Chicken adipocyte fatty acid-binding protein knockdown affects expression of peroxisome proliferator-activated receptor γ gene during oleate-induced adipocyte differentiation

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ABSTRACT Adipocyte fatty acid-binding protein (A-FABP) is expressed in both adipose cells and macrophages. As one of the downstream genes of peroxisome proliferator-activated receptor γ (PPAR-γ), A-FABP plays an important role in the lipid metabolism of adipocytes in mammal and poultry. However, studies in A-FABP null macrophages of mice showed that A-FABP was a critical regulator of PPAR-γ and could affect the expression of PPAR-γ. The current study was designed to investigate whether the same mechanism as that in macrophages exists in chicken adipocytes. After transfection with interference and overexpression plasmids of A-FABP in chicken adipocytes for 24 h, oleate was added to the medium. Then, lipid accumulation, nonesterified fatty acids (NEFA) in the medium, and expression of lipid metabolism-related genes were detected. The results showed that in the A-FABP knockdown adipocytes, lipid accumulation was decreased at 6 h and NEFA in the medium was higher at 1 and 6 h compared with that in the control group. Moreover, gene expression levels of lipoprotein lipase, perilipin, and PPAR-γ were higher than that of the control group ($P < 0.05$). In the A-FABP overexpression adipocytes, lipid accumulation and expression of lipid metabolism related genes were similar to that of the control group. However, NEFA in the medium was significantly lower in the A-FABP overexpression group 1 h after adding oleate ($P < 0.05$). The present study suggested that the A-FABP knockdown might lead to decreased lipid accumulation and upregulated expression of PPAR-γ in chicken adipocytes.

Key words: adipocyte fatty acid-binding protein, peroxisome proliferator-activated receptor γ, chicken, adipocyte, lipid accumulation

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INTRODUCTION

Adipocyte fatty acid-binding protein (**A-FABP**) is also referred to as aP2 and adipocyte lipid-binding protein (ALBP). It is expressed at high levels not only in white and brown adipose cells, but also in monocytes and macrophages (Pelton et al., 1999). The nature of its role in the adipocyte, as well as its function in the macrophage, is emerging from recent studies of animals null for A-FABP.

Mice null for A-FABP had lower plasma insulin levels and BW on a high-fat feeding (Hotamisligil et al., 1996). Within mice adipose tissue, it has been confirmed that functions between A-FABP and epithelial fatty acid-binding protein (**E-FABP**) are compensatory (Storch and Corsico, 2008). In addition, the FABP mouse models show altered lipid metabolism, including misregulated lipolysis and lipogenesis (Coe et al., 1999; Scheja et al., 1999; Shaughnessy et al., 2000). Analysis of these pathways indicates that genes linked to lipolysis are downregulated and multiple genes involved in lipogenesis are upregulated in the A-FABP/aP2 null compared with the E-FABP transgenic adipose tissue. However, not all peroxisome proliferator-activated receptor γ (**PPAR-γ**)-regulated genes changed and changes were not observed in PPAR-γ. The mechanism(s) responsible for the coordinate reprogramming of gene expression response to the loss or gain of A-FABP remains obscure, and a hypothesis that may be linked to the activity of PPAR-γ has been proposed (Hertzel et al., 2006).

Interestingly, E-FABP was also present in macrophages and regulated in an essentially identical manner. Unlike the compensatory regulation in adipocytes, E- FABP did not appear to be significantly upregulated in macrophages derived from A-FABP–/– mice

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(Makowski et al., 2001). In the A-FABP/aP2 null macrophages, the PPAR-γ activity and expression of PPAR-γ-regulated genes was elevated (Makowski et al., 2005). The mechanism responsible for the coordinate reprogramming of gene expression response to the loss of A-FABP is that A-FABP is a critical regulator of the PPAR-γ pathway in mammalian macrophages.

Wang et al. (2008) showed that A-FABP was downregulated when PPAR-γ was knocked down in the chicken adipocytes, which indicated that A-FABP was one of the downstream genes of PPAR-γ. Although the results in mice adipocytes showed that expression of PPAR-γ-related genes, except PPAR-γ, were changed when the A-FABP gene was knocked out, there was no confirmative proof for the mechanism underlying this process (Hertzel et al., 2006). The objective of the present study was to investigate whether the same mechanism as that in mice macrophages exists in chicken adipocytes.

