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Expression pattern of L-FABP gene in different tissues and its regulation of fat metabolism-related genes in duck

Jun He · Yong Tian · Jinjun Li · Junda Shen · Zhengrong Tao · Yan Fu · Dong Niu · Lizhi Lu

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Abstract Liver fatty acid binding protein (L-FABP) is a member of intracellular lipid-binding proteins responsible for the transportation of fatty acids. The expression pattern of duck L-FABP mRNA was examined in this study by quantitative RT-PCR. The results showed that duck L-FABP gene was expressed in many tissues, including heart, lung, kidney, muscle, ovary, brain, intestine, stomach and adipocyte tissues, and highly expressed in liver. Several lipid metabolism-related genes were selected to detect the regulation of L-FABP in duck. The expression of L-FABP and lipoprotein lipase was promoted by oleic acid. The L-FABP knockdown decreased the expression levels of peroxisome proliferator-activated receptor α (PPARα), fatty acid synthase and lipoprotein lipase by 61.1, 42.3 and 53.7 %, respectively ($P < 0.05$), but had no influences on the mRNA levels of PPARγ and leptin receptor. L-FABP might function through the PPARα to regulate the fat metabolism-related gene expression and play important roles in lipid metabolism in duck hepatocytes.

Keywords Duck · L-FABP · SiRNA · Fat metabolism-related gene · Oleic acid

J. He · Y. Tian · J. Li · J. Shen · Z. Tao · L. Lu (✉)
Institute of Animal Husbandry and Veterinary Science, Zhejiang Academy of Agricultural Sciences, 198 Shiqiao Road, Hangzhou 310021, Zhejiang, China
e-mail: lulizhi18@yahoo.com

J. He · Y. Fu · D. Niu (✉)
College of Animal Sciences, Zhejiang University, 388 Yuhangtang Road, Hangzhou 310058, Zhejiang, China
e-mail: nndd72@yahoo.com

Introduction

Fatty acid-binding proteins (FABPs) are members of intracellular lipid-binding proteins (iLBPs) that is involved in the transportation of fatty acids from plasma membrane to the sites of β oxidation and/or triacylglycerol or phospholipids synthesis [1, 2]. There are 12 FABPs found with similar functions and distinct structures in different tissues [3]. Liver-type fatty acid-binding protein (L-FABP) is the first member of iLBPs that is cloned from recombinant sources. One L-FABP molecule could bind two fatty acids, whereas other types of FABP could only bind one [4–11]. L-FABP functions widely and is expressed in various tissues. In human, L-FABP is expressed higher than other FABPs, and can be detected in many tissues, including liver, intestine, kidney and so on [12]. Polymorphism of L-FABP gene is associated with abdominal fat content in chicken [13]. L-FABP knockdown in rats could significantly reduce the transportation of fatty acids [14]. Meanwhile, L-FABP is able to bind both fatty acids and hydrophobic ligands, including acyl-CoA, lyso-phosphatidylcholine, bilirubin, bile salts, prostaglandins, peroxisome proliferators and so on [15–19]. In duck, L-FABP is reported to be associated with the intramuscular fat content [20]. Whereas, the information about duck L-FABP is still very limited. The expression pattern and regulation of L-FABP in lipid metabolism are still not clear.

The objectives of this study were to detect the mRNA expression pattern of duck L-FABP gene in various tissues, examine whether fatty acid could stimulate its mRNA expression, and investigate its potential role in regulating the expression patterns of fat metabolism-related genes, such as fatty acid synthase (FAS), lipoprotein lipase (LPL), leptin receptor (LEPR), peroxisome proliferator-activated receptor γ (PPARγ) and PPARα in duck hepatocytes.

Meterials and methods

Sample collection

Eight 10-week-old Sheldrake ducks were selected for tissue mRNA abundance detections of duck L-FABP. Fifteen tissues of each individual were collected, including brain, liver, pancreas, lung, spleen, chest muscle, leg muscle, gizzard, heart, kidney, ovary, intestine, abdominal fat, sebum and proventriculus, frozen into lipid nitrogen immediately for 2 days, and then stored at −70 °C for RNA extraction. All experimental animals were slaughtered following ethical standards.

