

## Tissue expression characterization of chicken adipocyte fatty acid-binding protein and its expression difference between fat and lean birds in abdominal fat tissue<sup>1</sup>

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**ABSTRACT** Fatty acid-binding proteins are considered to be the carriers for the transportation of intracellular fatty acids and play an important role in the development of fatness traits. Adipocyte fatty acid-binding protein (A-FABP) is one of the family members. The current study was designed to analyze the tissue expression characterization of chicken A-FABP and its expression difference between the fat and lean males in abdominal fat tissue to reveal the possible relationship between the expression of A-FABP and abdominal fat tissue development and growth in chicken. First, fusion protein glutathione S-transferase/A-FABP was induced and purified, and then the antiserum containing specific polyclonal antibodies was obtained by immunizing healthy female rabbits using the purified fusion protein. Second, tissue expression characterization of A-FABP

was investigated by Western blot. Finally, A-FABP expression difference in abdominal fat tissue between the fat and lean males was investigated by real-time reverse transcription-PCR and Western blot methods. The results showed that A-FABP expressed specifically in abdominal fat tissue and the mRNA expression level of A-FABP in fat males was lower than that of lean males at 2, 3, 4, 6, 7, 9, and 10 wk of age ( $P < 0.05$ ), and the protein expression level of fat males was lower than that of lean males at 6 and 10 wk of age ( $P < 0.05$ ). These results suggested that chicken A-FABP might affect abdominal fat deposition through changing its expression level, and the possible mechanism may be that a high expression level of A-FABP induced the high lipolytic rate and led to the decreased abdominal fat mass accordingly.

**Key words:** adipocyte fatty acid-binding protein, expression characterization, expression difference, fat and lean males

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### INTRODUCTION

Fatty acid-binding protein (**FABP**) belongs to a superfamily of lipid-binding protein and occurs intracellularly in invertebrates and vertebrates (McArthur et al., 1999). Tissues with high rates of fatty acid (**FA**) metabolism, such as intestine, liver, adipose, and muscle, have high levels of FABP that parallel FA uptake and utilization (Storch and Corsico, 2008). Adipocyte FABP (**A-FABP**) is traditionally thought to be a cytosolic FA chaperone expressed in adipocytes (Xu et al., 2006). It appears to bind only long-chain FA with high affinity. This ligand specificity has led to

the broad hypothesis that A-FABP plays an important role in triglyceride storage and release in cells. The precise physiological role of A-FABP has been recognized only upon the development of genetic models in mice (Maeda et al., 2003, 2005; Makowski and Hotamisligil, 2004). Mice lacking A-FABP are healthy and exhibit only minor alterations in their steady-state lipid metabolism (Hotamisligil et al., 1996). There was no difference in the rate of FA influx or esterification in adipocytes between wild-type and A-FABP null mice, but basal lipolysis was decreased approximately 40% in A-FABP null mice (Coe et al., 1999; Jenkins-Kruchten et al., 2003). In poultry, such as chicken, there was almost no information about the precise physiological role of A-FABP.

The objective of the present study was to reveal the possible relationship between the expression of A-FABP and abdominal fat tissue development and growth through elucidating the tissue expression characterization of A-FABP and analyzing the expression difference of A-FABP in abdominal fat tissue between fat and lean males.

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## MATERIALS AND METHODS

### Experimental Birds

Ninety males, derived from the 11th generation population of the Northeast Agricultural University broiler lines divergently selected for abdominal fat content (Wang et al., 2007b), were used in this study and their abdominal fat percentage (AFP) was calculated (Figure 1). The fat males used here were the offspring of the families with the highest AFP according to their slaughtered sib information, and the lean males used here were the offspring of the families with the lowest AFP according to their slaughtered sib information. All males were kept in the same environmental conditions and had free access to feed and water. Commercial corn-soybean-based diets that met all NRC requirements were provided to the birds. From hatching to 3 wk, the males received a starter feed (21% CP and 3,100 kcal of ME/kg), and from 4 wk to slaughter, they were fed with a grower diet (19% CP and 3,000 kcal of ME/kg).

