Active immunization of fatty acid translocase specifically decreased visceral fat deposition in male broilers

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ABSTRACT Lipid accumulation of avian adipocytes is mainly dependent upon the fatty acid transmembrane uptake process mediated by membrane proteins, such as fatty acid translocase (FAT/CD36), fatty acid transport protein 1, and caveolin-2. To examine the effects of FAT/CD36 on spatial-specific fat deposition, 60 broiler chickens were randomly allocated to 2 groups by sex. Each male or female group contained 2 subgroups (n = 14–15) inoculated by intramuscular injection with chicken FAT/CD36 or BSA (control) immunogens at 34, 49, and 63 d. The subcutaneous and visceral fat deposits were measured, as were levels of plasma triglyceride and free fatty acid. Serum antibody titer was measured by ELISA. The mRNA expression levels of fatty acid transport-related genes in the adipose tissue of the male broilers were investigated to reveal the relationships among various fatty acid transporters. The results showed that active immunization with FAT/CD36 could significantly decrease the visceral fat of the male broilers by up to 40%, but it had no effect on subcutaneous fat stores of male broilers or on either site of fat deposition in female broilers. The concentration of plasma free fatty acids increased in the experimental groups for both male and female broilers. After the FAT/CD36 immunization, very low density lipoprotein receptor mRNA expression was upregulated in both the subcutaneous and visceral fat of male broilers, whereas peroxisome proliferator-activated receptor γ, FAT/CD36, and acyl-CoA binding protein mRNA expression levels were upregulated only in the visceral fat of male broilers. These results indicated a novel role of chicken FAT/CD36 in fat deposition, with sex- and spatial-specific effects.

Key words: yellow-feathered broiler, fat deposition, fatty acid translocase, active immunization

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INTRODUCTION

During the past few decades, the growth performance of broilers has been greatly accelerated by genetic breeding accompanied with excessive abdominal fat deposits, which not only attenuates the carcass quality but also reduces the feed conversion efficiency. However, a certain amount of subcutaneous fat is necessary for the flavor of the carcass. Therefore, the differential regulations of subcutaneous and visceral fat deposition are important to improve carcass quality of broilers.

The mechanism of spatial-specific fat deposition has remained unclear until now. In avian models, the liver, but not adipose tissues, is the main site of de novo fatty acid synthesis (Griffin et al., 1992). Therefore, fatty acids are obtained chiefly from the liver or the intestine in the form of chylomicrons and very low density lipoproteins (Mossab et al., 2002). The free fatty acids (**FFA**) are released by lipoprotein lipase and then facilitated transported by several membrane or cytoplasm proteins, including fatty acid translocase (**FAT/CD36**), fatty acid transport protein (**FATP**), and long-chain acyl-CoA synthetase (**ACSL**; Hamilton et al., 2002; Kampf and Kleinfeld, 2007).

First identified by Abumrad et al. (1999), FAT/CD36 was expressed by tissues with an active long-chain fatty acid (**LCFA**) metabolism, such as adipose, heart muscle, and skeletal muscle (Brinkmann et al., 2002; Feng et al., 2007; Shu et al., 2008). A series of evidence supports that FAT/CD36 could promote the synthesis of triglycerides in adipocytes and the clearance of chylomicrons from plasma (Drover and Abumrad, 2005; Pohl et al., 2005) as well as the lipid metabolism and fatty acid transport (Febbraio et al., 2002; Bonen et al., 2004; Holloway et al., 2008, 2009). Though FAT/CD36 was considered to be a fatty acid transporter because of the function on the fatty acids cross-membrane transportation, the transport mechanism of the process remains unknown and the function of FAT/CD36 during the process is still controversial in that FAT/

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CD36 has only 2 transmembrane domains and a larger extracellular domain, unlike other typical transporters with multiple transmembrane domains (Hajri and Abumrad, 2002). Furthermore, another opinion suggested that FAT/CD36 may act as sensor on the tongue for fat taste (Laugerette et al., 2005).

To reveal the multifunction of FAT/CD36, we previously cloned the cDNA for FAT/CD36 from the yellow-feathered broiler and found that the tissue distribution of FAT/CD36 mRNA was the same as mammals with the highest level in visceral fat tissue (Feng et al., 2007). Ontogenetic study revealed that FAT/CD36 mRNA was upregulated in association with increased visceral fat deposition in male (but not female) broilers, which indicated that FAT/CD36 might play an important role in site- and sex-specific fat deposition (Feng et al., 2007).