MATERIALS AND METHODS

Birds

Thirty Arbor Acres male birds were kept in the same environmental conditions and had free access to food and water. Commercial corn-soybean-based diets that met all National Research Council requirements (NRC, 1994) were provided to the birds. All treatments of animals were in accordance with the Institute for Laboratory Animal Research (ILAR) Guide for Care and Use of Laboratory Animals.

Chicken Primary Adipocyte Culture

Chicken preadipocytes were isolated in a pool of abdominal adipose tissues from five 13-d-old Arbor Acres male birds for 1 experiment (interference or overexpression). The results in the current study were obtained from 3 independent interference and overexpression experiments. The cells were seeded at a density of 1×10^6 cells/cm^2 in medium and cultured at 37°C in a humidified, 5% CO_2 atmosphere according to previously published methods (Cryer et al., 1987; Ramsay and Rosebrough, 2003) with several modifications.

Transfection

Chicken preadipocytes were plated on 6-well or 12-well plates in Dulbecco's modified Eagle's medium F12 without antibiotics. Transfection was carried out according to the directions for Lipofectamine 2000 (Invitrogen, Carlsbad, CA) when cells were at >80% confluence. The transfection was conducted with the interference and overexpression plasmids of A-FABP, which were constructed according to our previous work (Shi et al., 2010a). After transfection for 24 h, oleate at 180 μ*M* was added to the medium. Then, the expression of A-FABP, cell lipid accumulation, nonesterified fatty acids (**NEFA**) in medium, and expression of genes related to lipid metabolism were detected at 1, 6, 12, 24, and 48 h after oleate induction.

Western Blot Analysis

Cultured cells were washed with PBS. Aliquots of detergent-solubilized cells were separated by 12% SDS-PAGE and transferred to an Immun-Blot PVDF membrane (Millipore, Billerica, MA). To block nonspecific binding, the membrane was incubated in blocking buffer (PBS with 5% nonfat dry milk) for 1 h at room temperature. The membrane was first immunoblotted with rabbit anti-chicken A-FABP antiserum (1:3,000; Shi et al., 2010b) for 1 h at room temperature. After being washed with PBS with 0.05% Tween-20 (**PBST**), the membrane was immunoblotted with goat anti-rabbit IgG conjugated with horseradish peroxidase (1:5,000; ZSGB-Bio, Beijing, China) for 1 h at room temperature. After washing with PBST, the immunoreactive protein on the membrane was visualized using enhanced chemiluminescence and exposed to x-ray film (Kodak, New York, NY). Mouse anti-chicken glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**) antibody (Beyotime Institute of Biotechnology, Jiangsu, China) and peroxidase-conjugated AffiniPure goat anti-mouse IgG (H^+L; ZSGB-Bio) were used to detect GAPDH. Immunoreactive protein levels were determined semiquantitatively by densitometric analysis using the UVP system Labworks TM3.0 (UVP, Upland, CA). Results were expressed as the relative quantity of A-FABP/GAPDH.

Detection of Cell Lipid Accumulation, NEFA in Medium, and Gene Expression

Lipid accumulation, NEFA in medium, and expression level of genes related to lipid metabolism were detected after adding oleate.

Lipid accumulation was measured by oil red O staining extraction assay (Sen et al., 2001). Cultured cells were fixed with 10% fresh formalin for 30 min at room temperature, rinsed twice in PBS, and incubated in filtered oil red O staining solution [1% (wt/vol) oil red O dye in isopropanol] for 40 min. After the staining solution was removed, oil red O was extracted from the cells using 100% isopropanol and then the isopropanol was measured at 500 nm using an UV Spectrophotometer 1000 (Pharmacia, Piscataway, NJ).

Nonesterified fatty acids in medium were detected according to the methods of Coe et al. (1999) with several modifications using the NEFA kit (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China). Results were expressed as follows, where OD_{440} = optical density at 440 nm:

Table 1. Primer sequences (F = forward, R = reverse) of real-time, reverse-transcription PCR

