were detected in triplicate using the SYBR Green I dye. The qRT-PCR mixture consisted of cDNA (1 μL), PCR-Master Mix (5 μL), PCR-F-Primer (0.5 μL), PCR-R-Primer (0.5 μL), and RNase-free H2O (3 μL) in a total volume of 10 μL. The reaction conditions were as follows: 95°C denaturation for 1 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s, and extension at 72°C for 15 s. The data obtained from the Eppendorf qRT-PCR instrument (Hamburg, Germany) were analyzed using the 2^-△△CT method.

**Primary Hepatocytes Isolated and Cultured In Vitro**

After sterilizing the 15-d embryonic embryos, opened the abdominal cavity and isolated the liver, soak them in PBS containing double antibodies for 3 times, and put them into 1 mL 0.25% Collagenase Type II lyophilized (ThermoFisher SCIENTIFIC, 17101015), then ground the liver tissue mass until there was no obvious tissue mass, digested them at 37°C. 5% CO2 incubator for 25 min, then added 0.5 mL trypsin for 10 min. Filter the cell suspension through a 70-µm cell sieve, added DMEM/F-12 (DF-12, Biological Industries) with Fetal Bovine Serum (FBS, Chenghe) to terminate the digestion, centrifuge at 800 g/min for 5 min, discarded the supernatant, then seeded into 6-well plates with the cell density of 10^6·mL^-1, incubated at 37°C.

**miR-375 mimic NC, miR-375 mimic, miR-375 Inhibitor Transfection**

Primary hepatocytes cells were plated two days before transfection at density of approximately 1 × 10^6 cells/

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**Table 1.** The genes and related primers for qRT-PCR.

| Gene       | Primer sequence (5’-3’) | Annealing temperature (°C) | Product size (bp) |
|------------|-------------------------|-----------------------------|-------------------|
| U6         | RT: CGCTTCACAGAATTTGCGTCAT | /                           | /                 |
| miR-375    | RT:CTCAACTGGTGCCGAGAATTCAGTTGAGTAA | 60                          | 72                |
| miR-375    | F:ACACTCCAGCCTGTTTCTCGGCTC | R:CTCAAGTCTTGAGTCGGCAA       | 60                | 80                |
| miR-375    | R:CTCAATGTTGAGTCGGCAA | /                           | /                 |
| U6         | F:GCCTTCCGACACATATACTAATAAT | 60                          | 60                |
| miR-375    | R:CGCTTCACAAATTGCGTCAT | /                           | /                 |
| RBPJ       | F: CATAACACCGAAACCGAGG | T: TGTTGGATTGGGGCTGACAG     | 73                | 60                |
| β-actin    | R: TGCAGAGGTGTTGACATCA | /                           | /                 |
| miR-375    | F:GAGAAATTTGCGGGTGACATCA | /                           | /                 |
| PMP22      | R: CCTGAACCTCTCATGGGCA   | /                           | /                 |
| IGF-1      | F:GCACTTCTACGTTGCGCAATTCAGTTGAGTAA | 60                          | 152               |
| IGF-2      | R:GCCCTTCAGGTGCAACACACTC | /                           | /                 |
| IGFBP-5    | F: GAGAAATTTGCGGGTGACATCA | /                           | /                 |

Abbreviations: IGF-1, insulin-like growth factor-1; IGF-2, insulin-like growth factor-2; IGFBP-5, insulin-like growth factor binding protein-5; PMP22, peripheral myelin protein 22; RBPJ, recombination signal binding protein for immunoglobulin kappa J region.
well into 6-well culture plates. miR-375 mimic NC and miR-375 inhibitor NC served as the control for miR-375 mimic and miR-375 inhibitor, respectively. All transfection sequences were transfected at a final concentration of 150 nM by use of Lipofectamine 2000 (ThermoFisher SCIENIFIC, Waltham, MA). After 48 h transfection, the cells were used to extract total RNA. Each transfection was performed in triplicate, and three independent experiments were conducted.

**siRBPJ and RBPJ Overexpression Plasmid Transfection**

After the primary hepatocytes isolated in vitro were cultured for 48 h, the medium was changed to DF-12, and then siRBPJ and RBPJ plasmids (the final concentration is 5 μg) were added with 3 μL Lipofectamine 2000 (ThermoFisher SCIENIFIC). Then the cells were cultured 48 h for subsequent detection.