Cell culture and siRNA transfection

Duck hepatocytes were obtained from 1-week-old Sheldrake ducks following the perfusion method [21]. After anaesthesia and sterile dissection, preheated buffer (D-Hank's, 20 ml/min) was firstly perfused into the liver through portal vein, then followed by the preheated digesting perfusate (D-Hank's, 0.5 mg/ml collagenase, 20 ml/min). The liver was isolated carefully and torn in serum-free medium (medium/RPMI1640, 10^{-7} M insulin, 10^{-8} M dexamethasone, 100 U/ml penicillin and 100 μg/ml streptomycin). The hepatocytes were cultured in incubator for 30 min (5 % CO_2, 37 °C), then filtered, centrifuged, and seeded on 6-well (35 mm) cell culture dishes (Sangon, Shanghai, China) at a density of $3 \times 10^5/cm^2$ with complete medium (medium/RPMI1640, 10 % FBS, 10^{-7} M insulin, 10^{-8} M dexamethasone, 100 U/ml penicillin and 100 μg/ml streptomycin) and cultured in incubator (5 % CO_2, 37 °C).

Oleic acid was added in the medium to assess its effects on the expression of duck L-FABP, PPARα, PPARγ, FAS, LEPR and LPL. Prepared siRNA for duck L-FABP (Genepharma, Shanghai, China) was transfected into the hepatocytes with liposome (TransGen Biotech Co., China). After 24 h of transfection, the hepatocytes were harvested for total RNA extraction and quantitative RT-PCR.

Total RNA extraction and reverse transcription

Total RNA was extracted with Trizol reagent (Takara, Osaka, Japan) and the cDNA was synthesized with M-MLV reverse transcriptase (Takara, Osaka, Japan) following the manufacturer's protocols.

Quantitative RT-PCR

Quantitative RT-PCR analyses of the selected tissues and hepatocytes were performed on ABI 7300 (Applied Biosystems, Foster City, CA, USA). The 20 μl reaction mixture containing 10 μl 2 × SYBR qPCR Mix, 2 μl cDNA template, 0.4 μM forward/reverse primer (Table 1), and 0.4 μl ROX reference dye (TransGen Biotech Co., China). The cycling protocol was 94 °C for 3 min, 40 cycles of denaturation at 94 °C for 10 s, annealing and extension at 60 °C for 30 s. All detections were performed with three repetitions.

Quantitative RT-PCR efficiency determination and statistical analysis

The amplification efficiency (E) of each pair of primers for quantitative RT-PCR in this study was estimated by serial dilution of liver cDNA (ten fold dilution for five times). The formula to calculate the amplification efficiency was $E = 10^{-1/\text{slope}} - 1$.

The relative expression levels of duck L-FABP, PPARα, PPARγ, FAS, LEPR and LPL genes were indicated by $2^{-\triangle Ct}$, and the $\triangle Ct = Ct_{\text{target gene}} - Ct_{\beta\text{-actin}}$. Data was shown as mean ± SD. Statistical significance was determined using the Student's t test. Difference was considered significant when $P < 0.05$.

Results

Expression pattern of duck L-FABP mRNA in different tissues

The expression of duck L-FABP gene in different tissues was determined by quantitative RT-PCR (Fig. 1). No

Table 1 Sequences of primer pairs and L-FABP siRNA

Primer	Nucleotide sequences (5′ → 3′)	Accesion No.	Size (bp)
L-FABP	F: actgcccccactgcgtt	HQ640427	184
	R: cgtcaccacaaagtcgtctcct		
FAS	F: tcaaccttctgctgaagc	AY613443	204
	R: agccatcagtgttactcc		
PPARα	F: cagagtcatccttgcagg	EF534215	229
	R: gtcaagattggagaagcc		
PPARγ	F: ttccaactccctttatggc	EF546801	232
	R: ggcattgtgtgacattcc		
LEPR	F: aggatctgctggtctgac	EU807932	208
	R: tccagtcactccagaacc		
LPL	F: acaatgtccacttgctgg	FJ859348	184
	R: taggtgtgtaggacatcc		
β-actin	F: atgtcgccctggatttcg	EF667345	165
	R: cacaggactccatacccaagaa		
siRNA	cccaagcaaucuguaacuatt		
	uaguuacagauugcuugggtt		