Gene name[1]	Accession no.	Sequence
GAPDH	K01458	F 5′-TGACGTGCAGCAGGAACAC-3′ R 5′-CAGTTGGTGGTGCACGATG-3′
FAS	NM205155	F 5′-AAGGAGGAAGTCAACGG-3′ R 5′-TTGATGGTGAGGAGTCG-3′
ACC	NM205505	F 5′-TTCCTACCAAGACTCCCTAT-3′ R 5′-GGTTTCTACGGCAACTACTC-3′
LPL	NM205282	F 5′-GGTCCGGGCCATGTTGA-3′ R 5′-CAGGTTGGTGCGGGTGA-3′
PPAR-γ	AF163811	F 5′-TACATAAAGTCCTTCCCGCTGACC-3′ R 5′-TCCAGTGCGTTGAACTTCACAGC-3′
Perilipin	NM_001127439	F 5′-GGGGTGACTGGCGGTTGTA-3′ R 5′-GCCGTAGAGGTTGGCGTAG-3′
ATGL	EU240627	F 5′-TCTACTGTGGGCTGATACCT-3′ R 5′-GTGGAACTGTCTCGTGGG-3′
E-FABP	BI394974	F 5′-CAAATGGTGCCTGGTCTC-3′ R 5′-CAGTCTTCTTGCCATCCC-3′

[1]GAPDH = glyceraldehyde-3-phosphate dehydrogenase; FAS = fatty acid synthase; ACC = acetyl-CoA carboxylase; LPL = lipoprotein lipase; PPAR-γ = peroxisome proliferator-activated receptor γ; ATGL = adipose triglyceride lipase; E-FABP = epithelial fatty acid-binding protein.

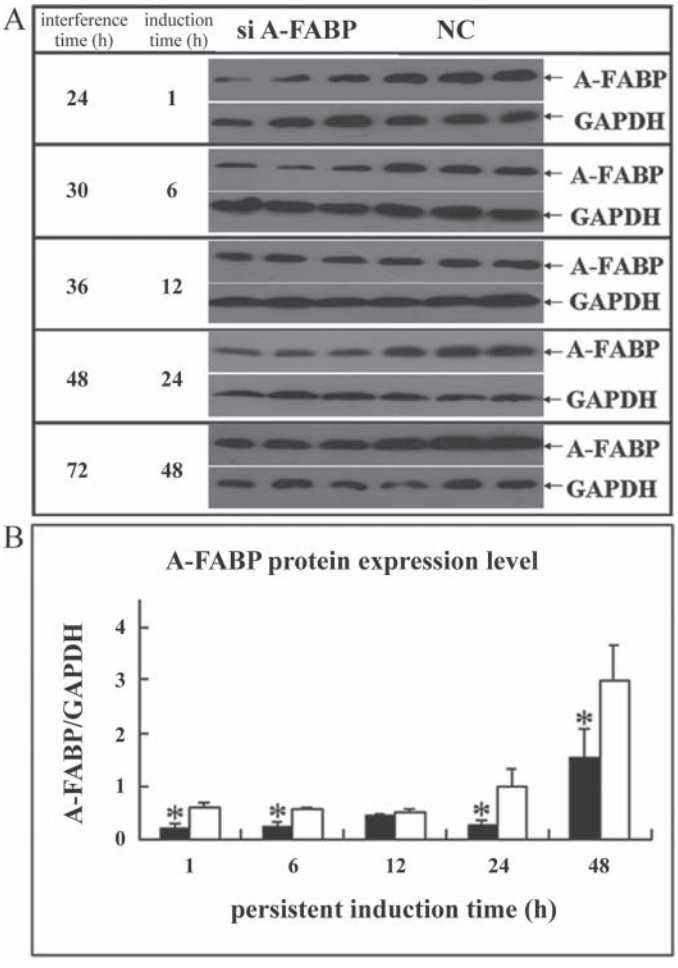

Figure 1. Western blot analysis of adipocyte fatty acid-binding protein (A-FABP) level. A) Western blot analysis of A-FABP in adipocytes from A-FABP interference (si A-FABP, which were transfected with the A-FABP interference plasmids) and irrelevant interference groups (NC); B) quantitation of A-FABP from Western blot analysis. Graph depicts the A-FABP levels in si A-FABP and irrelevant interference groups, plotted with the mean value and standard deviation (n = 3). The black columns represent si A-FABP groups, and the white columns represent irrelevant interference groups. *Significant downregulation ($P < 0.05$). GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