**Triglyceride Content Detection**

After hepatocytes were cultured and transfected with miR-375 mimic NC, miR-375 mimic, miR-375 inhibitor, siRBPJ, and RBPJ overexpression plasmids for 48 h, triglyceride (TG) content was measured by triglyceride (TG) enzymatic method Test kit E1013 (APPLYGEN, Beijing) separately.

**Oil Red O Staining**

To explore the role of miR-375 and RBPJ on lipid formation, after miR-375 mimic NC, miR-375 mimic, miR-375 inhibitor, siRBPJ, and RBPJ overexpression plasmids transfection for 48 h, the hepatocytes were stained with Oil Red O detection kit (Solarbio LIFE SCIENCE, Beijing).

**Data Analysis**

The data were presented as the means ± S.D. All data represent 3 independent experiments. Two-sided Student’s t test was used to calculate P-values. Significance is displayed as *P ≤ 0.05 or **P ≤ 0.01.

**RESULTS**

**Different Expressed miRNA Analysis in Liver Tissue**

After removal of low-quality and adaptor containing reads, the clean reads were aligned to the swine reference sequences by Bowtie (setting allows one mismatch) and the distribution and coverage of reads on reference genes were calculated (Table 2). We identified that 26 miRNAs were significantly differentially expressed (DE) (Table 3), in which 14 miRNAs (gga-miR-375, gga-miR-1a-3p and gga-miR-2188-3p et al.) were upregulated and 12 miRNAs (gga-miR-222a, gga-miR-221-3p, and gga-miR-155 et al.) were downregulated in fatty liver group, respectively. Among these, miR-375 was chosen for further analysis based on having the most significant fold change and highly expressed DE one in fatty liver group.

Next, target genes of miR-375 were used to performed in KEGG pathway enrichment and GO analysis to unravel the miRNA-mediated biological processes associated with development of fatty liver. In the results of KEGG enrichment analysis, the target genes targeted by miR-375 are involved in many related pathways. For example, the signaling pathways related to cellular processes mainly include regulation of Notch signaling, MAPK signaling, and glycerolipid metabolism pathways include fatty acid elongation, Citrate cycle (TCA cycle) and so on (Figure 1).

In GO enrichment analysis, the biological process involves in pathways that the target gene targeted by miR-375 are mainly participate in positive regulation of cell proliferation and cell activation. Cellular component participates in cytoplasm, intracellular part. And the molecular function related to fatty-acyl-COA binding and carbohydrate derivative binding pathways (Figure 2).

**Target Gene Prediction and Validation of miR-375**

In order to prove the targeting relationship between miR-375 and gene RBPJ, the binding site was analyzed (Figure 3A) with the software TargetScan (http://www.targetscan.org/vert_72/), and result indicated that miR-375 can target binding RBPJ. To investigate the potential targeting of RBPJ by miR-375, luciferase activity assay was used. The RBPJ-3’UTR-luciferase reporter plasmid and reporter plasmid with mutated 3’UTR sequence (Mut) were constructed (Figure 3B). The result shown that miR-375 inhibit the luciferase activity of the RBPJ-3’UTR reporter (P < 0.01), instead of the Mut reporter with the altered sequence (P > 0.05; Figure 3B). Together, these data suggest that miR-375 may affect the expression of RBPJ by targeting the RBPJ-3’UTR.

In order to prove the regulation of miR-375 on its targeted genes, the RBPJ express levels were detected after transfecting miR-375 mimic NC, miR-375 mimic, miR-375 inhibitor NC, and miR-375 inhibitor for 48 h in primary hepatocytes cultured in vitro. The results showed that compared with NC group, the expression of miR-375 was significantly upregulated after miR-375 mimic

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**Table 2. The quality of the data.**

| Sample | Reads  | Error rate | Q20   | Q30   | GC content |
|--------|--------|------------|-------|-------|------------|
| C_m1R1 | 14438306 | 0.01%      | 99.29% | 98.09% | 48.97%     |
| C_m1R2 | 17474283 | 0.01%      | 99.60% | 98.80% | 49.31%     |
| C_m1R3 | 15108013 | 0.01%      | 99.57% | 98.82% | 48.62%     |
| C_m1R4 | 15384176 | 0.01%      | 99.74% | 99.30% | 50.15%     |
| FL_m1R1 | 13501813 | 0.01%      | 99.61% | 98.85% | 49.57%     |
| FL_m1R2 | 14973000 | 0.01%      | 99.57% | 98.75% | 49.31%     |
| FL_m1R3 | 13145104 | 0.01%      | 99.31% | 98.66% | 50.34%     |
| FL_m1R4 | 13188263 | 0.01%      | 99.58% | 98.86% | 50.65%     |