New Zealand rabbits and Kunming White mice were purchased from Harbin Medical University, Heilongjiang, China.

### Cloning and Expression of Chicken A-FABP cDNA

Chicken abdominal fat tissue was collected and homogenized in 1 mL of Trizol reagent, and the abdominal fat tissue total RNA sample was extracted according to the instruction of the manufacturer (15596-026, Invitrogen, Carlsbad, CA). The concentration of RNA was estimated by measuring the absorbance at 260 nm by UV spectrophotometer (Ultrospec 1000, Biochrom Ltd., Cambridge, UK). Reverse transcription was performed according to the directions of the Takara RNA PCR Kit (version 3.0, Takara, Dalian, China; Wang et al., 2008). The coding region of chicken A-FABP gene (GenBank accession no. NM\_204290) was amplified with a pair of specific primers (forward, 5'-ga gaattc

ATGTGCGACCAGTTTGTGGGC-3'; reverse, 5'-cg ctcgag CATGAAGACGGCTTCCTCATGCTC-3'), which contain *Eco*RI and *Xho*I sites, respectively. The PCR products were digested with *Eco*RI and *Xho*I and then inserted into PGEX 4T-1 vector. The plasmid PGEX 4T-A-FABP was transformed into an *Escherichia coli* BL21 (DE3) strain to produce glutathione S-transferase (GST) fusion protein (GST/A-FABP).

### Expression and Purification of Recombinant A-FABP

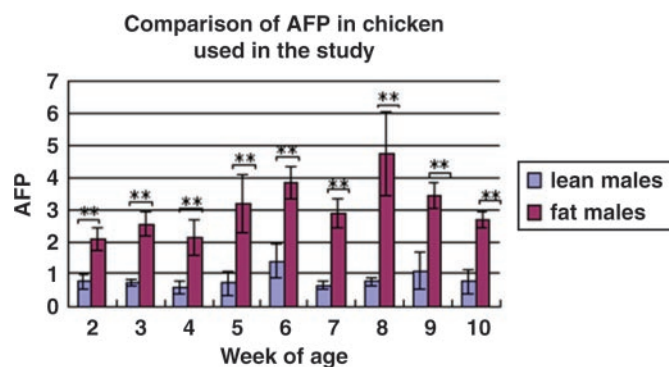
The target protein was expressed as soluble protein induced by isopropyl  $\beta$ -D-1-thiogalactopyranoside for 4 h at 37°C. The product was obtained by centrifugation and sonication and was then applied to Glutathione Sepharose 4B column according to the instruction of the manufacturer (17-0756-01, Amersham, Uppsala, Sweden).

### Immunization of Rabbit

A basic immunization was done by injecting 100  $\mu$ g of pure GST/A-FABP as the immunogen, emulsified with Freund's complete adjuvant. Two successive boosts were carried out after the first immunization with an interval of 2 wk between each boost. For each boost, 100  $\mu$ g of antigen and 1 mL of Freund's incomplete adjuvant were used. For the fourth immunization, 200  $\mu$ g of antigen and 1 mL of Freund's incomplete adjuvant were used. Antiserum was collected 7 d after the last boost.