The purpose of this study was to investigate whether the block of FAT/CD36 by active immunity could affect chicken growth performance and specifically decrease visceral fat deposition. To further examine the effect, some proteins such as FATP1, acyl-CoA synthase of long-chain fatty acid 1 (**ACSL1**), and caveolin-2, which are involved in the uptake of fatty acid, were also put into transcriptional evaluation.

MATERIALS AND METHODS

Preparation of the Antigen

We had previously constructed the extramembrane domain of the FAT/CD36 prokaryotic expression vector and optimized the expression parameters. After renaturation and concentration, the fusion protein was used with Freund's adjuvant to prepare the FAT/CD36 antigen, of which the final concentration was 1 mg/mL. Bovine serum albumin (Biotech, Nanchang, China) was used as the control. Both were stored at 4°C for future study.

Birds and Active Immunization

The procedure of the experiment was conducted in accordance with "The Instructive Notions with Respect to Caring for Laboratory Animals" issued by the Ministry of Science and Technology of the People's Republic of China. Sixty 1-d-old yellow-feathered chicks were housed in a fan-ventilated house. Birds were grown under standard management conditions from 1 d to approximately 21 d of age and fed a starter diet. All of the birds were fed water and diet ad libitum. After the initial period, chicks were fed a grower diet and were randomly divided into 2 groups according to sex. Diets met NRC (1994) requirements. Each male or female group contained 2 subgroups (experimental and control group) with 15 birds independently housed in cages. Experimental group birds were inoculated by intramuscular injection with 1 mg of the chicken FAT/CD36 at 34, 49, and 63 d, whereas control group birds were treated with BSA immunogens. At each time point, serum was isolated from wing vein blood samples before injection and was stored at −20°C for further study.

Growth Performance and Sampling

During the trial, food intake was measured every week. The ADG was measured in 3 phases (35–48, 49–63, and 64–77 d). At 77 d of the trial (i.e., when birds were aged 77 d), all birds were killed for the evaluation of fat deposition. Subcutaneous fat thickness was measured. Abdominal fat and fat outside the gizzard were isolated and weighed to determine the percentage of visceral fat. At the same time, the samples of subcutaneous and visceral fat were collected immediately, immersed in liquid nitrogen, and then stored at −80°C.

Serum Antibody Titer

The serum antibody titer was determined by ELISA. The 96-well plate was coated with chicken FAT/CD36 fusion protein; serum samples were added afterward. Blocking solution was then added, followed by the addition of the secondary antibody. After 1 h of incubation, a 30-min color reaction was carried out. The optical density at 450 nm was detected after the termination reaction.

Serum Triglyceride and FFA Concentrations

Total triglyceride (**TG**) content in serum was determined using the Triglyceride Assay Kit (Biosino Bio-Technology and Science Inc., Beijing, China). The FFA content was determined by colorimetry using the Nonesterified Free Acids Assay Kit (Nanjing Jiancheng, Nanjing, China).

Total RNA Extraction

Total RNA of the subcutaneous and visceral fat from male broilers was extracted according to the instructions that accompany the TRIZOL Reagent (Beijing Saibaisheng Gene Technology Co. Ltd., Beijing, China) and treated with DNase I (Tiangen Biotech Co. Ltd., Beijing, China) to eliminate trace pollution with genomic DNA. The concentration of each RNA sample was then quantified by measuring the absorbance at 260 nm in a biophotometer (Eppendorf, Hamburg, Germany). Ratios of absorption (260/280 nm) were between 1.8 and 2.0 for all preparations. Aliquots of RNA samples were subjected to electrophoresis through agarose gels to verify their integrity.