**Abbreviations:** C, control group; FL, fatty liver group.
| miRNA         | FL.miR1 | FL.miR2 | FL.miR3 | FL.miR4 | FL.miR | C.miR1 | C.miR2 | C.miR3 | C.miR4 | C.miR |
|--------------|---------|---------|---------|---------|--------|--------|--------|--------|--------|--------|
| **Upregulated** |         |         |         |         |        |        |        |        |        |        |
| gga-miR-375  | 1,881   | 6,773   | 3,012   | 7,679   | 4,959  | 1,313  | 863    | 1,342  | 1,510  | 1,454  |
| gga-miR-1a-3p| 948     | 4178    | 7,427   | 3,525   | 4,115  | 5,802  | 1,278  | 515    | 1,576  | 1,493  |
| gga-miR-2188-3p| 8       | 7       | 6       | 3       | 1      | 4      | 1      | 1      | 2      | 1.529  |
| gga-miR-22-3p | 3,523   | 5,004   | 5,333   | 3,092   | 4,659  | 6,672  | 2,974  | 1,806  | 3,564  | 3,018  |
| gga-miR-219a | 219     | 357     | 425     | 397     | 377    | 698    | 272    | 128    | 241    | 249    |
| gga-miR-219b | 219     | 357     | 425     | 397     | 377    | 698    | 272    | 128    | 241    | 249    |
| gga-miR-140-3p| 874     | 1,224   | 1,133   | 1,108   | 1,194  | 1,366  | 902    | 490    | 1,065  | 809    |
| gga-miR-144-3p| 317     | 96      | 237     | 26      | 217    | 62     | 81     | 50     | 90     | 67     |
| **Downregulated** |       |         |         |         |        |        |        |        |        |        |
| gga-miR-221-3p| 336     | 760     | 632     | 389     | 562    | 3,399  | 1,115  | 534    | 1,891  | 1,272  |
| gga-miR-155  | 40      | 136     | 68      | 86      | 84     | 355    | 133    | 88     | 517    | 217    |
| gga-miR-34c-5p| 1       | 2       | 18      | 5       | 6.81   | 48     | 15     | 48     | 40     | 37.91  |
| gga-miR-221-5p| 123     | 298     | 299     | 130     | 226    | 867    | 371    | 217    | 589    | 403    |
| novel_194    | 1       | 0       | 1       | 0       | 0.66   | 11     | 6      | 1      | 8      | 4.813  |
| gga-miR-130b-5p| 29      | 96      | 30      | 71      | 58.19  | 295    | 123    | 48     | 131    | 110    |
| gga-miR-229b-5p| 0       | 2       | 1       | 3       | 1.456  | 12     | 5      | 2      | 21     | 7.82   |
| gga-miR-223   | 77      | 193     | 186     | 137     | 155    | 439    | 274    | 244    | 297    | 240    |
| gga-miR-222b-5p| 4       | 15      | 7       | 12      | 9.715  | 48     | 48     | 22     | 8      | 28.51  |
| gga-miR-363-5p| 2       | 10      | 6       | 8       | 6.55   | 40     | 10     | 7      | 25     | 15.05  |
| gga-miR-9-5p  | 114     | 291     | 192     | 320     | 240    | 1558   | 211    | 211    | 598    | 435    |

**Abbreviations:** C, control group; FL, fatty liver group.
Figure 1. The histogram of candidate target genes involved KEGG pathway. Histogram of candidate target genes involved KEGG pathway, where the vertical axis represents pathway name, the horizontal axis represents the number of candidate target genes in this pathway.