### Testing of the Chicken A-FABP Antiserum Activity and Specificity

The pcDNA3-A-FABP plasmid was constructed by insertion of the A-FABP coding region into the pcDNA3.1 vector. The plasmid DNA was transfected into mouse by hydrodynamics transfection according to the methods of Liu et al. (1999). Eight hours after transfection, the liver of mouse was dissected and washed in ice-cold PBS, minced, and homogenized at 4°C in ice-cold radioimmunoprecipitation assay buffer (150 mmol/L of NaCl, 50 mmol/L of Tris-HCl pH 7.5, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate) added with the protease inhibitor phenylmethylsulfonyl fluoride (BY12203, Sigma, St. Louis, MO) and then centrifuged at  $2,300 \times g$  for 20 min at 4°C to obtain total protein. The total protein was mixed with standard SDS-PAGE gel-loading buffer, heated at 100°C for 5 min, and then separated by 12% SDS-PAGE and transferred to an Immun-Blot polyvinylidene fluoride membrane (Millipore, Billerica, MA). After block nonspecific binding, the membrane was first immunoblotted with the rabbit anti-chicken A-FABP antiserum (1:3,000) for 1 h at room temperature. After being washed with PBS with 0.05% Tween-20, the membrane was then immunoblotted with Peroxidase-Conjugated AffiniPure Goat



**Figure 1.** Comparison of abdominal fat percentage (AFP) of chicken used in the study. Results were given with the mean value and SD (n = 5). \*\* $P < 0.01$ , highly significant difference between fat and lean males. Color version available in the online PDF.

Anti-Rabbit IgG (H<sup>+</sup>L) (1:5,000; ZB-2301, ZSGB-BIO, Beijing, China) for 1 h at room temperature. Finally, the immunoreactive protein on the membrane was visualized using enhanced chemiluminescence and was exposed to x-ray film (Bio-Rad, Hercules, CA).

### **Tissue Expression Characterization of Chicken A-FABP**

Chicken was killed and heart, liver, abdominal fat, muscle, muscle stomach, spleen, small intestine, lung, and kidney tissues were dissected. Total proteins of various tissues were obtained according to the methods described above.

The A-FABP protein was detected by Western blot with A-FABP antiserum and Peroxidase-Conjugated AffiniPure Goat Anti-Rabbit IgG (H<sup>+</sup>L) (ZB-2301, ZSGB-BIO). Mouse anti-chicken glyceraldehyde 3-phosphate dehydrogenase (**GAPDH**) antibody (AG019, Beyotime Institute of Biotechnology, Jiangsu, China) and Peroxidase-Conjugated AffiniPure Goat Anti-Mouse IgG (H<sup>+</sup>L) (ZDR-5307, ZSGB-BIO) were used to detect GAPDH. The procedure of Western blot was performed according to the methods described above (in the part of testing of chicken A-FABP antiserum activity and specificity).

### **The mRNA Expression Level of Chicken A-FABP in Abdominal Fat Tissue Between Fat and Lean Males**

Males were slaughtered at 2, 3, 4, 5, 6, 7, 8, 9, and 10 wk of age, respectively, and abdominal fat was isolated, immediately frozen in liquid nitrogen, and stored at -80°C.

For each week, there were 10 samples, 5 males from fat line and 5 males from lean line, and the AFP was calculated (Figure 1).

Total RNA was extracted from chicken abdominal fat tissue by using a Trizol reagent kit (15596-026, Invitrogen) according to the recommendations of the manufacturer. Reverse transcription was performed according to the directions of the Takara RNA PCR Kit (version 3.0, Takara). Real-time reverse transcription-PCR was used to detect A-FABP expression by using SYBR Premix Ex Taq (Takara) with A-FABP primers (AFF1: 5'-ATGTGCGACCAGTTTGT-3'; AFR1: 5'-TCACCATTGATGCTGATAG-3'). Chicken 18S rRNA was chosen as the internal reference and was detected with the 18S rRNA primers (18SF:5'-TAGATAACCTCGAGCCGATCGCA-3' and 18SR: 5'-GACTTGCCCTCCAATGGATCCTC-3'). One microliter of each RT reaction product was amplified in a 20- $\mu$ L PCR reaction system. 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 by Dissociation Curve 1.0

software (Applied Biosystems) for each PCR reaction to detect and eliminate possible primer-dimer artifacts. Results of real-time PCR were expressed as the relative quantity of A-FABP/18S.