Reverse-Transcription and Quantitative PCR

The reverse transcription reaction mixture (20 μL) contained 2 μg of total RNA and 5 μmol/L of oligo $dT_{(18)}$ (Takara, Dalian, China), which were incubated

Table 1. Real-time PCR primers

Gene[1]	GenBank accession no.	Primer sequence (5′–3′)
ACSL1	NM_001012578	F: AGACAAGCCTAATCCTAGAGGGG; R: GTCTTTCTTACGGTCTATGATCTGC
ACBP	NM_204576	F: ATGTCTGAGGCTGCGTTCCA; R: TTCCAGGCGTCCCACTTTG
β-Actin	NM_205518	F: CTCTTCCAGCCCTCCTTCCT; R: AGCACCGTGTTGGCGTAGAG
Caveolin-2	NM_001007086	F: CTGGTGTTGGGGATCGTCTT; R: CGGCCTACGCTATGAAAGAAT
FAT/CD36	DQ323177	F: GAGAATACGGACCAAGGGAGG; R: ACGCTGTAAGATGTCGGAGAAA
FATP1	DQ352834	F: CAGCAATCGCAGATCCTAAAAC; R: GTGTCAACCTGGGGTGAAAGA
LPL	NM_205282	F: TTTGAGTATGCTGATGCCCCTAT; R: CAACAGGCTTCTGAATCCCAAT
PPARγ	AF309648	F: GACCAAAGCCAAGGCAAGG; R: CCTCCACAGAGCGAAACTGAC
SREBP-1	AF278697	F: CCGAGGGAGACCATCTACAGC; R: CAACGCATCCGAAAAGCAC
VLDLR	NM_205229	F: CAGTGTTCTGGACCTGGCAAAT; R: GAGCATCCACCATTGTTGACC

[1]ACSL1: long-chain acyl-CoA synthase 1; ACBP: acyl-CoA binding protein; FAT/CD36: fatty acid translocase; FATP1: fatty acid transport protein 1; LPL: lipoprotein lipase; PPARγ: peroxisome proliferator-activated receptor γ; SREBP-1: sterol regulatory element-binding protein 1; VLDLR: very low density lipoprotein receptor.

at 70°C for 5 min and immediately chilled on ice before the addition of other reagents, including 4 μL of 5× RT buffer, 0.5 mmol/L of dNTP, 20 U of RNase inhibitor (Takara), and 200 U of MMLV reverse transcriptase (Promega, Madison, WI). The reaction mixture was incubated at 42°C for 1 h, followed by a final step of 80°C for 5 min. The products were diluted with 1× Tris-EDTA buffer and then stored at −20°C.

Quantitative PCR was used to detect the mRNA expression level, with β-actin as an internal standard. All of the primer sequences of PCR are shown in Table 1. The PCR amplification products of all genes were purified separately and cloned to the pMD18-T vector (Takara). The recombinant plasmids were diluted to gradient concentrations for use as templates for quantitative standard curves.

The reaction mixture (20 μL) of real-time PCR comprised 10 μL of 2× SYBR Green Master Mix (Toyobo, Tokyo, Japan), 4 μL of cDNA template, and 0.2 mmol/L of primers. The DNA amplification was performed at 95°C for 1 min, followed by 40 cycles at 95°C for 15 s, 60°C for 15 s, 72°C for 30 s, and 79°C for 35 s. Melting curves were analyzed after the reactions. According to the cycle threshold value and the standard curves, the original mRNA copy number of each sample was measured and normalized by the β-actin copy number. Primer sequences are shown in Table 1.

Statistical Analysis

Data were described as mean± SE and statistically analyzed with 1-way ANOVA and Duncan multiple comparison using SPSS 17.0 software (SPSS Inc., Chicago, IL). Significant difference was considered as $P < 0.05$.

RESULTS

Plasma Anti-FAT/CD36 Antibody

Plasma levels of antibody increased rapidly after intramuscular injection of the partial fragment of FAT/CD36 antigen and peaked after the second inoculation. Plasma antibody levels at 49, 63, and 77 d were significantly higher among the male and female broilers in the experiment group compared with those in the control group (Figure 1).