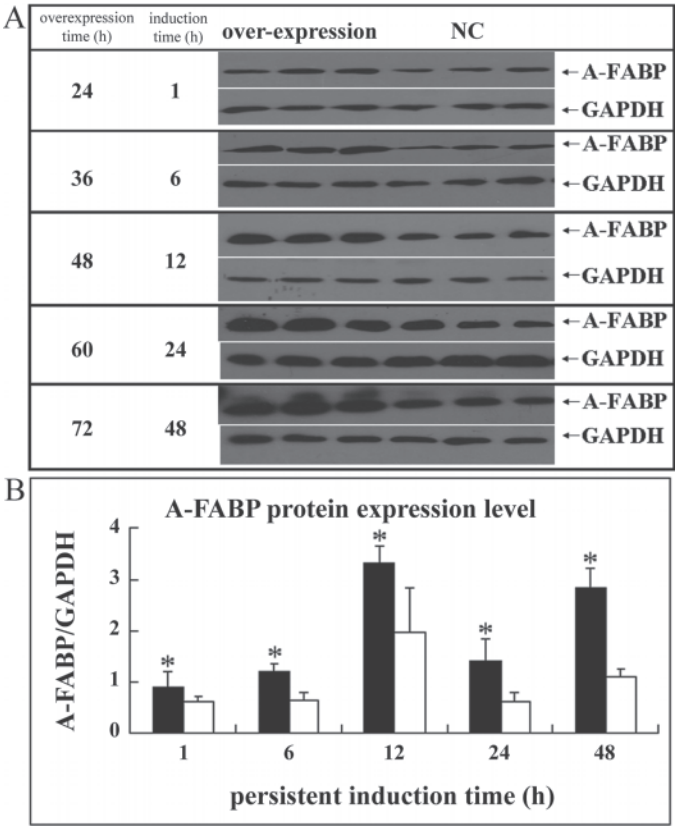

Figure 2. Western blot analysis of adipocyte fatty acid-binding protein (A-FABP) level. A) Western blot analysis of A-FABP in adipocytes from A-FABP overexpression and empty pcDNA3.1 vector groups (NC); B) quantitation of A-FABP from Western blot analysis. Graph depicting the A-FABP levels in A-FABP overexpression and empty pcDNA3.1 vector groups, plotted with the mean value and standard deviation (n = 3). The black columns represent the A-FABP overexpression groups, and the white columns represent empty pcDNA3.1 vector groups. *Significant upregulation ($P < 0.05$). GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

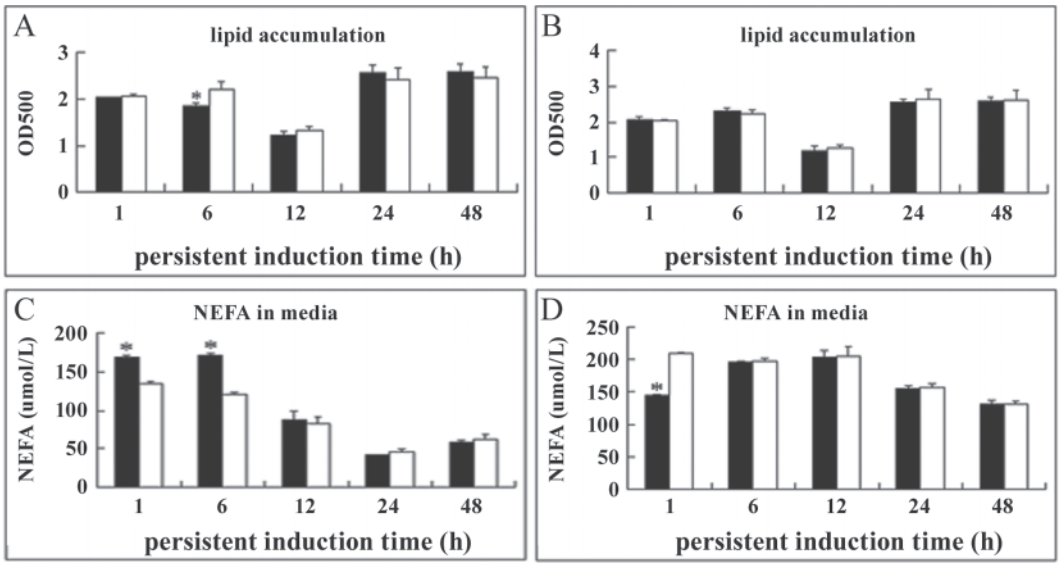

Figure 3. Analysis of lipid accumulation and nonesterified fatty acids (NEFA) in media of adipocytes. A) Analysis of lipid accumulation in adipocytes of si adipocyte fatty acid-binding protein (A-FABP, which were transfected with the A-FABP interference plasmids) and control groups (which were transfected with the A-FABP irrelevant interference plasmids); B) analysis of lipid accumulation in adipocytes of A-FABP overexpression and control groups; C) analysis of NEFA in media from adipocytes of si A-FABP and control groups; D) analysis of NEFA in media from adipocytes of A-FABP overexpression and control groups. Results are given with the mean value and standard deviation (n = 3). For A and C, the black columns represent the si A-FABP group, and the white columns represent the control group; for B and D, the black columns represent the A-FABP overexpression group, and the white columns represent the control group; OD = optical density. *Significant upregulation or downregulation ($P < 0.05$).