### **The Protein Expression Level of Chicken A-FABP in Abdominal Fat Tissue Between Fat and Lean Males**

Samples from fat and lean males used here were the same as those used for the detection of mRNA expression level. Total protein was extracted from abdominal fat tissue of fat and lean males according to the methods described above (in the part of testing of chicken A-FABP antiserum activity and specificity). For each sample, 40  $\mu$ g of total protein was used to analyze the expression level of A-FABP and GAPDH. The analyses of A-FABP expression level were performed with the A-FABP antiserum and Peroxidase-Conjugated AffiniPure Goat Anti-Rabbit IgG (H<sup>+</sup>L) ZB-2301, ZSGB-BIO). Mouse anti-chicken GAPDH antibody (AG019, Beyotime Institute of Biotechnology) and Peroxidase-Conjugated AffiniPure Goat Anti-Mouse IgG (H<sup>+</sup>L) (ZDR-5307, ZSGB-BIO) were used to detect GAPDH. Immunoreactive protein levels were determined semi-quantitatively by densitometric analysis using a laboratory imaging and analysis system (CA 91786, UVP, Upland, CA). Results were expressed as the relative quantity of A-FABP/GAPDH.

### **Statistical Analysis**

Data were subjected to *t*-test. Results are given as mean  $\pm$  SD. Difference was considered significant at *P* < 0.05 unless otherwise specified.

## **RESULTS**

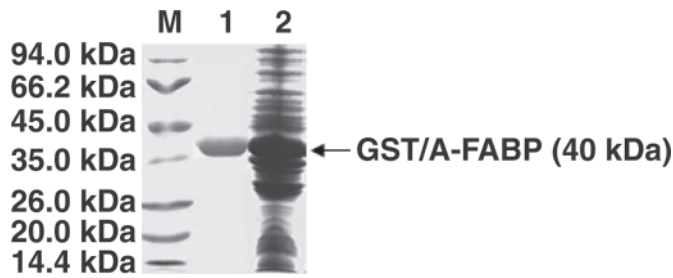
### **Expression and Purification of GST/A-FABP Protein**

Chicken A-FABP cDNA were cloned into PGEX 4T-1 and confirmed with *Eco*RI and *Xho*I digestion and by DNA sequencing. The PGEX 4T-A-FABP plasmid was transformed into *E. coli* BL21 (DE3) and the corresponding protein was expressed as N-terminal fusion protein with GST. The supernatants containing the soluble GST/A-FABP fusions were purified using Glutathione Sepharose 4B column and the homogeneity was assessed by SDS-PAGE. The fusion protein GST/A-FABP was visible as a band of 40 kDa (14 kDa of A-FABP + 26 kDa of GST; Figure 2).

### **Activity and Specificity of A-FABP Antiserum**

The A-FABP protein, which was used to check the activity and specificity of A-FABP antiserum, was ex-





**Figure 2.** Adipocyte fatty acid-binding protein (A-FABP) expressed in *Escherichia coli* and purified by Glutathione Sepharose 4B (Amersham, Uppsala, Sweden). M = protein molecular weight marker; lane 1 = purified glutathione S-transferase (GST)/A-FABP; lane 2 = isopropyl  $\beta$ -D-1-thiogalactopyranoside-induced expression of GST/A-FABP in *E. coli*.

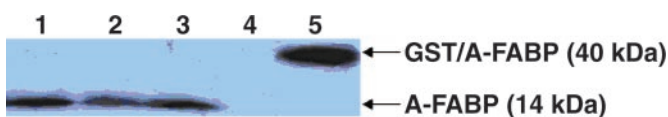
pressed successfully by transfecting pcDNA3-A-FABP plasmid into the liver of mouse. The activity and specificity of the antiserum was tested by Western blot. Adipocyte FABP is visible as a band of 14 kDa both in the mice livers injected with pcDNA3-A-FABP plasmid and in the chicken abdominal fat tissue. The fusion protein GST/A-FABP was visible as a band of 40 kDa (Figure 3). The result showed that the A-FABP antiserum can recognize the chicken A-FABP with excellent activity and specificity.