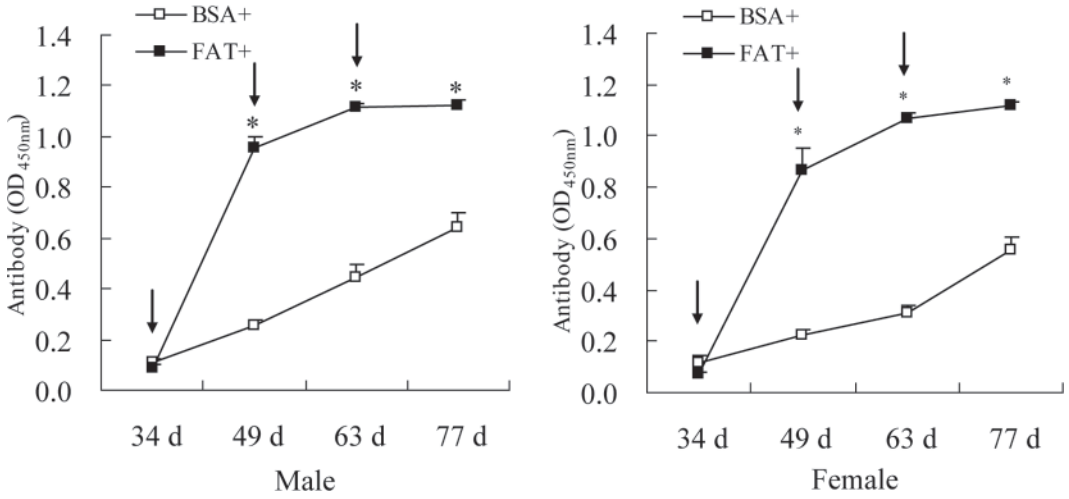

Figure 1. Plasma anti-fatty acid translocase (FAT/CD36) antibody level of yellow-feathered broiler in the FAT/CD36 active immunization experiment (n = 15). An asterisk (*) indicates significant difference ($P < 0.05$). Arrowheads indicate antigen was injected. BSA+: BSA inoculation control; FAT+: FAT/CD36 antigen inoculation group. OD = optical density.

Table 2. Food intake (g; mean ± SE) of yellow-feathered broilers at different phases

Group[1]	Phase 1 (35–48 d)	Phase 2 (49–63 d)	Phase 3 (64–77 d)
Male			
BSA+	707.92 ± 16.47	871.59 ± 24.84	959.73 ± 17.43
FAT+	649.45 ± 19.99*	892.65 ± 28.08	1,014.98 ± 10.23*
Female			
BSA+	545.99 ± 20.21	738.79 ± 23.54	858.27 ± 11.65
FAT+	591.54 ± 21.90	735.56 ± 21.14	819.25 ± 23.28

[1]BSA+: BSA inoculation control; FAT+: fatty acid translocase antigen inoculation group.
*Significant difference between FAT+ and BSA+ (control) in the same phase.

Feed Intake and ADG

The food intake of the male broilers was significantly decreased by FAT/CD36 immunization during the first 2 wk (phase 1). Food intake gradually recovered in the experimental group. During the last 2 wk of the trial, FAT/CD36 immunization induced an appetitive effect among the male broilers. Remarkably, the effect of FAT/CD36 immunization on the food intake of female broilers was the reverse of that observed among male broilers (Table 2).

The ADG did not differ significantly between the FAT/CD36 and BSA control groups during phases 1 and 2. However, during the last 2 wk of the trial, FAT/CD36 immunization significantly decreased the ADG of male broilers but not that of female broilers (Figure 2).

Fat Deposition

After the active FAT/CD36 immunization, the visceral fat of the male broilers, but not female broilers, decreased by about 40%. This change was significant. The thickness of subcutaneous fat remained unchanged among both male and female broilers (Figure 3).

Serum Triglyceride and FFA Concentrations

The concentration of serum TG was constant after FAT/CD36 active immunization. The concentration of serum FFA increased significantly among both male and female broilers (Figure 4).

Fatty Acid Transport-Associated Gene Expression

In subcutaneous fat, only very low density lipoprotein receptor (**VLDLR**) mRNA expression was upregulated by FAT/CD36 immunization. No significant changes in the expression of any other gene were observed (Figure 5 A). In contrast, the genes expressed in visceral fat were more sensitive to FAT/CD36 immunization. Levels of VLDLR, peroxisome proliferator-activated receptor γ (**PPARγ**), FAT/CD36, and acyl-CoA binding protein (**ACBP**) mRNA expression were simultaneously upregulated by FAT/CD36 antigen inoculation. However, levels of ACSL1, sterol regulatory element-binding protein-1 (SREBP-1), caveolin-2, and lipoprotein lipase remained unaltered (Figure 5 B).