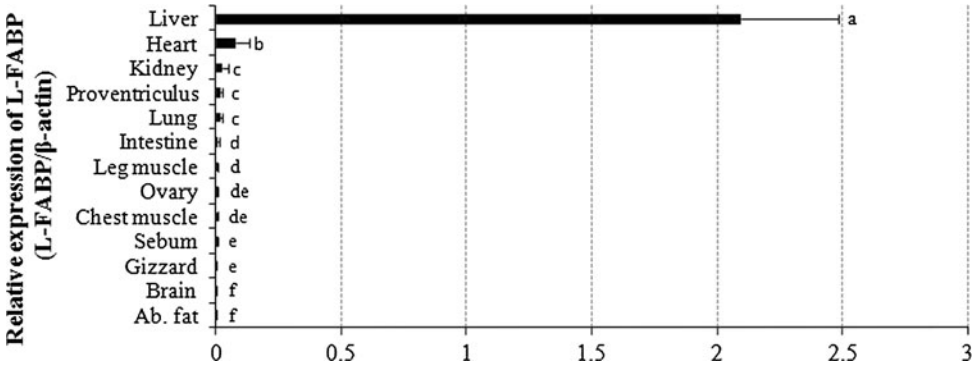

Fig. 1 Relative abundance of duck L-FABP mRNA in different tissues. mRNA expression levels were determined by quantitative RT-PCR in 15 tissues, and then normalized to that of β-actin. Data of 13 tissues are shown in the diagram, no expression was detected in pancreas and spleen. Each column represents the mean ± SD of 8 individuals. *Different letters* indicate significant difference at $P < 0.05$

significant difference was detected between male and female individuals ($P > 0.05$). The results showed that the L-FABP gene was highly expressed in liver, also expressed in other tissues except pancreas and spleen.

Effects of oleic acid on duck L-FABP gene expression

The duck primary hepatocytes were stimulated with oleic acid of different concentrations for 12 h to examine whether it could regulate L-FABP gene expression. Compared with the control, the L-FABP gene expression was increased by 3.15 times ($P < 0.05$) and 16.32 times ($P < 0.05$) when treated with 100 and 200 μM oleic acid, respectively (Fig. 2). The other data could not be obtained, as the hepatocytes treated with 500, 1,000 and 1,500 μM oleic acid appeared a rapid cell death. Most hepatocytes detached from the dishes, and no significant cell vitality was detected by MTT test.

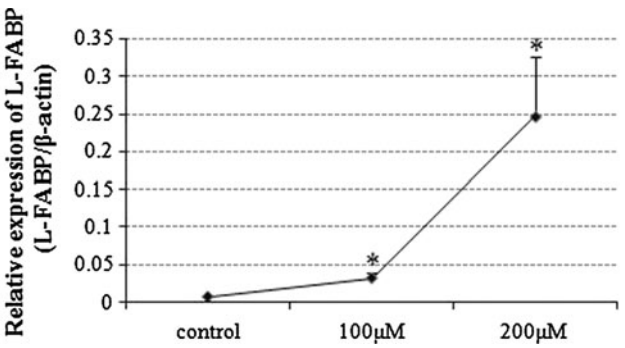

Fig. 2 Effects of oleic acid on duck L-FABP mRNA in hepatocytes. The mRNA abundances were determined by quantitative RT-PCR, normalized to that of β-actin. The results of 500, 1,000 and 1500 μM oleic acid treatment were not shown as those high concentrations of oleic acid led to rapid cell death of duck hepatocytes. Each spot represents the mean ± SD of three replicates. * indicates $P < 0.05$, ** indicates $P < 0.01$. (These also apply to the following figures)

Effects of duck L-FABP knockdown on the expression patterns of fat metabolism-related genes

