Arch Biol Sci. 2021;73(4):491-501 https://doi.org/10.2298/ABS210916043X

Effect of taurine intervention on oleic acid-induced primary hepatocyte steatosis in orange-spotted grouper (*Epinephelus coioides*)

Ruyi Xiao, Hanmo Feng, Xingjian Niu, Fakai Bai and Jidan Ye*

Xiamen Key Laboratory for Feed Quality Testing and Safety Evaluation, Fisheries College of Jimei University, Yindou Road 43, Jimei District, Xiamen, China

***Corresponding author**: yjyjd@jmu.edu.cn

Received: September 16, 2021; **Revised**: October 17, 2021; **Accepted**: October 19, 2021; **Published online**: November 2, 2021

Abstract: Examination of the molecular mechanism of taurine regulation of lipid metabolism in fish is limited. In this study, an oleic acid (OA)-induced hepatocyte steatosis model of orange-spotted grouper (*Epinephelus coioides*) was established for the first time. The model was used to test the effect of taurine on steatosis hepatocytes in Control, High-fat (0.4 mM OA) and Taurine (0.4 mM OA + 2 mM taurine) experimental groups of fish. Hepatocyte samples were subjected to transcriptome analysis. A total of 99634 unigenes was assembled, 69982 unigenes were annotated and 1831 differentially expressed genes (DEGs) in Control vs High-fat group, and 526 DEGs in the High-fat vs Taurine group were identified, of which 824 DEGs (Control vs High-fat) and 237 DEGs (High-fat vs Taurine) were observed to be upregulated, and 1007 DEGs (Control vs High-fat) and 289 DEGs (High-fat vs Taurine) were downregulated after taurine intervention. These genes are involved in peroxisome proliferator-activated receptor (PPAR) and 5' AMP-activated protein kinase (AMPK) signaling pathways, fatty acid elongation, primary bile acid biosynthesis, glycerophospholipid and glycerolipid metabolism. The findings provide new clues in understanding the regulatory role of taurine in lipid and fatty acid metabolism of fish. It is hoped that the obtained results will help in the design of feed formulations to improve grouper growth from the perspective of aquaculture nutrition.

Keywords: *Epinephelus coioides*; taurine; hepatocyte steatosis; differential expression gene; RNA-Seq

INTRODUCTION

Lipid is an indispensable macronutrient for the growth of fish, with essential fatty acids metabolized in fish to maintain their essential physiological functions [1]. The use of lipid in aquafeeds, as a non-protein energy source, can save protein for energy consumption to a certain extent [2,3]. Use of high-lipid feeds is widely accepted in intensive and large-scale aquaculture to reduce the use of high-quality proteins and the feed conversion ratio [4]. Despite these benefits, excessive lipid intake in fish can cause undesirable effects, such as visceral fat accumulation and fatty liver [5,6], accompanied by apoptosis and decreased immune function [7-10]. Fatty liver induced by high dietary lipid has become an important chronic liver disease closely associated with nutritional metabolic syndrome in fish cultivation. It is necessary to understand the mechanism of fatty liver in fish caused by high-lipid feed. Therefore, there is an urgent need to find a suitable way to solve the problem of fatty liver, which has become a threat to aquaculture production.

Taurine (2-aminoethanesulfonic acid), a sulfur-containing amino acid, is the most abundant free amino acid in animal tissues, including fish, comprising 30-50% of the total free amino acid pool [11]. Taurine plays many important physiological roles such as bile salt synthesis, osmoregulation, lipid metabolism, antioxidation, immunoregulation [12,13]. Taurine is abundant in marine and terrestrial animals [14], and is synthesized by many vertebrates [12,15]. However, unlike most terrestrial animals, fish (especially carnivorous fish and larvae), have little ability to synthesize taurine. Because endogenous taurine synthesis in fish is insufficient to meet the needs of normal growth and is usually dependent on external (food) supply, it is regarded as a conditional essential amino acid in fish [15]. Taurine deficiency in feed causes growth retardation of fish [12], accompanied by the accumulation of

liver and body lipid [16]. Dietary taurine intervention could reduce blood cholesterol and insulin levels and body lipid [17,18], suggesting that taurine possesses a preventive effect on fatty liver by relieving abnormal lipid metabolism in animals, including fish [19-21].

Transcriptomics focuses on gene expression at the RNA level and offers genome-wide information of gene structure and gene function that can reveal the molecular mechanisms involved in specific biological processes. With the development of next-generation high-throughput sequencing technology, transcriptome analysis has progressively improved our understanding of RNA-based gene regulatory networks [22]. RNA-Seq-based transcriptomics have been widely applied in various aspects of aquatic animals such as growth, nutrition, immunity, metabolism, disease, and in genetic studies [23-26].

The orange-spotted grouper (*Epinephelus coioides*) has become an economically important marine carnivorous fish species that is reared in Southeast Asian countries, including China. Increasing attention has been devoted to research in nutrition and feed for groupers in the past decades [27,28]. Dietary taurine supplementation could improve the growth of grouper [29,30], and its abundance and deficiency are closely related to lipid deposition and lipid metabolism in fish [31]; however, the underlying metabolic mechanism of taurine intervention on the development of fatty liver in fish is not wholly understood. To further clarify the effect of taurine on fatty liver in fish, grouper liver was selected as the target material and a primary hepatocyte steatosis model induced by oleic acid (OA) was exposed to taurine; cell samples were assessed using transcriptome analysis to obtain differentially expressed genes. The objective of the present study was to construct a metabolic network of taurine involvement in lipid metabolism to clarify its regulatory role in liver lipid metabolism of grouper *in vitro*.

MATERIALS AND METHODS

Fish maintenance

Juvenile groupers with an average weight of 50 g were obtained from a commercial fish farm in Zhaoan County, Fujian province, China, and transported to the Aquaculture Experimental Center of Jimei University. The fish were stocked in a closed recirculating system consisting of two 1000-L fiberglass tanks with a PolyGeyser bead filter (Aquaculture Systems Technologies, LLC., USA) and were maintained on commercial grouper feed with one meal a day (17:00). Uneaten feed and feces were removed via siphoning 30 min after a meal. During the holding period of fish, the water temperature was maintained at 28±0.5°C, dissolved oxygen ≥6.0 mg/L and ammonia nitrogen was <0.02 mg/L.

Isolation and primary culture of hepatocytes

The isolation and primary culture of hepatocytes was performed according to the method described [32]. Briefly, fish sampled from the rearing tanks were kept in seawater containing 10000 units/mL of penicillin and 10000 μg/mL of streptomycin (Pen Strep, Thermo Fisher Scientific, Shanghai, China) for 24 h, followed by anesthesia with a 100-mg/L solution of tricaine methane sulfonate (MS-222, Sigma-Aldrich Shanghai Trading Co. Ltd., Shanghai, China). After disinfection with 75% (v/v) alcohol, the fish were bled by cutting the gill arches. The liver was aseptically removed and washed three times using ice-cold sterile phosphate-buffered saline (PBS) containing 1% (10 mg/mL) Pen-Strep (PBS, Thermo Fisher Scientific, Shanghai, China). After removal of PBS, the liver was cut into small pieces (about 1 mm^3) and washed with PBS three times to remove any red blood cells. The pieces were digested with 0.25% trypsin-EDTA (Thermo Fisher Scientific, Shanghai, China) at room temperature for 10 min. The digestion was terminated by adding fresh medium containing 1% Pen-Strep and 15% (v/v) fetal bovine serum (FBS, Thermo Fisher Scientific, Shanghai, China). The cell suspension was collected after centrifugation (500 × *g*, 4°C, 5 min). The harvested cells were treated with 1 mL of red cell lysis solution (Biosharp Life Sciences, Hefei, China) for 5 min. After centrifugation (500 × *g*, 4°C, 5 min), the cells were collected and resuspended in Leibowitz's L-15 medium (L-15, Thermo Fisher Scientific, Shanghai, China) with 1% Pen-Strep and 15% FBS.

