ASSOCIATION BETWEEN mRNA EXPRESSION LEVEL OF ATGL GENE AND TISSUE FAT CONTENT AT DIFFERENT DEVELOPMENTAL STAGES OF GUIZHOU MINI-PIG

Yong Yang[1, 2], Chunjiang Zhao[3], Shan Xiao[1], He Zhan[1], Changxin Wu[3], Changwei Ma[1 *]

[1] College of Food Science and Nutritional Engineering, China Agricultural University, Beijing, 100083, China.
[2] College of Food Science, Sichuan Agricultural University, Yaan, 625014, China
[3] College of Animal Science and Technology, China Agricultural University, Beijing, 100094, China
[*]Changwei Ma (phone: +86-10-62737643; fax: +86-10-62737643; e-mail: chwma@cau.edu.cn)

Abstract—Adipose triglyceride lipase (ATGL) was recently identified as a novel lipase that performed the initial step in triacylglycerol hydrolysis. The objective of this study was to investigate the relationship between the mRNA expression level and tissue fat content at different developmental stages of Guizhou mini-pig. Real-time quantitative PCR showed that pig ATGL gene was highly expressed in adipose tissue while poorly expressed in muscle, liver, heart and kidney in three developmental stages. Tissue fat content decreased significantly from 90 d to 180 d and increased significantly from 180 d to 270 d in adipose tissue and muscle. Correlation analysis displayed that the expression level of ATGL gene closely and negatively correlated with tissue fat content in adipose tissue and *Longissimus dorsi* muscle. It suggests that ATGL is most probably a key enzyme responsible for fat deposition in swine, and it may have great impact on meat quality by regulating intramuscular fat content during development.

***Index Terms*—ATGL gene (or PNPLA2), mRNA expression level, tissue fat content, development and pig**

I. INTRODUCTION

Adipose triglyceride lipase (ATGL), also called patatin-like phospholipase domain-containing 2 (PNPLA2), was recently identified as a novel lipase that performed the initial step in triacylglycerol (TG) hydrolysis (Zimmermann et al., 2004) and therefore seemed to play a pivotal role in the lipolytic catabolism of stored fat in adipose tissue and non-adipose tissues (Lass et al., 2006; Smirnova et al., 2006). For many years, hormone-sensitive lipase (HSL) has been considered to be the key enzyme responsible for regulating lipid mobilization in adipose tissue (Raben & Baldassare, 2005). However, HSL-knockout mice were not obese and accumulated diacylglycerol (DG) instead of TG (Haemmerle, et al., 2002). Over-expression of ATGL increased the lipolysis in adipocytes, and inhibition markedly decreased total adipose acyl-hydrolase activity (Zimmermann et al., 2004). In another study (Smirnova et al., 2006), over-expression of wild-type ATGL caused a marked decrease in lipid droplets (LDs) size, and depletion of ATGL by RNA interference did the opposite. Similarly, genetic inactivation of ATGL in mice increased adipose mass and led to TG deposition in multiple tissues (Haemmerle, et al., 2006). Recently, mutation analysis in the human ATGL gene indicated that the individuals with neutral lipid storage disorders exhibited severe defect in TG degradation in fibroblasts and marked TG storage in liver, muscles and other visceral cells (Fischer et al., 2007). The above-mentioned studies provide strong evidence that ATGL is a rate-limiting enzyme in TG hydrolysis in mammalian cells. Thus, it is likely that ATGL is an important gene for tissue fat content in swine. Very recently, ATGL gene was reported highly expressed in adipose tissue and the expression level was increased dramatically during the development. However, the ATGL expression level was reported higher in Landrace pig (Deiuliis et al., 2008) and lower in Duroc × Landrace × Yorkshire pig (Shan et al., 2008). These work implicate that ATGL gene is an important gene for tissue fat content in swine, and ATGL expression pattern maybe different among different pig breeds. Guizhou mini-pig is a traditional pig breed and much differed from commercial pigs either in the figure or in the developmental characteristics. The objective of this study was aimed to investigate the relationship between the mRNA expression level of ATGL gene (or PNPLA2) and tissue fat content in multiple tissues of these mini-pigs during the development.