$$\text{NEFA } (\mu\text{mol/L}) = \frac{\text{OD}_{440}\ \text{sample} - \text{OD}_{440}\ \text{blank}}{\text{OD}_{440}\ \text{standard} - \text{OD}_{440}\ \text{blank}} \times 1,000.$$

Total RNA was isolated from cells using the RNAfast200 purification kit (Fastagen Biotech, Shanghai, China). Reverse transcription (**RT**) was performed according to the directions of the RNA PCR Kit (TaKaRa, Dalian, China). The expression levels of chicken lipid metabolism related genes were tested by real-time RT-PCR using SYBR Premix Ex Taq (TaKaRa). Reaction mixtures were incubated in an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) programmed to conduct 1 cycle at 95°C for 10 s and 40 cycles at 95°C for 5 s and at 60°C for 34 s. Dissociation curves were analyzed with Dissociation Curve 1.0 software (Applied Biosystems, Foster City, CA) for each PCR reaction to detect and eliminate possible primer-dimer artifacts. The expression of GAPDH was chosen as the internal reference. The sequences of the primers used to analyze the genes expression level are shown in Table 1.

Statistical Analysis

Data were subjected to *t*-test. Results were presented as the mean value and standard deviation. Differences were considered significant at $P < 0.05$ unless otherwise specified.

RESULTS

Knockdown and Overexpression of A-FABP in Adipocytes

Western blot was performed to detect A-FABP protein expression level after knockdown and overexpression of A-FABP in adipocytes. The results demonstrated the expected decreased expression of A-FABP in the interference group at 1, 6, 24, and 48 h. However, there was no significant difference between the interference and negative control groups at 12 h after adding oleate (Figure 1). The expected increased expression of A-FABP in the overexpression group was detected at all of the 5 detection times (Figure 2).

Effect of A-FABP on Lipid Accumulation in Adipocytes

Oil red O staining extraction assay was used to analyze the difference in lipid accumulation among the groups. The lipid accumulation of adipocytes from the A-FABP knockdown group was decreased significantly compared with that of the negative control group at 6 h after adding with oleate ($P < 0.05$). There was no significant difference of lipid accumulation in adipocytes between the A-FABP overexpression and control groups at any of the 5 detection time points (Figure 3).