Hepatocyte steatosis treated with taurine

The isolated hepatocytes were seeded into a 6-well culture plate at a density of 5×10^5 cells/mL. After

incubation for 24 h at 25°C, the supernatant was discarded. A 100-μL aliquot of the L-15 medium was added into each well. The experiment was divided into three groups: normal hepatocyte group (Control), hepatocyte steatosis group (control+0.4 mM OA; High-fat group), taurine intervention group (control+0.4 mM OA+2 mM taurine; Taurine group). Oleic acid and taurine were purchased from Sigma-Aldrich Trading Co., Ltd., Shanghai, China. After incubation for 48 h, the cells were collected for subsequent analysis. The viability of cells was assessed prior to each experiment using a Cell Counting Kit-8 (CCK-8, Dojindo Chemical Technology Co., Ltd., Shanghai, China). A 10-μL aliquot of CCK-8 was added, incubated for 4 h, and the OD450 was measured using a microplate reader (Thermo Fisher Scientific, MA, USA). The cell viability rate (%) was determined as follows: (experiment well-blank well)/(control well-blank well)×100%. The activities of glutamic-pyruvic transaminase (GPT) and glutamic oxaloacetic transaminase (GOT), triglyceride (TG) and total cholesterol (TC) in hepatocytes were determined according to the manufacturer's instructions (Jiancheng Bioengineering Institute, Nanjing, China). Oil red O staining was performed using Oil Red O staining kits according to the manufacturer's instructions (Jiancheng Bioengineering Institute, Nanjing, China). Slides were examined under a light microscope (Leica DM5500B, Germany) to visualize and capture images.

cDNA library construction and high throughput sequencing

Total RNA was extracted from the hepatocytes of the control, hepatocyte steatosis and taurine intervention groups using the E.Z.N.A.® total RNA kit II (Omega Bio-Tek Inc., Norcross, GA, USA) according to the manufacturer's protocol. A Nano Drop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and 1% agarose-gel electrophoresis were used for the determination of RNA integrity and quality. Four biological replicates for each treatment were subjected to cDNA library construction. The cDNA was synthesized using the Prime Script RT reagent kit (Thermo Fisher Scientific, Wilmington, DE, USA) according to the manufacturer's protocol. The cDNA libraries were constructed using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. The complete library was sequenced by Haiyi Genomic Technology Co., LTD (Shenzhen, China) using the Illumina HiSeq™ 2000 system (Illumina, San Diego, CA, USA).

Sequence data processing and analysis

Raw reads were filtered to remove low-quality sequences using the method from the program written by Haiyi Genomic Technology Co., LTD. RNA-Seq *de novo* assembly was carried out using Trinity software (https://github.com/trinityrnaseq/trinityrnaseq/wiki, trinityrnaseq_r20140717) and the Tgicl software (https://help.rc.ufl.edu/doc/Tgicl, version 2.1). The longest transcripts were regarded as unigenes after removing repetitive assemblies. BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to align unigene sequences with the Cluster of Orthologous Groups of Protein Database (COG, http://www.ncbi.nlm.nih.gov/COG/), NCBI Non-redundant Nucleotide Sequence Database (NR, ftp://ftp.ncbi.nih.gov/blast/db), NCBI Non-redundant Protein Sequence Database (NT, ftp://ftp.ncbi.nih.gov/blast/db), Swiss-Prot Protein Database (Swiss-Prot, http://www.uniprot.org/), Gene Ontology Database (GO, http://geneontology.org), and Kyoto Encyclopedia of Gene and Genomes Database (KEGG, http://www.kegg.jp/).

Analysis of differentially expressed genes

The Python toolkit HTseq (http://htseq.readthedocs.io/en/master) was used to accurately map the sequencing reads to reference genomes. The expression level of each gene was calculated from the fragment per kilobase of exon model per million mapped read (FPKM) values [33]. DESeq2 software was used to analyze and filter DEGs among different groups [34], and unigenes with |log2 (Fold Change) | >1.2 and false discovery rate (FDR)≤ 0.05 were defined as DEGs. Functional annotations of DEGs by GO were performed using the Blast2GO software (http://www.blast2go.com/), followed by GO functional classifications using WEGO (Web Gene Ontology Annotation Plot) software (http://wego.genomics.org.cn/document) to view the distribution of gene functions in fish at the macro level by visualizing, comparing, and plotting GO annotation results [35]. GO terms with a P value≤0.05 were considered as significantly enriched in DEGs.

Pathway enrichment analysis was carried out using KEGG that is available from the NCBI Short Read Archive. Pathways with P≤0.05 were designated as significantly enriched pathways for DEGs.

Validation of DEGs by quantitative reverse transcription PCR

To validate the gene expression data obtained by RNA-Seq, the relative expression levels of eight selected genes (*PPAR-α*, *SORBS1*, *HSD17B12*, *DGK*, *ACOT8*, *CAMKK2*, *PIAS1* and *MKP*) were confirmed by quantitative reverse transcription PCR (qRT-PCR). The primers were designed with Primer v. 5.0, with the β-actin gene of the fish used as the internal reference for the qPCR analysis (Table S1). qRT-PCR reactions were prepared with ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China). Quantitative reactions were performed on a Real-Time PCR Detection System (ABI 7500, Applied Biosystems, USA). The PCR cycling conditions were as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 15 s and 60°C for 15 s, and then cycles at 95°C for 15 s, 60°C for 60 s, and 95°C for 15 s. To check reproducibility, the qRT-PCR reaction for each sample was performed in four biological replicates. The relative expression of genes was calculated using the $2^{-\Delta\Delta Ct}$ method [36].

Statistical analysis

Data are presented as mean and standard errors of the mean (SEM). Data were subjected to one-way ANOVA and Student-Neuman-Keuls multiple comparison tests in SPSS Statistics 22.0 (SPSS, Michigan Avenue, Chicago, IL, USA). P<0.05 was considered statistically significant.

RESULTS

Effect of taurine on OA-induced hepatocyte steatosis

To confirm that taurine could mitigate the OA-induced cell steatosis, we pre-incubated cells with 0.4 mM OA for 48 h and then treated them with 2 mM taurine for another 48 h. The viability of cells was higher in the Taurine than in the High-fat group (Supplementary Fig. S1). There was a reduction of TG and TC levels in the Taurine vs the High-fat group, and the values were comparable to that of the Control group. Similarly, the activities of GOT and GPT were lower in the Taurine than in the High-fat group (Supplementary Fig. S2). The results of Oil Red O staining showed that lipid droplets of the cells in the Taurine group were reduced compared with those in the High-fat group (Fig. 1).