Figure 2. The histogram of GO enrichment of candidate target genes. The histogram of GO enrichment of candidate target genes. The horizontal axis is GO term of the next level in the three major categories of GO, and the vertical axis is the number of candidate target genes annotated to this term (including sub-terms of this term) and the proportion of the number of candidate target genes in the total number of annotated candidate target genes. The three different classifications represent the three basic classifications of GO term (from left to right, cell components, biological processes, and molecular functions).
transfection ($P < 0.01$) and downregulated after miR-375 inhibitor transfection ($P < 0.05$). In addition, the expression levels of RBPJ have significant downregulated ($P < 0.001$) or upregulated ($P < 0.001$) after miR-375 mimic or miR-375 inhibitor transfection compared with the NC group, respectively (Figures 4A and 4B).

**The Expression Levels of miR-375 and RBPJ in Liver Tissues Among Different Generations**

To further verify the relationship of miR-375 and RBPJ with fatty liver formation and inheritance, the
expressions of miR-375 and RBPJ were detected between the control group and fatty liver group from F0 to F3 generation in liver tissue of Jingxing-Huang (JXH) chickens by qRT-PCR. The results shown that miR-375 have significantly higher expression in fatty liver groups from F0 to F3 generation (P < 0.05, P < 0.01, or P < 0.001) compared with control groups (Figure 5A). But the expression levels of RBPJ have the opposite trend from F0 to F3 generation (P < 0.01 or P < 0.001; Figure 5B). Therefore, we conduct that miR-375 may play important role during fatty liver formation and inheritance depending on RBPJ in chicken.

**The Triglyceride Content and Lipid Droplets Deposition in Primary Hepatocytes Cultured In Vitro**

After transfection of siRBPJ and RBPJ plasmids, the RBPJ expression levels were significantly downregulated (P < 0.01) and upregulated (P < 0.01) respectively compared with NC group in primary hepatocytes cultured in vitro (Figure 6A).

The triglyceride content is significantly upregulated (P < 0.01 or P < 0.05) after siRBPJ or miR-375 mimic transfection. While over-expressed RBPJ or inhibited miR-375, the triglyceride content is significantly downregulated compared with the NC group (P < 0.01; Figures 6B and 6C). In addition, the lipid droplets increased significantly when the expression of RBPJ was knocked down in primary hepatocytes cultured in vitro, while the lipid droplets decreased after the gene RBPJ was overexpressed compared with the NC group. And the lipid droplets in miR-375 mimic or inhibitor transfection group were the same as knocked down or overexpressed RBPJ group (Figure 6D). Therefore, we concluded that miR-375 may regulate triglycerides formation and lipid metabolism by targeting RBPJ gene.

**Cell Proliferation Related Genes Detection in Primary Hepatocytes Cultured In Vitro**

The expression levels of cell proliferation related genes including PMP22, IGF-1, IGF-2, and IGFBP-5 were detected in primary hepatocytes cultured in vitro. The results shown that overexpression or knock-down of RBPJ could upregulate or downregulate the levels of PMP22 (P < 0.05), IGF-1 (P < 0.05), IGF-2 (P < 0.05), and IGFBP-5 (P < 0.05; Figures 7A–7D). Collectively, our findings demonstrate that RBPJ has an impact on hepatocyte proliferation.

**DISCUSSION**

The fatty liver is a metabolic disease caused by lipid metabolism disorder such as lipid absorption, lipid synthesis, β-oxidation, or lipoprotein transport (Adams et al., 2005). Fatty liver disease can further develop into nonalcoholic hepatitis, liver cirrhosis and hepatocellular carcinoma induced by nutritional, environmental, and genetic factors (Friedman et al., 2018), and negative impact on human health and animal production.

Fatty liver disease includes different stages of liver injury, from early steatosis or nonalcoholic fatty liver disease to nonalcoholic steatohepatitis related liver fibrosis (Brea and Puzo, 2013) regulated by related genes and signaling pathways. Researches shown that Notch signaling in collaboration with other tumor-promoting signaling pathways, including p53/Snail/IKKα/FOXA2/NUMB, induces liver cancer (Strazzabosco and Fabris, 2012; Zhou et al., 2012). And also, RBPJ-mediated Notch signaling regulates cell differentiation and plasticity in cooperation with other pathways (Hayward et al., 2008; Kopan and Ilagan, 2009). At present, the researches on RBPJ mainly focuses on the mechanism to HCC, and little research has done to the occurrence and genetics of fatty liver in
Figure 6. Gene expression levels, TG content and Oil red O staining in different groups. Gene expression levels, TG content and Oil red O staining in different groups. (A and B) represent RBPJ expression levels and TG content in siRBPJ NC, siRBPJ, pcDNA3.1 (+)-GFP and pcDNA3.1 (+)-RBPJ groups. (C) represents TG content in miR-375 mimic NC, miR-375 mimic, miR-375 inhibitor NC, miR-375 inhibitor group. (D) shows lipid droplets in different transfected groups, a represents siRBPJ NC group, b represents siRBPJ group, c represents pcDNA3.1 (+)-GFP group, d represents pcDNA3.1 (+)-RBPJ group, e represents miR-375 mimic NC group, f represents miR-375 mimic group, g represents miR-375 inhibitor NC group, h represents miR-375 inhibitor group. * Indicates $P \leq 0.05$ and ** indicates $P \leq 0.01$.