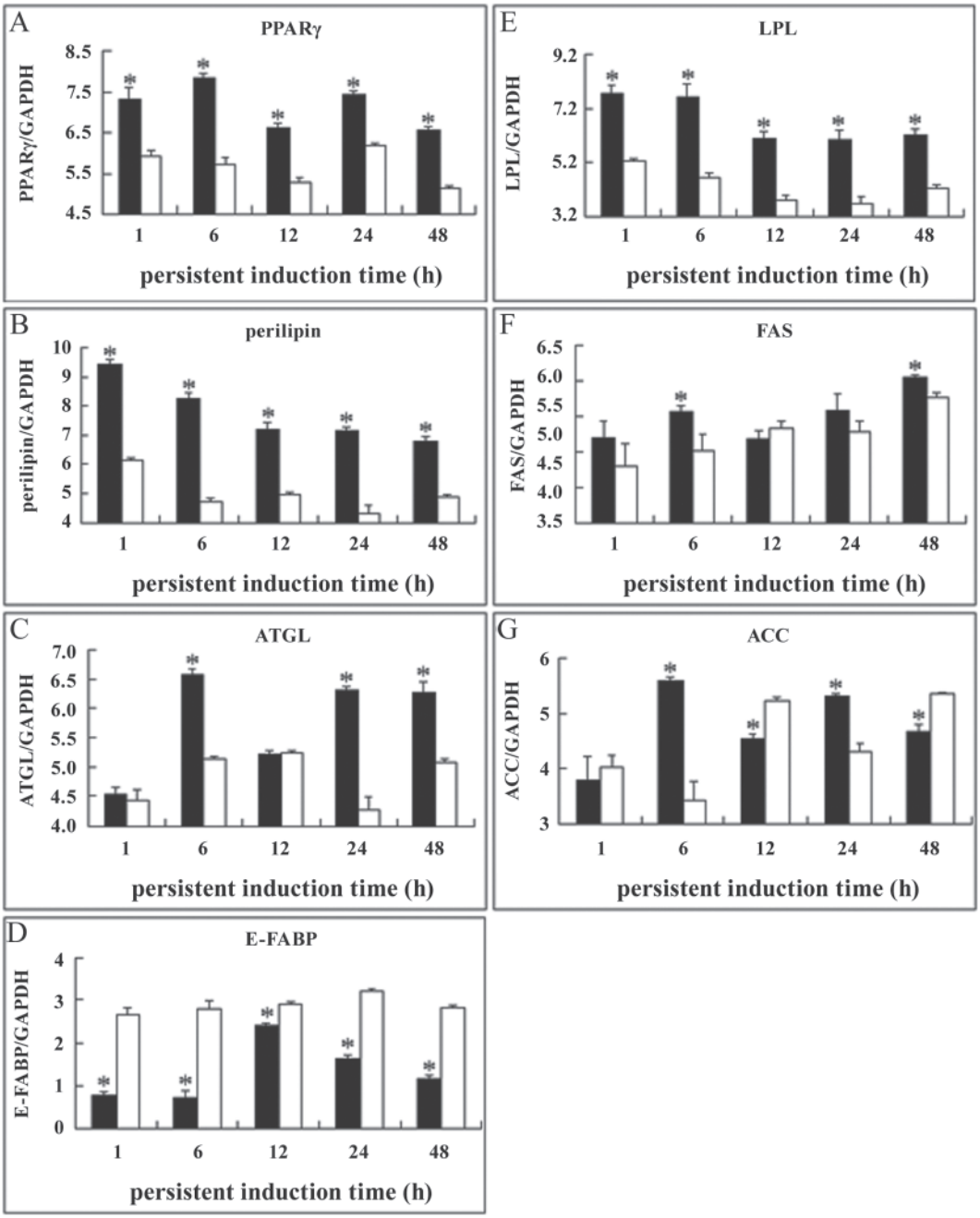

Figure 4. Analysis of gene expression levels in the adipocytes of si A-FABP (which were transfected with the A-FABP interference plasmids) and control groups (which were transfected with the A-FABP irrelevant interference plasmids). A) peroxisome proliferator-activated receptor γ (PPAR-γ); B) perilipin; C) adipose triglyceride lipase (ATGL); D) epithelial fatty acid-binding protein (E-FABP); E) lipoprotein lipase (LPL); F) fatty acid synthase (FAS); G) acetyl-CoA carboxylase (ACC). The expression levels of each gene were determined by real-time reverse-transcription PCR, and the expressed RNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a ratio of gene of interest: GAPDH mRNA levels. Results are given with the mean value and standard deviation (n = 3). The black columns represent the si A-FABP group, and the white columns represent the control group. *Significant upregulation or downregulation ($P < 0.05$).

Effect of A-FABP on NEFA in Medium of Adipocytes

To determine whether the changes of A-FABP expression level in adipocytes could be linked to oleate uptake, the level of NEFA in medium was evaluated in the A-FABP knockdown, overexpression, and control groups. The amount of NEFA in medium in the A-FABP knockdown adipocytes was higher than that of the control group at 1 and 6 h after adding oleate ($P < 0.05$). The amount of NEFA in medium in the A-FABP overexpression adipocytes was lower than that of the control group at 1 h after adding oleate ($P < 0.05$; Figure 4).