Total RNAs of the knockdown and the control hepatocytes were isolated and analyzed by quantitative RT-PCR. Relative standard curves of all primers were shown in Fig. 3. As the results showed, with the siRNA transfection, the expression level of duck L-FABP gene in hepatocytes was reduced by 58.9 % ($P < 0.05$, Fig. 4a). Meanwhile, the expression levels of duck FAS, PPARα and LPL genes were down-regulated by 42.3 % ($P < 0.01$, Fig. 4b), 61.1 % ($P < 0.05$, Fig. 4c) and 53.7 % ($P < 0.05$, Fig. 4d), respectively. No significant difference was detected on the LEPR and PPARγ mRNA expression between the knockdown and the control group ($P > 0.05$).

The knockdown and the control hepatocytes were then treated with oleic acid (100 μM) for 8 h. The results showed that the L-FABP knockdown prevented the oleic acid–induced expression of PPARα and LPL genes (Fig. 5b, 5c, $P < 0.05$), but had no influence on the FAS gene expression (Fig. 5a, $P > 0.05$).

Discussion

Fatty acid-binding proteins play key roles in fatty acid transportation [1, 2], and fat traits in duck are related to health, nutritive value and feed conversion efficiency. It has been reported that L-FABP is associated with abdominal fat content and fatty acid transportation in chicken [13] and mouse [14]. In pig, the allele frequency of one SNP (C > T) in L-FABP gene of Chinese native breeds was detected to be significantly different from that of Yorkshire, a foreign breed famous for its high lean meat percentage and low fat content [22]. For studying the regulation of L-FABP in hepatocytes, the β-actin was