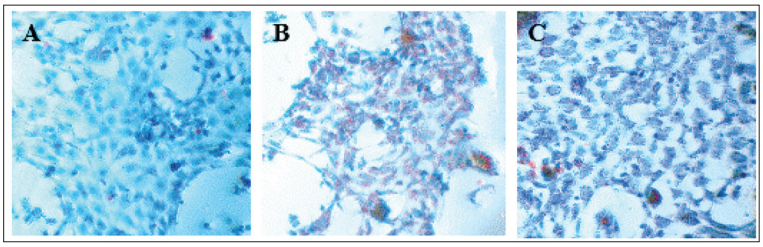

Fig. 1. Representative images of Oil Red O staining of primary cultured hepatocytes of *E. coioides* after three different treatments of oleic acid (OA) and taurine. **A –** Control group: hepatocytes without treatment with OA or taurine for 48 h; **B –** High-fat group: hepatocytes treated with 0.4 mM OA alone for 48 h; **C –** Taurine group: hepatocytes treated with both 0.4 mM OA and 2 mM taurine for 48 h.

Illumina sequencing and *de novo* assembly

According to the sequencing results of the transcriptome assay, we obtained 87057383400 clean bases and 580382556 clean reads, with Q30>90% (Supplementary Table S2). From the assembly results, 99634 unigenes were obtained with the N50 of unigenes being 4033 bp (Table 1). The length distribution of all unigenes is shown in Supplementary Fig. S3. After short and low-quality sequences were excluded, 69982 unigenes were identified and annotated by matching them against the COG, GO, KEGG, NT, Swiss-Prot and NR databases (Table 2).

Table 1. Summary statistics of the *de novo* transcriptome assembly of primary cultured hepatocytes of *E. coioides*.

Unigene number	Min length (bp)	Max length (bp)	Mean length (bp)	N50 (bp)	N90 (bp)
99634	200	50752	1890	4033	878

Min length – the minimum sequence length in a unigene set; Max length – the maximum sequence length in a unigene set; Mean length – average sequence length of a unigene set; N50/N90 – the unigenes were calculated by ordering all sequences, the length of unigenes was then collected one by one from the longest to the shortest until 50%/90% of the total length was attained.

Table 2. Summary of functional annotations of assembled unigenes of primary cultured hepatocytes of *E. coioides*.

Unigene number	NT	NR	GO	COG	KEGG	Swiss-Port
69982	18326	65773	43082	16393	46273	48342
100%	18.39%	66.01%	43.24%	16.45%	46.44%	48.52%

COG – cluster of orthologous groups of protein database; GO – gene ontology database; KEGG – Kyoto encyclopedia of genes and genome database; NR/NT – NCBI non-redundant protein/nucleotide sequence database; Swiss-Prot – protein sequence database.

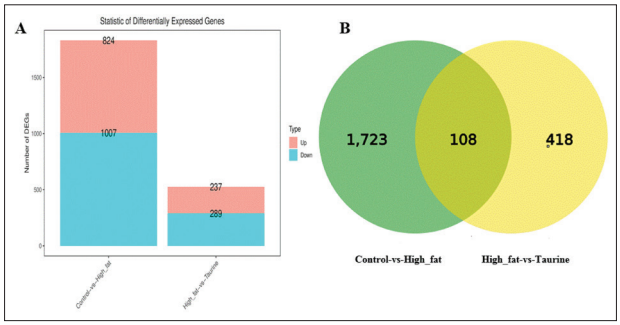

Fig. 2. Statistics and Venn diagram of differentially expressed genes (DEGs) of primary cultured hepatocytes of *E. coioides* of the Control vs the High-fat group, and of the High-fat vs the Taurine group. **A** – the statistics of DEGs; **B** – Venn diagram of DEGs. Control group: hepatocytes without treatment with OA or taurine for 48 h for 48 h; High-fat group: hepatocytes treated with 0.4 mM OA alone for 48 h; Taurine group: hepatocytes treated with both 0.4 mM OA and 2 mM taurine for 48 h.

Identification of DEGs

To identify DEGs in hepatocyte steatosis after taurine intervention, three digital gene expression libraries from the Control, High-fat and Taurine groups were constructed. There were 1831 DEGs identified in the comparison of the Control vs the High-fat group, of which 824 were upregulated and 1007 were downregulated (Fig. 2A). In the comparison of the High-fat and Taurine groups, 526 DEGs were identified, of which 237 were upregulated and 289 were downregulated (Fig. 2A). There were 108 DEGs shared between the two pairwise comparisons (Fig. 2B).

Unigene function annotation and analysis

Unigene sequences were annotated by searching the nonredundant NCBI protein database using BLAST. A total of 69982 distinct sequences (70.23% of the total unigenes) matched known genes (Table 2). Based on gene ontology (GO) classification, 43082 (43.24%) unigenes were mapped and clustered into biological processes, cellular components and molecular function categories. In biological processes, 18408, 15058 and 8406 unigenes were classified into cellular processes, metabolic processes and biological regulation categories, respectively. In the cellular component, 9513, 9513, and 6367 unigenes were, respectively, classified into cell, cell part and organelle GO. Regarding molecular function, 26298, 13719, and 1928 unigenes were clustered into binding, catalytic activity and transporter activity categories, respectively (Supplementary Fig. S4). The results of two pairwise comparisons showed that DEGs were consistent with their molecular functions (Fig. 3); in the comparison of the Control with the High-fat, and the High-fat with the Taurine group, 214 and 62 DEGs were involved in the protein binding.

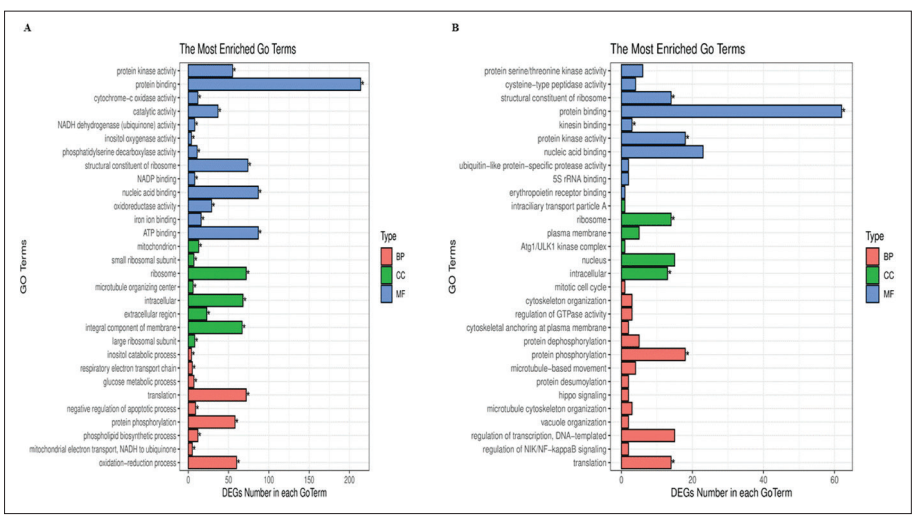

Fig. 3. Gene ontology (GO) classification of differentially expressed genes (DEGs) of primary cultured hepatocytes of *E. coioides* between the Control vs the High-fat group, and between the High-fat vs the Taurine group. **A** – GO significant enrichment analysis for DEGs of the Control vs the High-fat group; **B** – GO significant enrichment analysis for DEGs of the High-fat vs the Taurine group. Control group: hepatocytes without treatment with OA or taurine for 48 h; High-fat group: hepatocytes treated with 0.4 mM OA alone for 48 h; Taurine group: hepatocytes treated with both 0.4 mM OA and 2 mM taurine for 48 h.