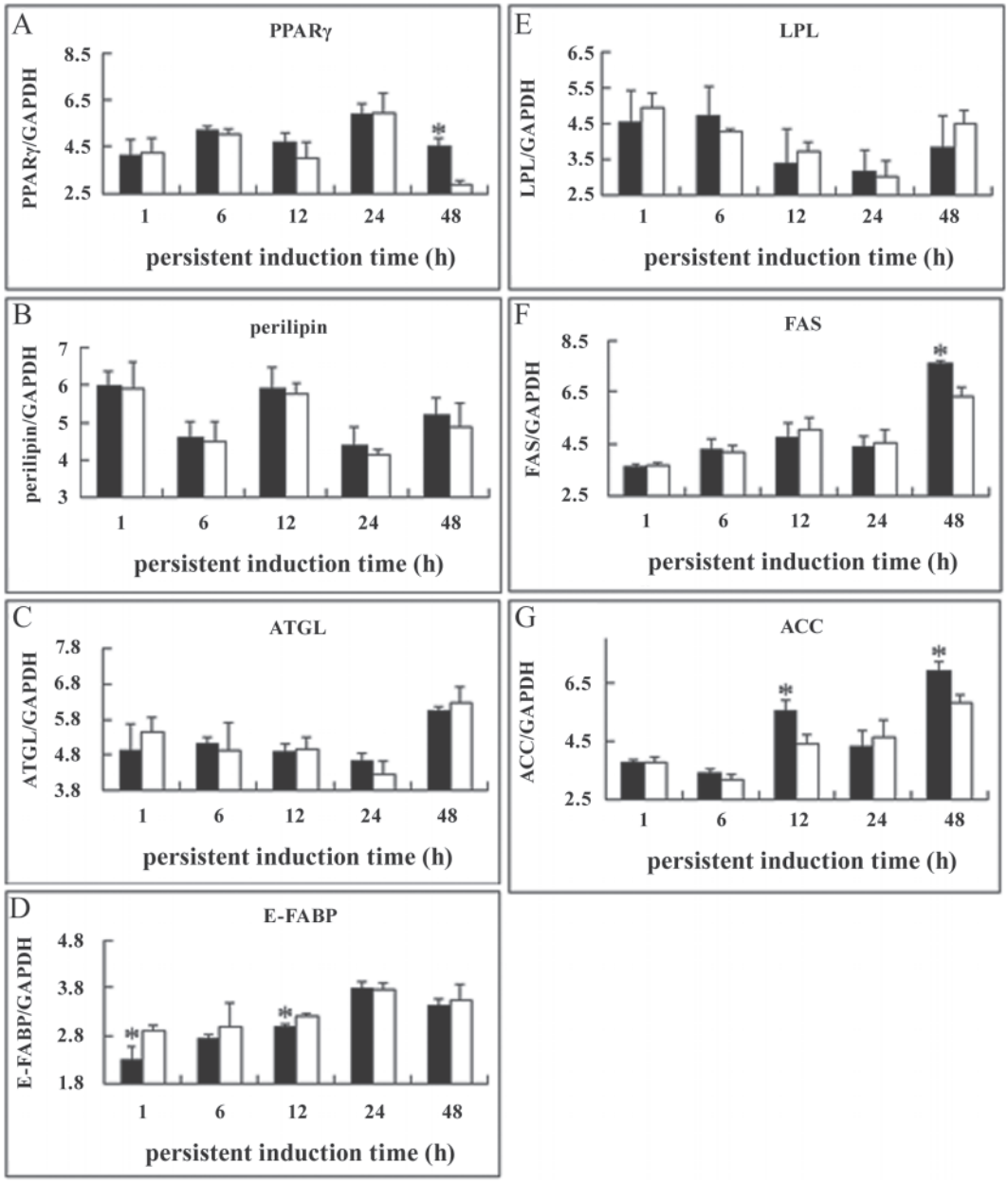

Figure 5. Analysis of gene expression levels in the adipocytes of A-FABP overexpression and control groups. A) peroxisome proliferator-activated receptor γ (PPAR-γ); B) perilipin; C) adipose triglyceride lipase (ATGL); D) epithelial fatty acid-binding protein (E-FABP); E) lipoprotein lipase (LPL); F) fatty acid synthase (FAS); G) acetyl-CoA carboxylase (ACC). The expression levels of each gene were determined by real-time reverse-transcription PCR, and the expressed RNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a ratio of gene of interest: GAPDH mRNA levels. Results are given with the mean value and standard deviation (n = 3). The black columns represent the A-FABP overexpression group, and the white columns represent the control group. *Significant upregulation or downregulation ($P < 0.05$).

Effect of A-FABP on the Expression of Genes Related to Lipid Metabolism in Adipocytes

The expression levels of PPAR-γ, perilipin, adipose triglyceride lipase (**ATGL**), E-FABP, lipoprotein lipase (**LPL**), fatty acid synthase (**FAS**), and acetyl-CoA carboxylase (**ACC**) were detected by real-time RT-PCR. When A-FABP was downregulated, the expression of PPAR-γ, perilipin, and LPL increased significantly relative to that of the negative control group at 1, 6, 12, 24, and 48 h after adding oleate (Figure 4, panels A, B, and E). The expression of ATGL, FAS, and ACC genes did not change when A-FABP was knocked down (Figure 4, panels C, F, and G). Expression of E-FABP was decreased when the A-FABP gene was knocked down at 1, 6, 12, 24, and 48 h after adding oleate (Figure 4, panel D).