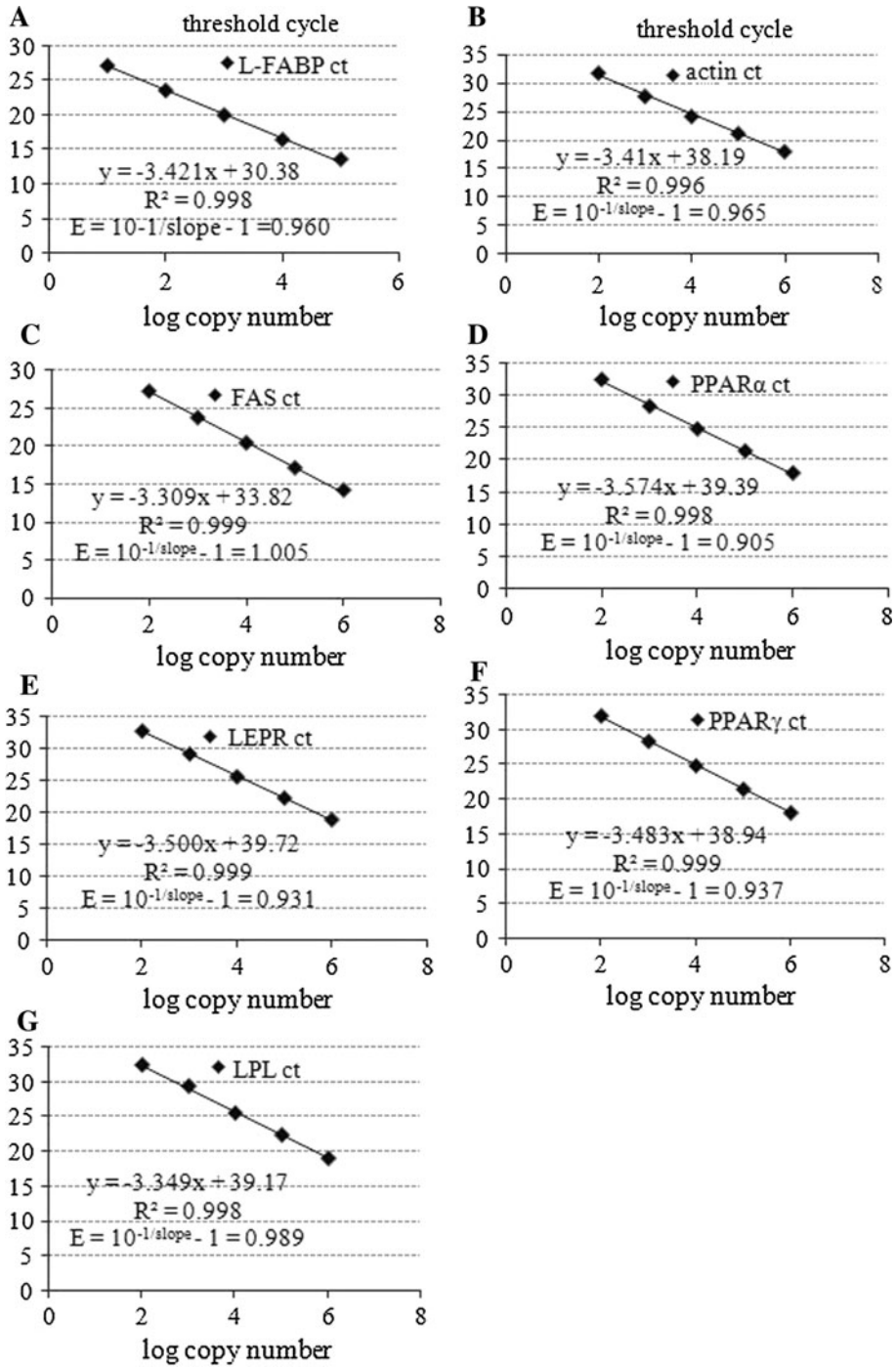

Fig. 3 Relative standard curves of the primers used in this study. The standard curves of all primers used in the paper were generated by plotting cycles at threshold fluorescence (*Ct*) against logarithmic values of liver cDNA amounts (mean ± SD; $n = 3$). Quantities of liver cDNA were expressed as dilution factors of the cDNA preparation (1, 1/10, 1/100, 1/1,000, 1/10,000). Data are shown in the diagram, including correlation coefficients (R^2) and amplification efficiencies (*E*)

chosen as the reference gene for mRNA expression normalization as this gene was reported to be the most used house-keeping gene. We also examined the expression of *β*-actin in different tissues and cultured hepatocytes, and no significant difference was detected. Our results showed that the L-FABP gene was highly expressed in liver, and liver is considered as the most important site for lipid metabolism in vivo. The L-FABP gene was found to be also expressed in various tissues, indicating it might have other potential functions besides fatty acid transportation. Binas et al. [23] found that L-FABP gene knockdown could reduce the content of long-chain fatty acid and suppress insulin resistance. Haluzík et al. [24] reported a similar conclusion in type-II diabetes. L-FABP was considered to be an important marker for type-II diabetes development [25].

However, the regulation of L-FABP gene has not been studied clearly yet. Peroxisome proliferator-activated receptors (PPARs) were reported to be able to regulate the