II. MATERIALS AND METHODS

2.1 Animals and tissue samples

Twelve male Guizhou mini-pig with the same birthday were selected to start our study. All pigs were raised according to the uniform feeding rules in one sty in the Chinese Experimental Mini-pig Breeding Center, China Agricultural University. Pigs were castrated at 40 day (d). Experimental animals were slaughtered in three successive phases when they were 90 d, 180 d and 270 d after birth, and in each time, three pigs with approximate the same weight (± 2 kg) were selected for slaughtering and sampling. Pigs were killed by electrical stun and exsanguinations. Subcutaneous adipose tissues, *Longissimus dorsi* muscle (from the last thoracic vertebra to the last lumbar vertebra), liver, heart, and kidney were removed and dissected as quickly as possible. Then, all tissue samples were immediately dipped into liquid nitrogen and stored at -80°C until RNA isolation and fat extraction.

2.2 RNA isolation and purification

Total RNA was isolated from different tissue samples using TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions. The integrity of the RNA was verified by ethidium bromide staining of the ribosomal RNA

on 0.8% agarose gels. The contaminated genomic DNA in RNA samples were eliminated by DNase I (Invitrogen, USA) before the first-strand cDNA synthesis. RNA samples were dispensed and stored at -80°C.

2.3 Quantitative analysis of the mRNA expression level of ATGL gene (PNPLA2)

First-strand cDNA was synthesized using the SuperScriptTM III First-Strand Synthesis System for RT-PCR. The housekeeping gene β-actin was used as an endogenous control to normalize the template level. The primers for pig PNPLA2 were 5'-AGGAGCTCATCCAGGCCAAC-3' (forward) and 5'-AGATGCCACCGTCCACGTAG-3' (reverse), and the product size is 102 bp. The primers for pig β-actin (GenBank: AY550069) were 5'-AGGTCATCACCATCGGCAAC-3' (forward) and 5'-CGTCGCACTTCATGATGGAGT-3' (reverse), and the product size is 123 bp. Primer sequences were designed using Primer Express software v 2.0 (Applied Biosystems, USA). PCR amplification of PNPLA2 and β-actin was performed in a 25 μL final volume containing 11.25 μL of 2.5 × RealMasterMix / 20 × SYBR solution (TIANGEN, China), 200 nm of each primer, 1 μL of cDNA and 12.15 μL of water. All amplifications were carried out in optical-grade 96-well plates on ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, USA) with an initial step at 50°C for 2 min and at 94°C for 3 min, followed by 40 cycles of 94°C for 30 sec, 58°C for 40 sec and 68°C for 20 sec, and then a final step of 95°C for 15 sec, 60°C for 15 sec, 95°C for 15 sec for dissociation analysis. Pooled RNA from all tissue samples of Guizhou mini-pig at 90 d was used to generate cDNA by reverse transcription (3 μL of pooled RNA added to each reaction tube in a final volume of 20 μL and following the instruction manual of the SuperScriptTM III First-Strand Synthesis System for RT-PCR), and the cDNA was applied to construct standard curves by 2-fold serial dilutions in 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 along with a non-template control. In each 96-well plate, two series of dilutions of the standard cDNA were added to generate standard curves of PNPLA2 and β-actin respectively at the same time and in the same way, and the unknown samples for quantification of PNPLA2 and β-actin were also added to the same plate. All real-time PCR amplification reactions were repeated three times independently to ensure the reproducibility of the results.

2.4 Tissue fat extraction and fat content determination

Tissue fat was extracted from the frozen samples (stored at -80°C) using a 17-fold dilution of tissue in 2:1 chloroform/methanol (vol:vol) according to the method established by Folch et al in 1957. The extracts were taken to dryness under vacuum on a rotary evaporator. Tissue fat content was expressed as grams per 100 grams of wet tissue (g/100 g), and was determined by the average lipid content of a specific tissue obtained from different individuals.

2.5 Data analysis

Elementary analysis of Q-PCR was performed using SDS2.2 (Applied Biosystems, USA) and Microsoft Excel (Microsoft Corporation). mRNA quantity of PNPLA2 and β-actin were calculated by their own standard curve. Relative mRNA expression levels of PNPLA2 were subsequently normalized by the amount of β-actin to correct the differences in RNA amounts and reverse transcription reactions. All statistical comparisons and correlation analysis were carried out by SAS 8.2 package (SAS Institute Inc., USA). Data were presented as means ± standard deviation (SD).