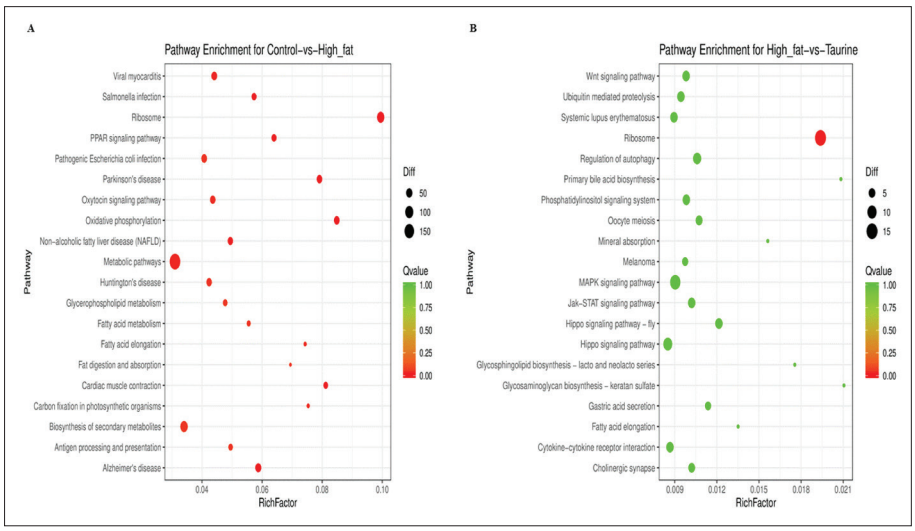

Fig. 4. Bubble chart of significantly enriched KEGG pathways in the differentially expressed genes (DEGs) of primary cultured hepatocytes of *E. coioides* between the Control vs the High-fat group, and between the High-fat vs the Taurine group. **A** – pathway enrichment of DEGs of the Control vs the High-fat group; **B** – pathway enrichment of DEGs of the High-fat vs the Taurine group. The vertical axis represents the pathway categories, the horizontal axis shows the enrichment factor. The point size shows the number of DEGs enriched in the KEGG pathway. The point color shows different Q values as indicated on the right. Control group: hepatocytes without treatment with OA or taurine for 48 h; High-fat group: hepatocytes treated with 0.4 mM OA alone for 48 h; Taurine group: hepatocytes treated with both 0.4 mM OA and 2 mM taurine for 48 h.

In the biological processes category, 72 DEGs were involved in translation after comparison of the Control vs the High-fat group, 18 DEGs were involved in protein phosphorylation in after comparison of the High-fat vs the Taurine group. KEGG analysis showed that the matched 46273 (46.44%) unigenes were assigned to 410 pathways, of which the top 20 are depicted in Fig. 4. We established that the PPAR signaling pathway was enriched after comparison of the Control vs the High-fat group and the ribosome was enriched in the High-fat vs the Taurine group.

Change in gene expression related to lipid metabolism

Based on the above analysis, several signaling pathways of taurine mediated lipid metabolism were clustered in Supplementary Table S3 and mainly included primary bile acid biosynthesis, glycerophospholipid metabolism, glycerolipid metabolism, fatty acid elongation, sphingolipid metabolism, steroid hormone biosynthesis, biosynthesis of unsaturated fatty, the PPAR signaling and AMPK signaling pathways. Furthermore, the signaling pathway network related to taurine-mediated lipid metabolism in hepatocytes was constructed and is presented in Fig. 5.

qRT-PCR validation of DEGs

To validate the reliability of RNA-Seq data, eight randomly selected DEG expression profiles in Control, High-fat and Taurine samples were examined by qRT-PCR. As shown in Fig. 6, the fold-changes obtained by qRT-PCR were consistent with the values obtained by RNA-seq for the selected genes, suggesting our RNA-Seq data and the results based on RNA-Seq data analysis were reliable.

DISCUSSION

The technique of primary cell culture of fish hepatocytes has been used in many fields of biology, such as nutrition, pharmacology, toxicology [37-41]. OA-induced lipid accumulation is assumed to be an important mechanism involved in lipotoxicity [42]. Taurine is effective in alleviating fatty acid-induced hepatocyte steatosis [20]. In this study, OA-induced hepatocyte steatosis was used to investigate the effect of taurine on fatty liver of fish. We established the OA-induced hepatocyte steatosis experimental model using 0.4 mM OA and a 48-h culture time window. Treatment with 2 mM taurine in the hepatocyte steatosis model resulted in a reduction of TG and TC concentrations and GOT and GPT enzyme levels. The results revealed an effect of taurine on hepatocyte steatosis.

Herein we present the first transcriptomic analysis of taurine intervention on hepatocyte steatosis in grouper *in vitro* in an attempt to understand the regulatory mechanism of taurine on OA-induced hepatocyte steatosis. The analysis of DEGs in normal hepatocytes, hepatocytes exhibiting steatosis and taurine-treated