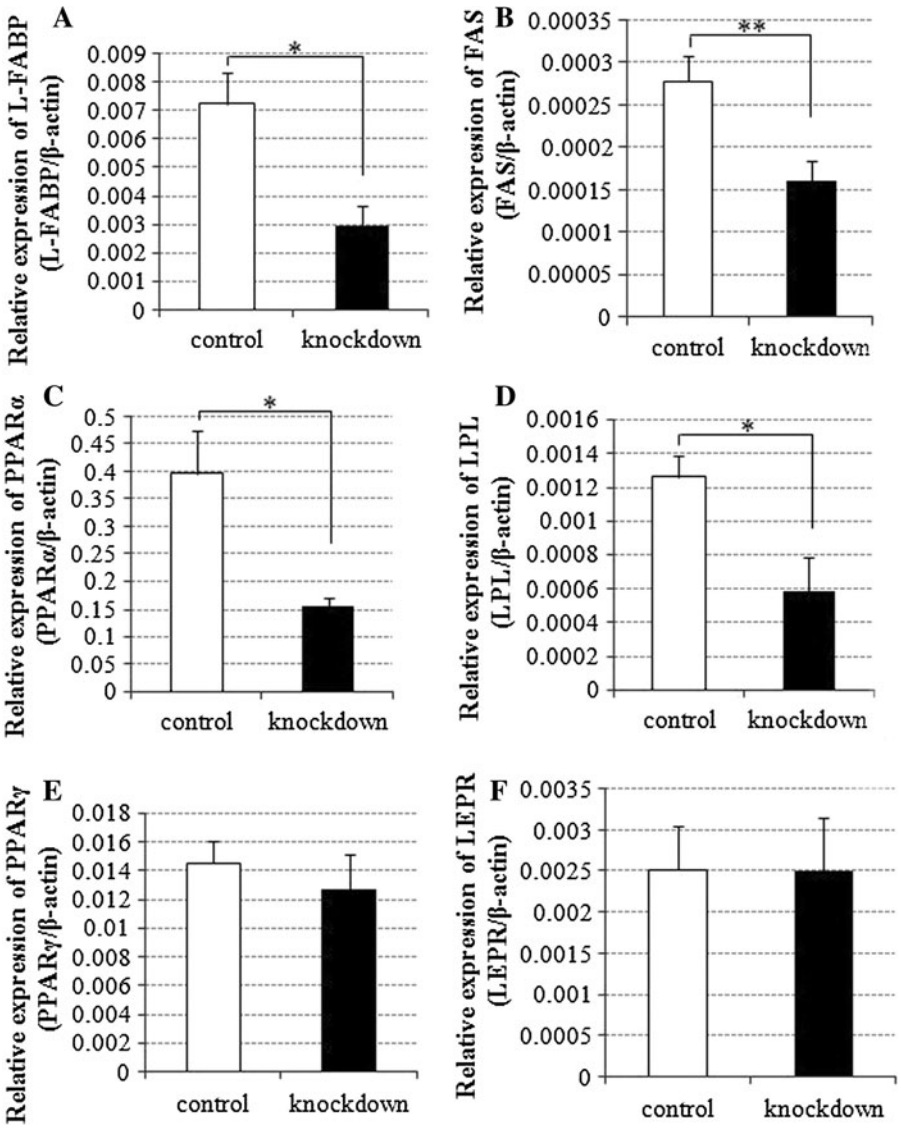

Fig. 4 Effects of L-FABP knockdown on the mRNA expression of L-FABP (**a**), FAS (**b**), PPARα (**c**), LPL (**d**), PPARγ (**e**) and LEPR (**f**) in duck hepatocytes. The control cells were transfected with negative siRNA that targets no gene. Significant differences were detected on the expression of L-FABP, FAS, PPARα and LPL, and no significant differences was found on the PPARγ and LEPR mRNA expression ($P > 0.05$)

expression of fat-related genes [26, 27]. FAS, LPL and PPARγ were demonstrated to be related to adipocyte differentiation in chicken [28]. Moreover, oleic acid could promote the expression of LEPR, LPL, PPARγ and FAS genes in goose adipocytes [29]. We tried the similar treatments in duck hepatocytes, and the results showed that the expression of L-FABP and LPL genes could be up-regulated by oleic acid stimulation. We also studied the regulation of duck L-FABP gene in lipid metabolism in hepatocytes by siRNA transfection. L-FABP gene knockdown significantly suppressed the expression of L-FABP, FAS, PPARα and LPL genes. And the oleic acid-treated knockdown group showed increased expression of FAS and LPL genes, no significant difference was detected for the PPARα expression (Fig 5). The influence of L-FABP knockdown on LEPR and PPARγ genes appeared not significant. It has been reported that PPARα is highly expressed in liver, kidney and epithelium, and PPARγ mainly functions in adipocyte and immunocyte [30]. The PPARα might act as a regulator to influence the expression levels of fat metabolism-related genes in hepatocytes. L-FABP knockdown significantly decreased the level of PPARα, implying that L-FABP might directly or indirectly regulate the PPARα, which further regulates its downstream fat-related genes in hepatocytes. PPARα was proven to be able to regulate the expression of LPL gene to reduce fat content [31]. Our previous study on adipocyte showed that adipocyte fatty acid-binding protein (A-FABP) gene knockdown significantly decreased the expression of PPARγ, LEPR, FAS and LPL (data not shown), therefore we also examined the same genes in hepatocytes, and found that PPARγ and LEPR were not influenced by L-FABP knockdown. PPARγ and LEPR might not be regulated by L-FABP in hepatocytes as they were proven to mainly function in adipocyte [25]. These results implied that the L-FABP gene might play important roles in