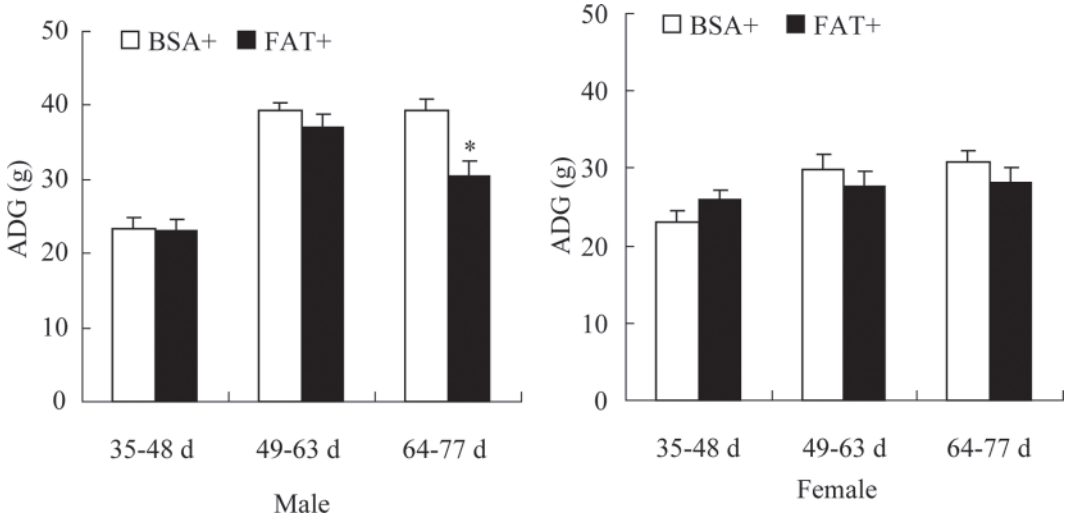

Figure 2. The ADG of yellow-feathered broilers in the fatty acid translocase (FAT/CD36) on different days in the experiment (n = 14–15). Data are mean ± SE. BSA+: BSA inoculation control; FAT+: FAT/CD36 antigen inoculation group. An asterisk (*) indicates significance difference between FAT+ and BSA+ in the same phase.

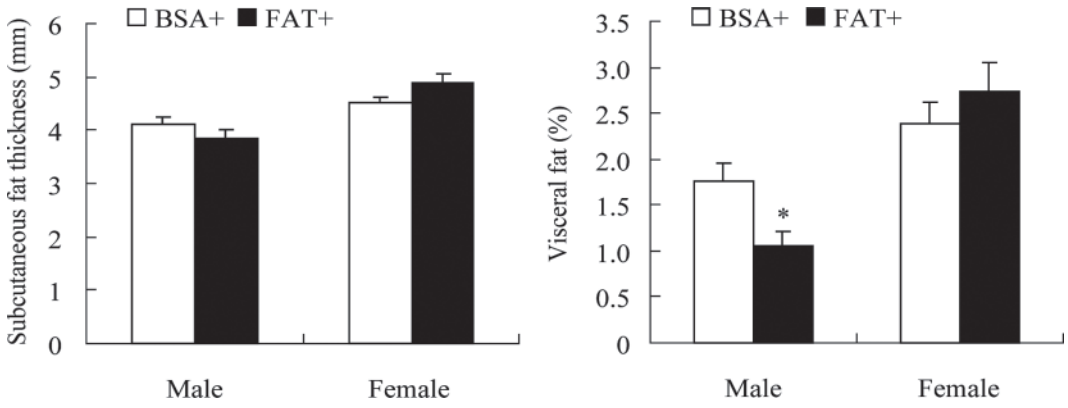

Figure 3. Subcutaneous fat thickness and the percentage of visceral fat of yellow-feathered broilers in the fatty acid translocase (FAT/CD36) active immunization experiment (n = 15). Data are mean ± SE. BSA+: BSA inoculation control; FAT+: FAT/CD36 antigen inoculation group. An asterisk (*) indicates significance difference between FAT+ and BSA+.