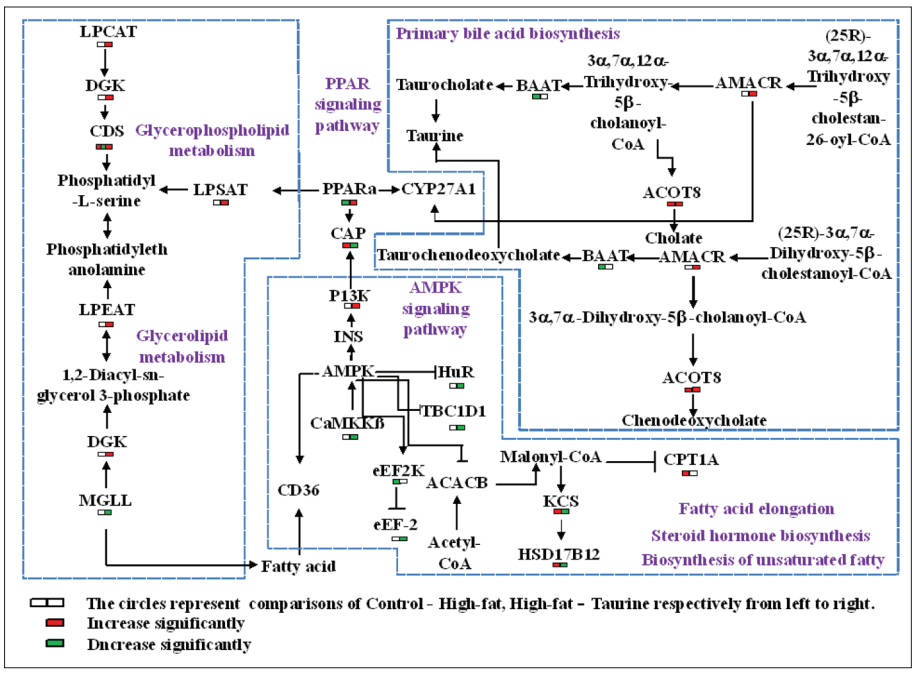

Fig. 5. Signal pathway network diagram of taurine-mediated lipid metabolism of primary cultured hepatocyte steatosis of *E. coioides*. Abbreviations: ACACB – acetyl-CoA carboxylase/biotin carboxylase 2; ACOT8 – acyl-CoA thioesterase 8; AMACR – alpha-methylacyl-CoA racemase; AMPK – 5'-AMP-activated protein kinase; BAAT – bile acid-CoA:amino acid N-acyltransferase; CaMKK2 – calcium/calmodulin-dependent protein kinase kinase 2; CAP – sorbin and SH3 domain containing protein 1; CDS – phosphatidate cytidylyltransferase; CYP27A1 – cholestanetriol 26-monooxygenase; DGK – diacylglycerol kinase (ATP); eEF2K – elongation factor 2 kinase; HuR – ELAV-like protein 1; INS – insulin; KCS – 3-ketoacyl-CoA synthase; LPCAT – lysophosphatidylcholine acyltransferase; LPEAT – lysophospholipid acyltransferase; LPSAT – lysophospholipid acyltransferase; MGLL – acylglycerol lipase; P13K – phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha/beta/delta; PPARα – peroxisome proliferator activated receptor α; TBC1D1 – TBC1 domain family member 1.

hepatocyte steatosis identified many genes and signaling pathways related to lipid metabolism. A total of 1831 DEGs was obtained when comparing the Control and High-fat groups, while 526 DEGs were obtained when comparing the High-fat and Taurine groups. GO annotation and KEGG pathway analysis of DEGs showed that most DEGs were enriched in cell component and biological process genes. The discovery of these genes and signaling pathways should contribute to an improved understanding of the molecular mechanism of regulation of taurine in fish lipid metabolism.

PPARs, as the key regulators of adipocyte differentiation, accumulation and phenotypes, are considered as being primarily involved in regulating lipid homeostasis [43-46]. In this study, the *PPARα* gene was found to be significantly upregulated in the High-fat vs the Taurine group, which was similar to that of white fat tissue of mice fed with a high-fat diet [47], indicating the inhibitory effect of taurine on adipogenesis.

The bile acids (BAs) are endocrine and metabolic signaling molecules that affect host physiology via activation of BA receptors, such as Farnesoid X receptor (FXR) and Takeda G protein-coupled receptor 5 (TGR5) [48]. BA synthesis is the major pathway for catabolism of cholesterol, which occurs in the liver and requires 17 enzymatic steps in different subcellular organelles [49]. Acyl-CoA thioesterase 8 (ACOT8) is a peroxisome protein that with BA coenzyme A regulates the process of bile acid metabolism [50,51]. Alpha-methylacyl-CoA racemase (AMACR) is an enzyme presumed to be essential for BA synthesis and a participant in the degradation of methyl-branched fatty acids [52]. The significant upregulation of these enzyme genes in the High-fat vs the Taurine group suggests that taurine could alleviate hepatocyte steatosis by promoting BA synthesis.

Glycerophospholipids are important structural constituents of biological membranes in animals that have important functions in signal transduction, regulation of membrane transport or control of cell cycle progression [53,54]. Diacylglycerol kinases (DGKs) are a family of enzymes that phosphorylate diacylglycerol (DAG) to form phosphatidic acid (PA) [55]. Both DAG and PA are important signaling molecules involved in many signal transduction pathways [56,57]. The carnitine palmitoyltransferase (CTP) is a rate-limiting enzyme that mediates the transport of long-chain FAs into the mitochondria for subsequent β-oxidation [58,59]. The role of DGKs in lipid metabolism was investigated and it was shown that the

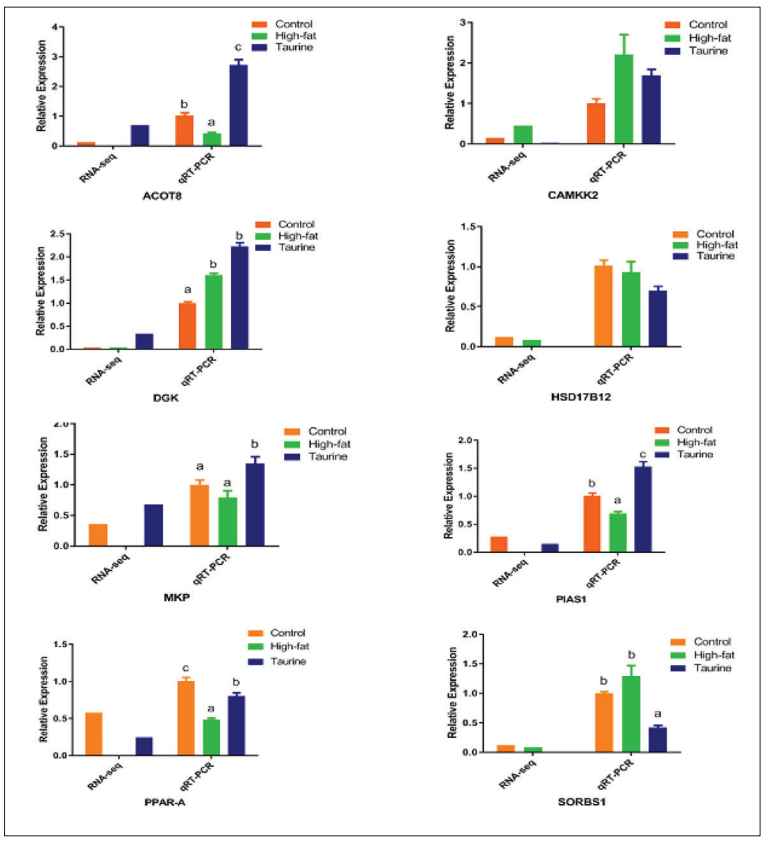

Fig. 6. Validation of RNA-seq data using qRT-PCR in primary cultured hepatocytes of *E. coioides* in Control, High-fat and Taurine groups. To validate the data from RNA-seq analysis, relative mRNA levels of eight selected differentially expressed genes (*PPAR-α*, *SORBS1*, *HSD17B12*, *DGK*, *ACOT8*, *CAMKK2*, *PIAS1* and *MKP*) from the primary cultured hepatocytes of Control, High-fat and Taurine groups were examined by RT-qPCR. mRNA levels are presented as fold changes when compared with the Control group after normalization against β-actin. The relative mRNA levels from the RNA-seq analysis were calculated as FPKM values. Control group: hepatocytes without treatment with OA or taurine for 48 h; High-fat group: hepatocytes treated with 0.4 mM OA alone for 48 h; Taurine group: hepatocytes treated with both 0.4 mM OA and 2 mM taurine for 48 h. Bars with different letters are significantly different (P<0.05).