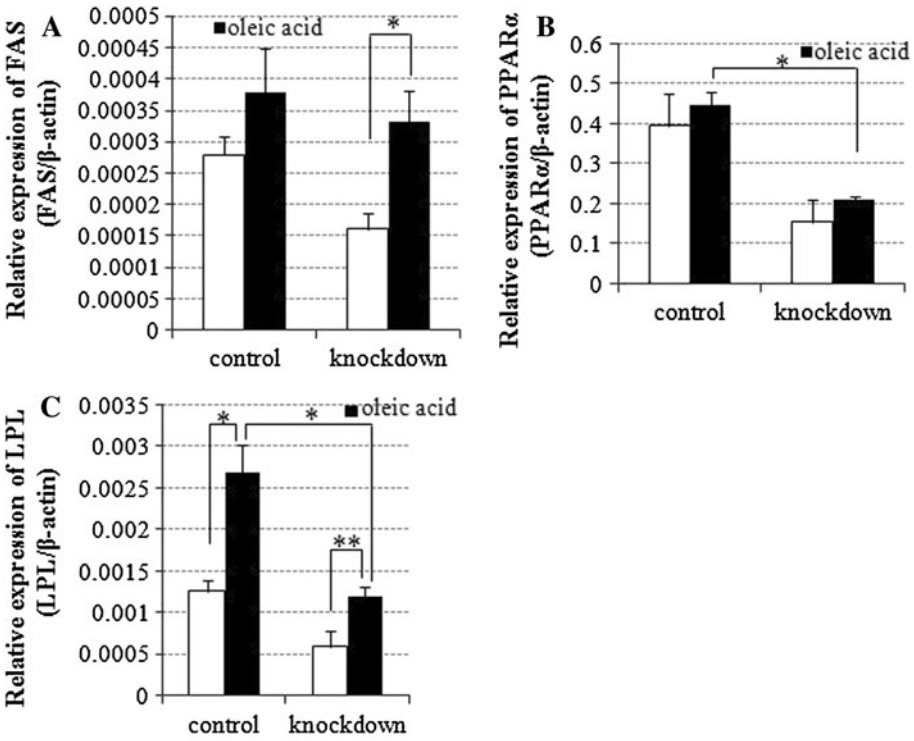

Fig. 5 Effects of oleic acid on the mRNA expression of FAS (**a**), PPARα (**b**) and LPL (**c**) in duck hepatocytes with or without L-FABP knockdown. The control cells were transfected with negative siRNA that targets no gene. Both the control and knockdown groups were treated with 100 μM oleic acid for 12 h. No significant differences were detected on the LEPR and PPARγ mRNA expression

lipid metabolism in hepatocytes. Meanwhile, we found that high concentration of oleic acid appeared toxic to duck hepatocytes, when it was above 200 μM in medium, the hepatocytes went to a rapid cell death. The mechanisms how duck L-FABP genes regulates lipid metabolism in liver and its other functions in different tissues still need to be further studied.

In conclusion, duck L-FABP genes was detected to be expressed in heart, lung, kidney, muscle, ovary, brain, intestine, stomach, adipocyte tissues, and highly expressed in liver. No expression was observed in pancreas and spleen. In the cultured duck hepatocytes, the mRNA expression of duck L-FABP and LPL genes were promoted by oleic acid. The L-FABP gene knockdown inhibited the mRNA expression of PPARα, FAS, LPL and L-FABP, but didn't influence the expression of PPARγ and LEPR genes. The L-FABP might function through the PPARα to regulate the fat metabolism-related gene expression.

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