### Tissue Expression Characterization of Chicken A-FABP

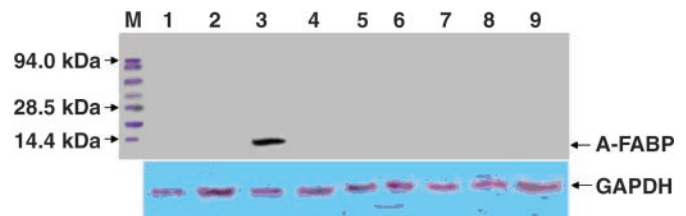
The expression characterization of A-FABP was tested by Western blot. The result showed that A-FABP protein was expressed only in abdominal fat tissue and there was no detectable signal in heart, liver, muscle, muscle stomach, spleen, small intestine, lung, and kidney (Figure 4).

### The mRNA Expression Level of A-FABP in Abdominal Fat Tissue Between Fat and Lean Males

Real-time reverse transcription-PCR was used to detect A-FABP mRNA expression level in abdominal fat tissue. The results showed that there was significant difference in mRNA expression level of A-FABP in abdominal fat tissue between fat and lean males and mRNA expression level of the lean males was much



**Figure 3.** Analysis of adipocyte fatty acid-binding protein (A-FABP) antiserum activity and specificity. Lane 1 = abdominal fat tissue of chicken; lanes 2 and 3 = liver of mouse injected with pcDNA3/A-FABP plasmid; lane 4 = liver of mouse injected with isotonic Na chloride; lane 5 = purified glutathione S-transferase (GST)/A-FABP. Color version available in the online PDF.



**Figure 4.** Analysis of adipocyte fatty acid-binding protein (A-FABP) expression characterization in chicken tissues. M = protein molecular weight marker; lane 1 = heart; lane 2 = liver; lane 3 = abdominal fat; lane 4 = muscle; lane 5 = muscle stomach; lane 6 = spleen; lane 7 = small intestine; lane 8 = lung; lane 9 = kidney. GAPDH = glyceraldehyde 3-phosphate dehydrogenase. Color version available in the online PDF.

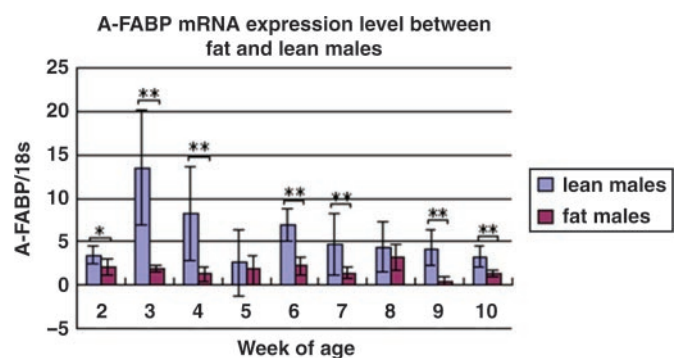
higher than that of the fat males at 2, 3, 4, 6, 7, 9, and 10 wk of age (Figure 5).