DISCUSSION

Membrane proteins associated with fatty acid transportation, such as FAT/CD36 (Coburn et al., 2001), FATP (Stahl, 2004), and caveolins (Okamoto et al., 1998), have been detected in recent years. Among these proteins, FAT/CD36 was found to be associated with diverse normal and pathologic processes (Febbraio et al., 2002). First, FAT/CD36 is highly expressed in adipose tissue and plays an important role in membrane binding and transport of LCFA (Sfeir et al., 1997; Abumrad et al., 1999; Coburn et al., 2000; Campbell et al., 2004; Ehehalt et al., 2008). The FAT/CD36-null mouse also had a 38% decrease in fat mass (Hajri et al., 2007). We previously found that the FAT/CD36 mRNA expression levels in visceral fat of male broilers increased gradually with age (Feng et al., 2007). In the present study, after blocking of the FAT/CD36, only the amount of visceral fat in male broilers was reduced significantly, whereas that of subcutaneous fat and both deposition sites in female broilers were unchanged. This result confirmed our previous hypothesis that FAT/CD36 has a site- and gender-specific function in broiler fat deposition.

The fat deposition for avians is determined by the de novo synthesis in liver and FFA cross-membrane transport. In our results, the serum FFA concentration was increased both in male and female birds, which indicated that FAT/CD36 blocking could decrease the utilization of FFA by peripheral tissues active in fatty acids metabolism such as skeletal muscle and fat. However, the de novo synthesis of TG in the liver was speculated to remain unchanged according to the constant serum TG concentrations we detected. Although both male and female birds had an increase in serum FFA concentration, their visceral fat deposition and food intake showed differently. Serum FFA concentration acts as a classic short-term appetite inhibitor (Shimabukuro, 2006). Thus, the food intake of female broilers was slightly decreased as expected. However, it was interesting to find that the food intake of male broilers was, on the contrary, significantly increased by FAT/CD36 blocking. These contradictory results might be explained by the decreased visceral fat deposition

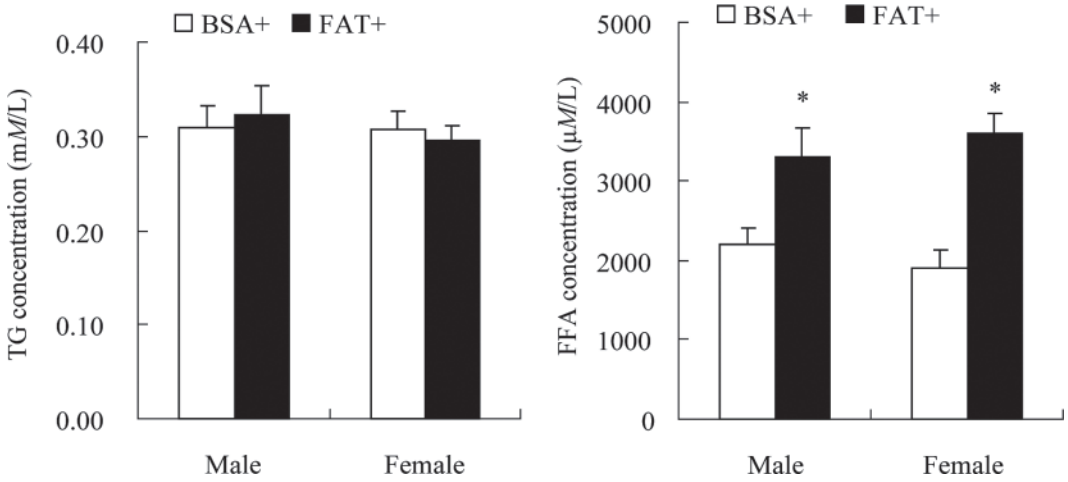

Figure 4. Plasma triglyceride (TG) and free fatty acid (FFA) concentrations of yellow-feathered broilers (n = 10–15). Data are mean ± SE. BSA+: BSA inoculation control; FAT+: fatty acid translocase (FAT)/CD36 antigen inoculation group. An asterisk (*) indicates significance difference between FAT+ and BSA+.