Figure 7. Cell proliferation related genes detection in primary hepatocytes cultured in vitro. Relative expression analysis results of cell proliferation related genes in different groups. (A–D) represent relative expression analysis results of PMP22, IGF-1, IGF-2, and IGFBP-5 in siRBPJ NC, siRBPJ, pcDNA3.1 (+)-GFP and pcDNA3.1 (+)-RBPJ groups, respectively. * Indicates $P \leq 0.05$ and ** indicates $P \leq 0.01$. Abbreviations: PMP22, peripheral myelin protein 22; IGF-1, insulin-like growth factor-1; IGF-2, insulin-like growth factor-2; IGFBP-5, insulin-like growth factor binding protein-5.
chicken. In the present study, we found that the expression levels of RBPJ in the liver of fat liver groups from F0 to F3 generation higher than those of control groups (P < 0.05 or P < 0.01), at the same time, we found that triglyceride content and lipid droplets increased or decreased when RBPJ was knocked down or overexpressed in primary hepatocytes cultured in vitro, respectively. So, we deduce that RBPJ could regulate hepatic lipid metabolism involved in occurrence and genetics of fatty liver in chicken.

Researches also shown that RBPJ can promote cell proliferation during HCC occurrence (Kopan and Ilagan, 2009), whether RBPJ has the same impact on cell proliferation in fatty liver is uncertain. In this study, we found that cell proliferation-related genes, including PMP22 (Hou et al., 2020), IGF-1, IGF-2, and IGFBP-5 (Lang et al., 2006; Aguiar et al., 2013; Jiao et al., 2013) were increased or decreased significantly after overexpression or knock-down RBPJ (P < 0.05 or P < 0.01), respectively. The results indicate that RBPJ could also promote cell proliferation in primary hepatocytes cultured in vitro. But the expression levels of RBPJ in fat liver groups were decreased compare with those in control groups, and cell proliferation was inhibited during fat liver in chicken. So, the other reason for formation and inheritance of fat liver, we speculate, is that cell proliferation was inhibited under the RBPJ regulation.

Increasing evidence points to pivotal roles for miR-375 in regulating fat accumulation and carcinogenesis. It has been proved that the expression levels of miR-375 in human hepatoma tissues and hepatoma cell lines were significantly lower than those in control groups (Li et al., 2018). Some studies shown that miR-375 further regulates the proliferation and migration of stem cells by regulating the transcription level of YAP, and then affects the occurrence and development of hepatocellular carcinoma (Liu et al., 2010). Our observation showed that RBPJ is target gene of miR-375, and the expression levels of miR-375 were higher in fatty liver groups compare with those in control groups from F0 to F3 generations (P < 0.05 or P < 0.01), and we also found that miR-375 could regulate triglycerides formation and lipid droplet deposition in primary hepatocytes cultured in vitro. These results indicate that miR-375 has a potential role in hepatic lipid metabolism via targeting RBPJ.

CONCLUSIONS

This study uniquely revealed that miR-375 could upregulated lipid synthesis and inhibited cell proliferation which may partly due to regulation of RBPJ expression, thereby involving in fatty liver formation and inheritance in chicken. Our findings will provide a valuable resource to insight into the regulation mechanism for the formation and inheritance of fatty liver with the point of epigenetics, and suggested that modulating miR-375 or RBPJ differential expression may represent a potential therapeutic strategy for fatty liver disease that could be used for disease-resistance selective breeding in chicken.

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Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

DISCLOSURES

The authors declare that they have no competing interests.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jspi.2022.102218.

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