DGK-knockout liver cancer cell line HepG2 exhibits increased mRNA levels of lipid synthesis-related genes (fatty acid synthase and *PPARγ*), and a decreased level of lipolytic gene *CPT1a* mRNA [60]. In the present study, significant upregulation of the *DGK* in the High-fat vs the Taurine group suggested that the *DGK* gene plays a key role in the control of lipid metabolism and signal transduction pathways.

The AMP-activated protein kinase (AMPK) is highly conserved in eukaryotic cells, acting as a metabolic sensor that monitors cellular AMP and ATP levels to respond to environmental and/or nutritional stress [61]. The calcium/calmodulin-dependent kinase kinase 2 (CaMKK2) is one of the major upstream kinases in the AMPK pathway, which is activated by increases in intracellular Ca^{2+} levels, thereby triggering transient activation of AMPK [62]. The loss of CaMKK2 protects mice from high-fat diet-induced obesity, insulin resistance and glucose intolerance [63]. We also observed significant downregulation of the *AMPK* gene in the High-fat vs Taurine group, indicating that taurine could activate the AMPK signaling pathway to protect hepatocytes from the influence of steatosis caused by OA.

CONCLUSIONS

In this study, an OA-induced primary hepatocyte steatosis model of orange-spotted grouper was established to investigate the regulatory role of taurine in liver lipid metabolism of fish. Transcriptomics technique was applied to analyze the DEGs in two pairwise comparisons: Control vs High-fat and High-fat vs Taurine. Reproducibility of the data was confirmed by the close correlation between the mRNA levels determined by RNA-Seq and RT-qPCR. A number of genes involved in hepatocyte steatosis displayed significant changes in expression after taurine treatment. A total of 2357 DEGs were identified as being related to lipid metabolism in the two pairwise comparisons. These genes belong to many signaling pathways and mainly include *PPARα*, *ACOT8*, *AMACR*, *DGK*, *CaMKK2*, and the AMPK and PPAR signaling pathways – genes involved in primary bile acid biosynthesis, glycerophospholipid metabolism, glycerolipid metabolism and fatty acid elongation. Taurine intervention *in vitro* significantly affected the expression of the identified genes, indicating that taurine plays a regulatory role in lipid metabolism and improves the functioning of steatosis hepatocytes. The results obtained may help in the design of feed formulations to improve grouper growth from the perspective of aquaculture nutrition.

Funding: This study was supported by the National Natural Science Foundation of China (Grant Nos. 31772861 and 31372546), and the Science and Technology Project of Fujian Province of China (No. 2020N0012).

Author contributions: RX: conceptualization, investigation, and writing, original draft preparation. HF: data curation and methodology. XN: formal analysis, validation and data curation. FB: software and visualization. JY: writing, reviewing and editing, supervision. All authors have read and agreed to the final version of the manuscript.

Conflict of interest disclosure: The authors declare no conflict of interest.

REFERENCES

1. Turchini GM, Torstensen BE, Ng WK. Fish oil replacement in finfish nutrition. Rev Aquac. 2009;1(1):10-57. https://doi.org/10.1111/j.1753-5131.2008.01001.x
2. Li X, Zheng S, Ma X, Cheng K, Wu G. Effects of dietary starch and lipid levels on the protein retention and growth of largemouth bass (Micropterus salmoides). Amino Acids. 2020;52:999-1016. https://doi.org/10.1007/s00726-020-02869-6
3. Welengane E, Sado RY, de Almeida Bicudo AJ. Protein-sparing effect by dietary lipid increase in juveniles of the hybrid fish tambatinga (♀ Colossoma macropomum × ♂ Piaractus brachypomus). Aquac Nutr. 2019;25(6):1272-80. https://doi.org/10.1111/anu.12941
4. Boujard T, Gelineau A, Coves D, Corraze G, Dutto G, Gasset E, Kaushik S. Regulation of feed intake, growth, nutrient and energy utilisation in European sea bass (Dicentrarchus labrax) fed high fat diets. Aquaculture. 2003;231(1):529-45. https://doi.org/10.1016/j.aquaculture.2003.11.010
5. Zhou Y, Guo J, Tang R, Ma H, Chen Y, Lin S. High dietary lipid level alters the growth, hepatic metabolism enzyme, and anti-oxidative capacity in juvenile largemouth bass Micropterus salmoides. Fish Physiol Biochem. 2020;46(1):125-34. https://doi.org/10.1007/s10695-019-00705-7
6. Li A, Yuan X, Liang X, Liu L, Li J, Li B, Fang J, Li J, He S, Xue M, Wang J, Tao Y. Adaptations of lipid metabolism and food intake in response to low and high fat diets in juvenile grass carp (Ctenopharyngodon idellus). Aquaculture. 2016;457:43-9. https://doi.org/10.1016/j.aquaculture.2016.01.014
7. Cao X, Dai Y, Liu M, Yuan X, Wang C, Huang Y, Liu W, Jiang G. High-fat diet induces aberrant hepatic lipid secretion in blunt snout bream by activating endoplasmic reticulum stress-associated IRE1/XBP1 pathway. BBA-Mol Cell Biol L. 2019;1864(3):213-23. https://doi.org/10.1016/j.bbalip.2018.12.005
8. Tang T, Hu Y, Peng M, Chu W, Hu Y, Zhong L. Effects of high-fat diet on growth performance, lipid accumulation and lipid metabolism-related MicroRNA/gene expression in the liver of grass carp (Ctenopharyngodon idella). Comp. Biochem Physiol B Biochem Mol Biol. 2019;234:34-40. https://doi.org/10.1016/j.cbpb.2019.04.006
9. Jia R, Cao L, Du J, He Q, Gu Z, Jeney G, Xu P, Yin G. Effects of high-fat diet on antioxidative status, apoptosis and inflammation in liver of tilapia (Oreochromis niloticus) via Nrf2, TLRs and JNK pathways. Fish Shellfish Immun. 2020;104:391-401. https://doi.org/10.1016/j.fsi.2020.06.025
10. Zhong Y, Pan Y, Liu L, Li H, Li Y, Jiang J, Xiang J, Zhang J, Chu W. Effects of high fat diet on lipid accumulation, oxidative stress and autophagy in the liver of Chinese softshell turtle (Pelodiscus sinensis). Comp Biochem Physiol B Biochem Mol Biol. 2020;240:110331. https://doi.org/10.1016/j.cbpb.2019.110331
11. Abdel FMES. Is dietary taurine supplementation beneficial for farmed fish and shrimp? a comprehensive review. Rev Aquac. 2014;6(4):241-55. https://doi.org/10.1111/raq.12042
12. Salze GP, Davis DA. Taurine: a critical nutrient for future fish feeds. Aquaculture. 2015;437:215-29. https://doi.org/10.1016/j.aquaculture.2014.12.006
13. Yang H, Tian L, Huang J, Liang G, Liu Y. Dietary taurine can improve the hypoxia-tolerance but not the growth performance in juvenile grass carp Ctenopharyngodon idellus. Fish Physiol Biochem. 2013;39(5):1071-8. https://doi.org/10.1007/s10695-012-9763-5
14. Caine JJ, Geracioti TD. Taurine, energy drinks, and neuroendocrine effects. Cleve Clin J Med. 2016;83(12):895-904. https://doi.org/10.3949/ccjm.83a.15050
15. Wang X, He G, Mai K, Xu W, Zhou H. Differential regulation of taurine biosynthesis in rainbow trout and Japanese flounder. Sci Rep. 2016;6(2):21231. https://doi.org/10.1038/srep21231
16. Espe M, Ruohonen K, El-Mowafi A. Effect of taurine supplementation on the metabolism and body lipid-to-protein ratio in juvenile Atlantic salmon (Salmo salar). Aquac Res. 2012;43(3):349-60. https://doi.org/10.1111/j.1365-2109.2011.02837.x
17. Chen W, Guo JX, Chang P. The effect of taurine on cholesterol metabolism. Mol Nutr Food Res. 2012;56(5):681-90. https://doi.org/10.1002/mnfr.201100799
18. Carina SS, Daniel F, Letícia MI, Talita R, Erika AR, Ana PA, Joseane M, Adriana ST, Everardo MC, Licio AV. Taurine enhances the anorexigenic effects of insulin in the hypothalamus of rats. Amino Acids. 2012;42(6):2403-10. https://doi.org/10.1007/s00726-011-1045-5
19. Kim S, Kim K, Kim K, Kim K, Son M, Rust M, Johnson R. Effect of dietary taurine levels on the conjugated bile acid composition and growth of juvenile Korean rockfish Sebastes schlegeli (Hilgendorf). Aquac Res. 2015;46(11):2768-75. https://doi.org/10.1111/are.12431
20. Murakami S, Ono A, Kawasaki A, Takenaga T, Ito T. Taurine attenuates the development of hepatic steatosis through the inhibition of oxidative stress in a model of nonalcoholic fatty liver disease in vivo and in vitro. Amino Acids. 2018;50(9):1279-88. https://doi.org/10.1007/s00726-018-2605-8
21. de Moura LB, Diogenes AF, Vasconcelos Campelo DA, Alves De Almeida FL, Pousao-Ferreira PM, Furuya WM, Peres H, Oliva-Teles A. Nutrient digestibility, digestive enzymes activity, bile drainage alterations and plasma metabolites of meagre (Argyrosomus regius) feed high plant protein diets