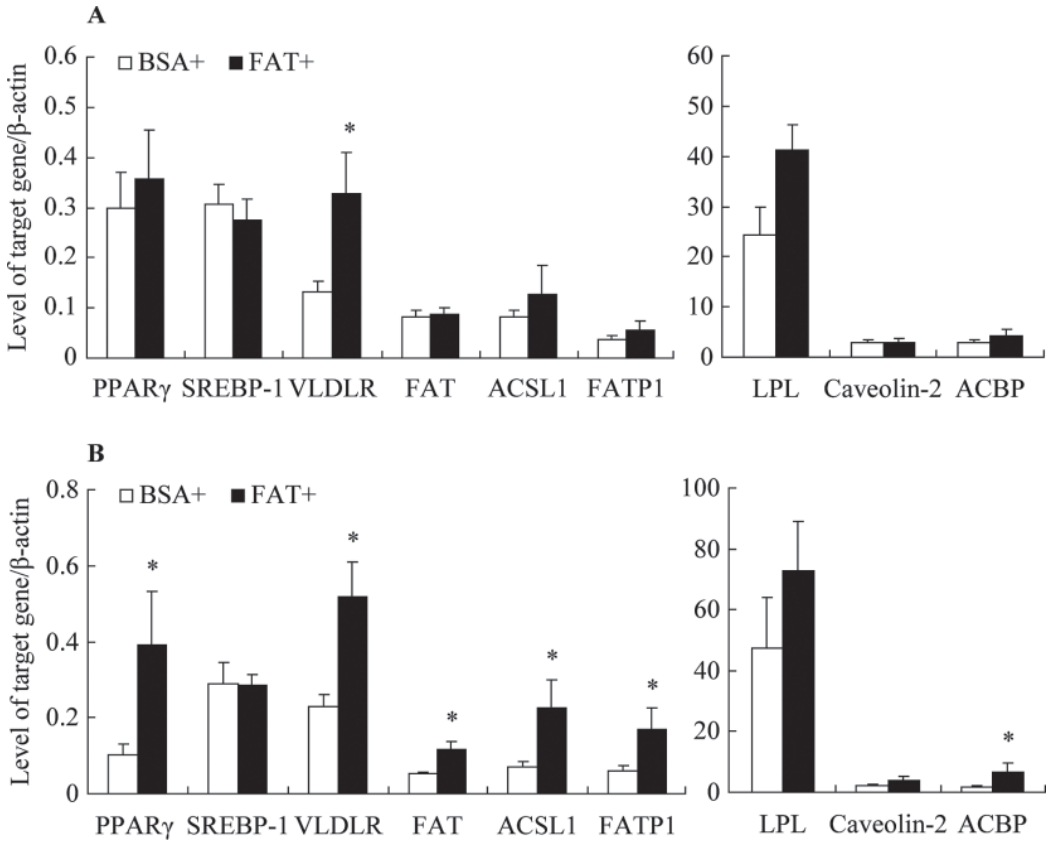

Figure 5. The mRNA expression of fatty acids transport-associated genes of male broilers (n = 6): A) subcutaneous; B) visceral. Data are mean ± SE. BSA+: BSA inoculation control; FAT+: FAT/CD36 antigen inoculation group. An asterisk (*) indicates significance difference between FAT+ and BSA+ of yellow-feathered broilers in the fatty acid translocase (FAT/CD36) active immunization experiment (n = 10–15). PPARγ: peroxisome proliferator-activated receptor γ; SREBP-1: sterol regulatory element-binding protein 1; VLDLR: very low density lipoprotein receptor; FAT: fatty acid translocase; ACSL1: long-chain acyl-CoA synthetase 1; FATP1: fatty acid transport protein 1; LPL: lipoprotein lipase; ACBP: acyl-CoA binding protein.

for the reason that several adipose-derived cytokines, such as leptin, have been reported to act as the primary depressors of food intake (Gao et al., 2007). Therefore, the increased food intake of male broilers might, at least partly, be owed to the prospective decrease of adipose-derived cytokines.

Increasing evidence supports that FAT/CD36 is a major protein associated with fatty acid uptake and it is preferentially found in lipid rafts, which can facilitate LCFA uptake (Pfeiffer et al., 2001; Mairhofer et al., 2002; Zeng et al., 2003; Pohl et al., 2005). However, an intense controversy exists concerning fatty acid uptake mechanism (Bonen et al., 2007; Kampf and Kleinfeld, 2007). Some researchers favor the view that adaptor molecules like FAT/CD36 at the plasma membrane might liberate LCFA from extracellular protein. And in this model, FATP, by providing CoA activity, are indirectly involved in the process (Ehehalt et al., 2006). One view holds that FAT/CD36 could help to increase the FFA concentration at the cell surface and therefore contributed in an indirect way to the process (Stahl et al., 2001; Stremmel et al., 2001). Others proposed that FAT/CD36 worked as a transporter at the plasma membrane (Bonen et al., 2002; Hajri and Abumrad, 2002).