The expression levels of the 7 lipid metabolism-related genes did not change significantly in the A-FABP over-

expression group relative to that of the control group, which was transfected with the empty pcDNA3.1 vector (Figure 5).

DISCUSSION

Previous research has shown that fatty acids are inducers of the A-FABP gene transcription in preadipocytes of mammals (Amri et al., 1991). In the current study, we detected the expected decrease in expression of A-FABP in the interference group at 1, 6, 24, and 48 h after adding oleate. However, there was no significant difference between the interference and negative control groups at 12 h after oleate induction. The results indicated that oleate could activate transcription of A-FABP in chicken adipocytes as in mammalian adipocytes. In mammals, the expression of many genes, such as PPAR-γ and A-FABP, is modulated by fatty acids. At the same time, PPAR-γ can upregulate the expression of A-FABP. According to the results of our study, we presumed that A-FABP was upregulated by at least 2 pathways, a fatty acid and PPAR-γ, which neutralized the effect of interference plasmid 12 h after oleate induction. At other time points, as the action of oleate weakened with time, we detected the expected different expression of A-FABP between the interference and negative control groups.

Fatty acids are energy-rich molecules that play important metabolic roles. They are also an integral part of cells as membrane components, which can influence fluidity and receptor or channel function. Clearly in cells, the signaling molecule is the free fatty acid (i.e., not bound to albumin), which is transported in and out of cells with the help of a membrane protein, the fatty acid transporter (**FAT**). There are 6 potential FAT candidates in mammals: fatty acid translocase (FAT-CD36), fatty acid transport protein, mitochondrial aspartate aminotransferase, caveolin, adipose differentiation-related protein, and fatty acid-binding protein (FABP, a cytosolic protein that can bind to membranes) (Duplus et al., 2000). In the current study, we detected decreased oleate uptake at 1 and 6 h after adding oleate in the A-FABP knockdown adipocytes. Based on the results in mammals, we presumed that chicken A-FABP was one of the FAT, and the expression level of A-FABP might affect oleate uptake during the differentiation of adipocytes induced by oleate. This suggests that the decrease in A-FABP levels might decrease oleate uptake by adipocytes. The decreased oleate uptake at 1 and 6 h after induction might, in turn, lead to decreased lipid accumulation in the A-FABP knockdown adipocytes at 6 h.

To analyze the possible mechanism underlying the phenotype of decreased oleate uptake and lipid accumulation in the A-FABP knockdown adipocytes, the expression of PPAR-γ, perilipin, ATGL, E-FABP, LPL, FAS, and ACC were detected. The results showed that in the A-FABP knockdown adipocytes, the expression of PPAR-γ, perilipin, and LPL genes were upregulated ($P < 0.05$) at 1, 6, 12, 24, and 48 h after adding oleate. Numerous studies have shown that perilipin and LPL are the downstream genes of PPAR-γ pathway (Robinson et al., 1999; Barbier et al., 2002; Shimizu et al., 2004; Brasaemle, 2007). In the current study, we detected altered expression of PPRA-γ, accompanied by altered expression of downstream genes of PPAR-γ, in the A-FABP knockdown adipocytes at all detection time points, containing the time point of 12 h in which A-FABP gene was not knocked down efficiently. We presumed that A-FABP is a potential regulator of PPAR-γ in chicken adipocytes. It might function as a trigger for the expression of PPAR-γ instead of affecting the expression of PPAR-γ constantly. However, specific mechanisms need to be investigated further.

In addition, the results of the present study showed that downregulated expression of E-FABP gene accompanied the knockdown of A-FABP ($P < 0.05$), which is inconsistent with results in mice (Hertzel et al., 2006). We presumed that the distinction might be attributed to species specificity.

In the overexpression experiment, we detected the increased oleate uptake only at 1 h after adding oleate. Lipid accumulation and expression of lipid genes in the overexpression adipocytes were similar to that of the control group. The reason might be that A-FABP was 1 of 6 limiting factors for oleate uptake, and its higher expression level could induce increased oleate uptake transiently. Then, under the effect of other factors, oleate uptake returned to normal.

In conclusion, the results of the A-FABP knockdown experiment showed that the A-FABP knockdown might lead to decreased lipid accumulation and upregulated expression of PPAR-γ in chicken adipocytes.

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