### The Protein Expression Level of A-FABP in Abdominal Fat Tissue Between Fat and Lean Males

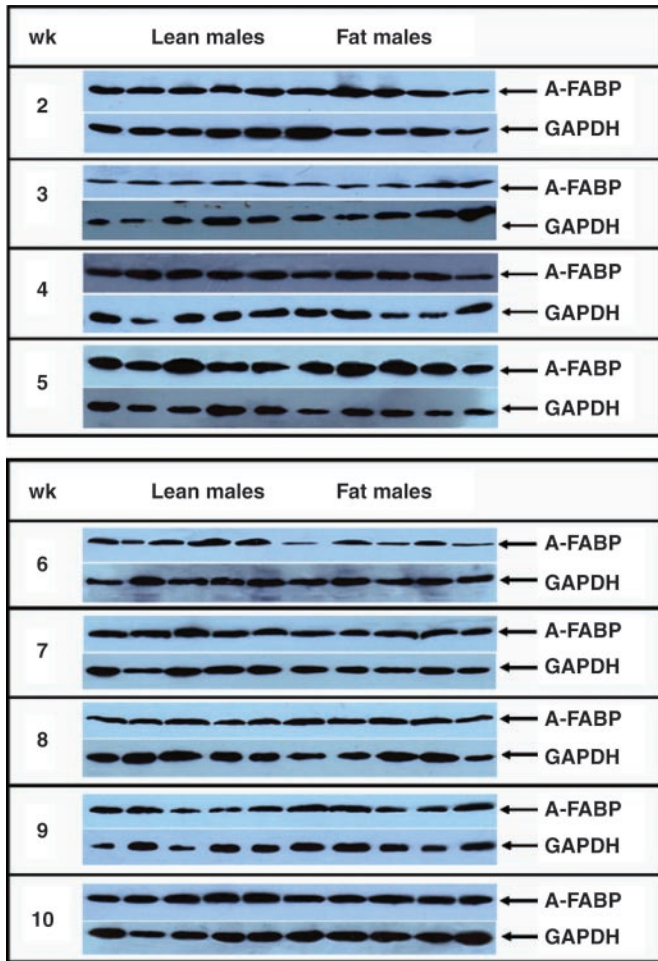
The protein expression level of A-FABP was detected by Western blot (Figure 6). The results showed that there were significant differences of A-FABP protein expression level between fat and lean males at 6 and 10 wk of age, and compared with the fat males, the A-FABP protein expression level of lean males was much higher. For other weeks of age, no significant difference was detected (Figure 7).

## DISCUSSION

The FABP specific occurrence in certain tissues or cells possibly results from adaptation to specific cellular needs (Zimmerman and Veerkamp, 2002). The results of gene expression profiling of chicken abdominal fat tissue demonstrated that the function of adipose tissue in chicken is important in lipid metabolism by directly or indirectly regulating the synthesis, transport, and



**Figure 5.** Analysis of adipocyte fatty acid-binding protein (A-FABP) mRNA expression level between fat and lean males. Results were given with the mean value and SD ( $n = 5$ ). \* $P < 0.05$ , significant difference between fat and lean males; \*\* $P < 0.01$ , highly significant difference between fat and lean males. Color version available in the online PDF.



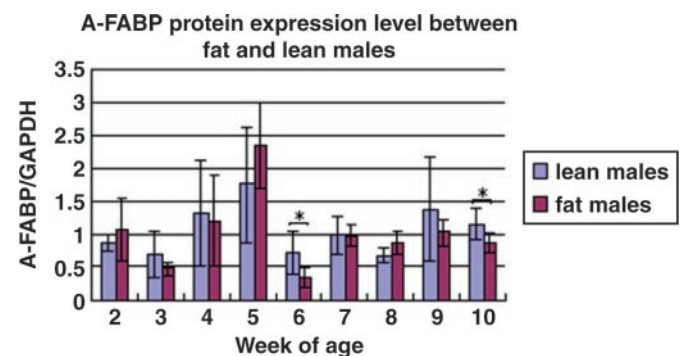
**Figure 6.** Western blot detection of adipocyte fatty acid-binding protein (A-FABP) expression in chicken abdominal fat tissues between fat and lean males. wk = week of age. GAPDH = glyceraldehyde 3-phosphate dehydrogenase. Color version available in the online PDF.

degradation of lipids (Wang et al., 2007a). The investigation of A-FABP gene polymorphism showed that the gene was associated with abdominal fat weight and percentage of abdominal fat (Wang et al., 2006). Similar to mouse A-FABP expression characterization (Pelton et al., 1999), the present study observed that the chicken A-FABP was expressed only in abdominal fat tissue, which was consistent with the result of Northern blot that the chicken A-FABP gene was expressed specifically in the abdominal fat tissue, and there were no detectable signals in brain, breast muscle, liver, kidney, heart, and lung (Wang et al., 2004). The abdominal fat tissue-specific expression has led to the hypothesis that chicken A-FABP plays an important role in lipid metabolism of abdominal fat tissue.