Fatty acid transport is a complicated process that involves a multitransporter family (Kampf et al., 2007; Ehehalt et al., 2008; Harasim et al., 2008; Goldberg et al., 2009). Previously, the cooperation or cross-talk between different transport proteins was rarely investigated. Researchers found that, in Chinese hamster ovary cells, FAT/CD36 expression alone did not affect uptake of oleic acid. And it is proposed that the ability of FAT/CD36 to mediate enhanced uptake of LCFA is dependent on coexpression of other proteins or factors that are lacking in Chinese hamster ovary cells (Eyre et al., 2008). In this paper, our results showed that, in contrast to the control group, most of the mRNA expression level of proteins involved in fatty acid transport in the experimental group had been markedly upregulated at varying degrees. It could be inferred that a compensatory mechanism might exist between different fatty acids transport-associated membrane proteins, or these genes were probably feedback regulated by extracellular FFA.

Caveolae, which have been suggested to be involved in LCFA uptake, are small surface invaginations that have been postulated to be formed by clustering of rafts at the cell surface (Kurzchalia and Parton, 1999; Stremmel et al., 2001). Caveolin-1 and caveolin-2 constitute a

framework of caveolae in nonmuscle cells. It is reported that caveolin-2, especially its β isoform, is targeted to the surface of lipid droplets (Fujimoto et al., 2001). Recent research showed that caveolin-1 and caveolin-2 can redistribute to lipid droplets in a regulated manner (Pol et al., 2004). Caveolin-1 was found to be present in detergent-resistant membrane fractions as well as FAT/CD36, which suggested the transport mechanism of the lipid raft-dependent LCFA uptake might be mediated by FAT/CD36 (Pohl et al., 2004). Instead of caveolin-1, we detected the mRNA expression level of caveolin-2 of the adipose tissue from male broilers. Surprisingly, however, its abundance was much lower than we had expected. Moreover, the injection of FAT/CD36 antigen had no significant effect on its transcriptional level of both the subcutaneous and visceral fat.

Among the FATP family, FATP1 was well studied for its tissue distribution and fatty acid transport function (Marotta et al., 2004). Several studies have given extensive evidence that FATP1 can activate LCFA to form acyl-CoA (Mashek et al., 2004; Pohl et al., 2004; Lobo et al., 2007). In this way, it may prevent the efflux of intracellular fatty acids by binding with fatty acid binding proteins or ACBP. So, FATP1 was also considered to be one of the ACSL family members, which has been suggested to play a crucial role in determining what happens to the cellular fatty acid pool (Coleman et al., 2002; Mashek et al., 2007). In the ACSL channeling hypothesis, mitochhondrial ACSL could provide activated fatty acids for esterification and subsequent β-oxidation (Digel et al., 2009). In connection with our result, the decreased visceral fat of male broilers might be partly attributable to the stimulated activities of the mitochondria localized ACSL1 when its mRNA expression was significantly upregulated. Consistent with the increase of the FATP1 and ACSL1 mRNA, the ACBP mRNA in the visceral fat of male broilers was also upregulated after the inoculation. This again confirms the interaction of these series of proteins involved in the uptake and metabolism of fatty acids.

Peroxisome proliferator-activated receptor γ is a key transcriptional regulator of adipocyte differentiation and lipid metabolism (Sato et al., 2008). Our results showed that the expression level of PPARγ mRNA also increased after the FAT/CD36 blocking, which was consistent with previous study. Drover et al. (2005) reported that the expression of PPAR was regulated by CD36-dependent fatty acid uptake. Compared with wild type mice, CD36-null mice had 5- to 10-fold increased PPAR mRNA in adipose tissue in the basal state. We also suggested that the increased PPARγ mRNA expression might contribute to the compensatory regulation of VLDLR, ACSL, FATP1, and ACBP gene expression as well as the lipid metabolic activities of visceral fat.

In summary, this study is the first to reveal a novel role of chicken FAT/CD36 in the visceral fat deposition of male broilers. The results also indicated that the mechanism of avian fat deposition has sex- and spatial-specific differences. Furthermore, various proteins involved in fatty acid uptake may not contribute equally to different sites of fat deposition, providing specific targets for site-specific regulation of fat deposition that could ultimately improve meat quality.

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