supplemented with taurine and methionine. Aquaculture. 2019;511:734231. https://doi.org/10.1016/j.aquaculture.2019.734231
22. McGettigan PA. Transcriptomics in the RNA-seq era. Curr Opin Chem Biol. 2013;17(1):4-11. https://doi.org/10.1016/j.cbpa.2012.12.008
23. Paneru BD, Al-Tobasei R, Kenney B, Leeds TD, Salem M. RNA-Seq reveals MicroRNA expression signature and genetic polymorphism associated with growth and muscle quality traits in rainbow trout. Sci Rep. 2017;7(1):9078. https://doi.org/10.1038/s41598-017-09515-4
24. Duan Y, Wang Y, Xiong D, Zhang J. RNA-seq revealed the signatures of immunity and metabolism in the Litopenaeus vannamei intestine in response to dietary succinate. Fish Shellfish Immun. 2019;95:16-24. https://doi.org/10.1016/j.fsi.2019.09.074
25. Fernandez I, Fernandes JMO, Roberto VP, Kopp M, Oliveira C, Riesco MF, Dias J, Cox CJ, Cancela ML, Cabrita E, Gavaia P. Circulating small non-coding RNAs provide new insights into vitamin K nutrition and reproductive physiology in teleost fish. Biochim Biophys Acta Gen Subj. 2019;1863(1):39-51. https://doi.org/10.1016/j.bbagen.2018.09.017
26. Abernathy J, Overturf K. Toward resolving long noncoding RNAs in fish: identification, mapping and association to disease using strand-specific RNA-seq in rainbow trout fed alternative diets. J Anim Sci. 2016;94(4):S67-8. https://doi.org/10.2527/jas2016.94supplement467a
27. He L, Qin Y, Wang Y, Li D, Chen W, Ye J. Effects of dietary replacement of fish oil with soybean oil on the growth performance, plasma components, fatty acid composition, and lipid metabolism of groupers Epinephelus coioides. Aquac Nutr. 2021;27(5):1494-511. https://doi.org/10.1111/anu.13292
28. Feng H, Yi K, Qian X, Niu X, Sun Y, Ye J. Growth and metabolic responses of juvenile grouper (Epinephelus coioides) to dietary methionine/cystine ratio at constant sulfur amino acid levels. Aquaculture. 2020;518:734869. https://doi.org/10.1016/j.aquaculture.2019.734869
29. Koven W, Peduel A, Gada M, Nixon O, Ucko M. Taurine improves the performance of white grouper juveniles (Epinephelus Aeneus) fed a reduced fish meal diet. Aquaculture. 2016;460:8-14. https://doi.org/10.1016/j.aquaculture.2016.04.004
30. Wang X, Zhou M, Huang Yan, Wang K, Ye J. Effects of dietary taurine level on growth performance and body composition of grouper (Epinephelus coioides) at different growth stages. Chinese J Anim Nutr. 2017;29(5):1810-20.
31. Shen G, Wang S, Dong J, Fen J, Xu J, Xia F, Wang X, Ye J. Metabolic effect of dietary taurine supplementation on grouper (Epinephelus coioides): a 1H-NMR-based metabolomics study. Molecules. 2019;24:2253. https://doi.org/10.3390/molecules24122253
32. Zhou W, Rahimnejad S, Tocher DR, Lu K, Zhang C, Sun, Y. Metformin attenuates lipid accumulation in hepatocytes of blunt snout bream (Megalobrama amblycephala) via activation of AMP-activated protein kinase. Aquaculture. 2019;499:90-100. https://doi.org/10.1016/j.aquaculture.2018.09.028
33. Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, Pachter L. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat Biotechnol. 2010;28:511-5. https://doi.org/10.1038/nbt.1621
34. Anders S, Huber W. Differential expression analysis for sequence count data. Genome Biol. 2010;11:R106. https://doi.org/10.1186/gb-2010-11-10-r106
35. Ye J, Fang L, Zheng H, Zhang Y, Chen J, Zhang Z, Wang J, Li S, Li R, Bolund L, Wang J. WEGO: a web tool for plotting GO annotations. Nucleic Acids Res. 2006;34:W293-7. https://doi.org/10.1093/nar/gkl031
36. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT Method. Methods. 2001;25(4):402-8. https://doi.org/10.1006/meth.2001.1262
37. Cao P, Huang G, Yang Q, Guo J, Su Z. The effect of chitooligosaccharides on oleic acid-induced lipid accumulation in HepG(2) cells. Saudi Pharm J. 2016;24(3):292-8. https://doi.org/10.1016/j.jsps.2016.04.023
38. Ellesat KS, Yazdani M, Holth TF. Species-dependent sensitivity to contaminants: an approach using primary hepatocyte cultures with three marine fish species. Mar Environ Res. 2011;72(4):216-24. https://doi.org/10.1016/j.marenvres.2011.09.003
39. Espe M, Xie S, Chen S, Pedro A, Holen E. Development of a fatty liver model using oleic acid in primary liver cells isolated from Atlantic salmon and the prevention of lipid accumulation using metformin. Aquac Nutr. 2019;25(3):737-46. https://doi.org/10.1111/anu.12905
40. Pesonen M, Andersson TB. Fish primary hepatocyte culture; an important model for xenobiotic metabolism and toxicity studies. Aquat Toxicol. 1997;37(2):253-67. https://doi.org/10.1016/S0166-445X(96)00811-9
41. Zhao X, Xue J, Xie M. Osthole inhibits oleic acid/lipopolysaccharide-induced lipid accumulation and inflammatory response through activating PPARα signaling pathway in cultured hepatocytes. Exp Gerontol. 2019;119:7-13. https://doi.org/10.1016/j.exger.2019.01.014
42. Huang W, Chen Y, Liu H, Wu S, Liou C. Ginkgolide C reduced oleic acid-induced lipid accumulation in HepG2 cells. Saudi Pharm J. 2018;26(8):1178-84. https://doi.org/10.1016/j.jsps.2018.07.006
43. Saponaro C, Gaggini M, Carli F, Gastaldelli A. The subtle balance between lipolysis and lipogenesis: a critical point in metabolic homeostasis. Nutrients. 2015;7(11):9453-74. https://doi.org/10.3390/nu7115475
44. Fernyhough ME, Okine E, Hausman G, Vierck JL, Dodson MV. PPARγ and GLUT-4 expression as developmental regulators/markers for preadipocyte differentiation into an adipocyte. Domest Anim Endocrinol. 2007;33(4):367-78. https://doi.org/10.1016/j.domaniend.2007.05.001
45. Lee HJ, Jang M, Kim H, Kwak W, Park WC, Hwang JY, Lee CK, Jang GW, Park MN, Kim HC, Jeong JY, Seo KS, Kim H, Cho S, Lee BY. Comparative transcriptome analysis of adipose tissues reveals that ECM-receptor interaction is involved in the depot-specific adipogenesis in cattle. PLoS One. 2013;8(6):e66267. https://doi.org/10.1371/journal.pone.0066267