It was found that the fat mass of epididymal, perirenal/retroperitoneal, interscapular/brown, axillary, and inguinal/dorsolumbar were increased in A-FABP null mice, and the lipolysis in high-fat-fed A-FABP null mice was decreased (Coe et al., 1999; Scheja et al., 1999; Shaughnessy et al., 2000; Baar et al., 2005; Hertz et al., 2006). Investigation of A-FABP expres-

sion level in obese people showed that the expression of A-FABP, both in mRNA level and protein level, was significantly higher in s.c. fat than omental adipose tissue, and the lipolysis rate of s.c. fat was significantly higher than omental fat, which implied the correlation between the high lipolytic rate and the high content of A-FABP (Fisher et al., 2002). The result of our present study showed that there was significant difference between the fat and lean males both in mRNA and protein expression level of A-FABP in abdominal fat tissue, and compared with the fat males, the A-FABP mRNA expression level of lean males was higher at 2, 3, 4, 6, 7, 9, and 10 wk of age and the A-FABP protein expression level was higher at 6 and 10 wk of age. According to the results of mice and obese people, we presumed that high expression level of A-FABP may induce the high lipolytic rate of abdominal fat tissue and lead to the decreased abdominal fat mass accordingly in chicken.

It was noted that there were significant differences of AFP between fat and lean males at 5 and 8 wk of age (Figure 1). However, the expected significant differences of A-FABP between the fat and lean males in mRNA expression level were not detected. In fact, adipose tissue development and growth is a complicated process that is related with multiple genes and pathways. Adipose tissue mass can be increased by multiplication of new fat cells through adipogenesis, by increased deposition of triglycerides in the cytoplasm, or both (Soukas et al., 2001). The results of comprehensive analysis of the chicken adipose tissue gene expression profile revealed that many genes involved in FA transport and degradation, including Spot 14, lipopolysaccharide, adrenomedullin, pyruvate dehydrogenase kinase, isoenzyme 4, the FABP family, and so on were highly expressed in adipose tissue (Wang et al., 2007a). Adipocyte FABP, one of the many important genes that contribute to the development and growth of chicken abdominal fat tissue, could make just partial contribution to the deposition of abdominal fat tissue. Therefore, for given



**Figure 7.** Analysis of adipocyte fatty acid-binding protein (A-FABP) protein expression level in chicken abdominal fat tissues between fat and lean males. Results were given with the mean value and SD (n = 5). \* $P < 0.05$ , significant difference between fat and lean males. GAPDH = glyceraldehyde 3-phosphate dehydrogenase. Color version available in the online PDF.

specific bird ages, at 5 and 8 wk of age here, no parallel relationship between A-FABP mRNA expression level and AFP may be reasonable, and the cause needs to be investigated further.

The mRNA expression level of A-FABP in abdominal fat tissue, although being coincident at 6 and 10 wk of age, was not accompanied overall by parallel changes in its protein expression level. This is consistent with the result of a porcine study, which showed that there was low correlation between the mRNA and protein expression levels of pig A-FABP (Gerbens et al., 2001). We presumed that the expression of chicken A-FABP is related with posttranscriptional modification.

In summary, our study confirmed that chicken A-FABP is specifically expressed in abdominal fat tissue. The possible mechanism of A-FABP that regulated abdominal fat tissue development and growth may be that high expression level of A-FABP induced the high lipolytic rate and led to the decreased abdominal fat mass accordingly. The low correlation between mRNA and protein expression levels implied posttranscriptional modification of chicken A-FABP. However, the precise mechanisms of A-FABP that regulate the development and growth of chicken abdominal fat tissue need to be investigated further.

## ACKNOWLEDGMENTS

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