46. Reddy JK, Hashimoto T. Peroxisomal β-oxidation and peroxisome proliferator-activated receptor α: an adaptive metabolic system. Annu Rev Nutr. 2001;21:193-230. https://doi.org/10.1146/annurev.nutr.21.1.193
47. Kyoung SK, Min JJ, Sungsoon F, Seul GY, Il YK, Je KS, Hyung-In Y, Dae HH. Anti-obesity effect of taurine through inhibition of adipogenesis in white fat tissue but not in brown fat tissue in a high-fat diet-induced obese mouse model. Amino Acids. 2019;51(2):245-54. https://doi.org/10.1007/s00726-018-2659-7
48. Chiang JYL, Ferrell JM. Up to date on cholesterol 7 alpha-hydroxylase (CYP7A1) in bile acid synthesis. Liver Res. 2020;4(2):47-63. https://doi.org/10.1016/j.livres.2020.05.001
49. Russell DW. The enzymes, regulation, and genetics of bile acid synthesis. Annu Rev Biochem. 2003;72:137-74. https://doi.org/10.1146/annurev.biochem.72.121801.161712
50. Tillander V, Alexson SHE, Cohen DE. Deactivating fatty acids: acyl-CoA thioesterase-mediated control of lipid metabolism. Trends Endocrinol Metab. 2017;28(7):473-84. https://doi.org/10.1016/j.tem.2017.03.001
51. Hunt MC, Siponen MI, Alexson SEH. The emerging role of acyl-CoA thioesterases and acyltransferases in regulating peroxisomal lipid metabolism. Biochim Biophys Acta. 2012;1822(9):1397-410. https://doi.org/10.1016/j.bbadis.2012.03.009
52. Schmitz W, Fingerhut R, Conzelmann E. Purification and properties of an α-methylacyl-CoA racemase from rat liver. Eur J Biochem. 1994;222(2):313-23. https://doi.org/10.1111/j.1432-1033.1994.tb18870.x
53. Zong L, Xing J, Liu S, Liu Z, Song F. Cell metabolomics reveals the neurotoxicity mechanism of cadmium in PC12 cells. Ecotoxicol Environ Saf. 2018;147:26-33. https://doi.org/10.1016/j.ecoenv.2017.08.028
54. Zufferey R, Pirani K, Cheung-See-Kit M LeeS, Williams TA, Chen DG, Hossain MF. The Trypanosoma brucei dihydroxyacetonephosphate acyltransferase TbDAT is dispensable for normal growth but important for synthesis of ether glycerophospholipids. PLoS One. 2017;12(7):e0181432. https://doi.org/10.1371/journal.pone.0181432
55. Shulga YV, Topham MK, Epand RM. Regulation and functions of diacylglycerol kinases. Chem Rev. 2011;111(10): 6186-208. https://doi.org/10.1021/cr1004106
56. Eichmann TO, Lass A. DAG tales: the multiple faces of diacylglycerol-stereochemistry, metabolism, and signaling. Cell Mol Life Sci. 2015;72(20):3931-52. https://doi.org/10.1007/s00018-015-1982-3
57. Mérida I, Torres-Ayuso P, Ávila-Flores A, Arranz-Nicolás J, Andrada E, Tello-Lafoz M, Liébana R, Arcos R. Diacylglycerol kinases in cancer. Adv Biol Regul. 2017;63:22-31. https://doi.org/10.1016/j.jbior.2016.09.005
58. Ipsen DH, Lykkesfeldt J, Tveden-Nyborg P. Molecular mechanisms of hepatic lipid accumulation in non-alcoholic fatty liver disease. Cell Mol Life Sci. 2018;75(18):3313-27. https://doi.org/10.1007/s00018-018-2860-6
59. Musso G, Gambino R, Cassader M. Recent insights into hepatic lipid metabolism in non-alcoholic fatty liver disease (NAFLD). Prog Lipid Res. 2009;48(1):1-26. https://doi.org/10.1016/j.plipres.2008.08.001
60. Zhang JJ, Zhao JL, Zheng XJ, Cai K, Mao QW, Xia HB. Establishment of a novel hepatic steatosis cell model by Cas9/sgRNA-mediated DGKθ gene knockout. Mol Med Rep. 2018;17(2):2169-76. https://doi.org/10.3892/mmr.2017.8140
61. Garcia D, Shaw RJ. AMPK: Mechanisms of cellular energy sensing and restoration of metabolic balance. Mol Cell. 2017;66(6):789-800. https://doi.org/10.1016/j.molcel.2017.05.032
62. Hurley RL, Anderson KA, Franzone JM, Kemp BE, Means AR, Witters LA. The Ca2+/calmodulin-dependent protein kinase kinases are AMP-activated protein kinase kinases. J Biol Chem. 2005;280(32):29060-6. https://doi.org/10.1074/jbc.M503824200
63. Anderson KA, Ribar TJ, Lin F, Noeldner PK, Green MF, Muehlbauer MJ, Witters LA, Kemp BE, Means AR. Hypothalamic CaMKK2 contributes to the regulation of energy balance. Cell Metabol. 2008;7(5):377-88. https://doi.org/10.1016/j.cmet.2008.02.011

Supplementary Material

The Supplementary Material is available at: http://www.serbiosoc.org.rs/NewUploads/Uploads/Xiao%20et%20al_7039_Supplementary%20Material.pdf