III. RESULTS AND DISCUSSION

3.1 Quantitative analysis of ATGL gene (or PNPLA2) mRNA expression level

Results showed that the transcripts of ATGL gene were expressed in all of the examined tissues in this study, and displayed a significant higher expression level of PNPLA2 mRNA in adipose tissue ($P<0.001$) than in muscle, liver, heart and kidney in three different developmental stages (Fig. 1, panel A, B and C). mRNA expression levels of PNPLA2 were very low in muscle, liver, heart and kidney, and there were not significant differences between them in each developmental stage. During the development of pig, the expression level of PNPLA2 in three selected time points tended to increase from 90 d to 180 d and decrease from180 d to 270 d in adipose tissue, muscle, liver and heart (Fig. 5, panel D). But, the expression level of PNPLA2 increased significantly ($P<0.05$) only in adipose tissue from 90 d to 180 d, and decreased significantly ($P<0.05$) in adipose tissue, muscle and heart from180 d to 270 d. As for kidney, the expression pattern of PNPLA2 was different and the expression quantities in the appointed time points were increased all along from 90 d to 270 d with significant differences between 180 d and 270 d ($P<0.05$) (Fig. 1, panel D). These results conform to previous results from human (Zimmermann et al., 2004; Langin et al., 2005), mice (Zimmermann et al., 2004; Villena et al., 2004; Lake et al., 2005; Kershaw et al., 2006; Shen, Patel, Yu, Jue & Kraemer, 2007) and swine (Shan et al., 2008). Most recently, ATGL expression pattern was reported to increase dramatically in pig subcutaneous adipose during development (Deiuliis et al., 2008), and ATGL reached a maximal expression level in adipose tissue in about 8 weeks old (Shan et al., 2008). But, our work displayed that the expression level of ATGL in the selected time points increased from 90 d to 180 d and decreased from180 d to 270 d in adipose tissue, muscle, liver and heart of Guizhou mini-pigs, and ATGL expression in kidney was increased all along from 90 day to 270 day in kidney.

3.2 Tissue fat content in different development stages

Total lipids content of different tissue samples in each development stages were given in Table 1. Obviously, adipose tissue contains much more total lipids than all other tissues during the development. Surprisingly, the tissue fat content did not increase continuously as supposed, and our results showed that tissue fat content decreased from 90 d to 180 d and increased from 180 d to 270 d in adipose tissue, muscle, liver and heart, but the significant decrease ($P<0.01$) and increase ($P<0.01$) were observed only in adipose tissue and muscle. The fat content decreased ($P<0.05$) from 90 d to 180 d in the heart, and increased ($P<0.05$) from 180 d to 270 d in the liver. Fat content seemed to be little changed in kidney. Data from White × Landrace × Duroc crossbred female pigs displayed that intramuscular fat content had a slight decrease from 16 weeks to 25 weeks in *Supraspinatis*, *Longissimus* and *Biceps femoris* (D''Souza et al., 2004). In

other species, total lipid content of the rabbit muscle was reported to decrease between 2 and 4 weeks (Gondret, Mourot & Bonneau, 1997). Intramuscular fat did not increase until the hot carcass weight up to 200 kg in British cattle and Japanese Black cattle (Pethick, Harper & Oddy, 2004). But, some other observations also demonstrated an increase in pig intramuscular fat content throughout life (Mayoral et al., 1999). Another report showed that bone developed at early stage, then muscle, and fat was the last tissue to develop in animals (Bruns, Pritchard & Boggs, 2004). According to the developmental characters, Guizhou mini-pig always grows faster from 90 d to 180 d after birth, but the most amount of fat deposition happens after 6 months. We think that the decrease of fat content in adipose tissue and muscle in early stage might attribute to the much more energy release from lipids to get faster growth rate in skeleton and muscle during this period, and also, the patterns of fat deposition during the development might be much differed between animal breeds (Albrecht, Teuscher, Ender & Wegner, 2006). Recently, it was suggested that not all adipose tissues were similar but each showed specific development and metabolism (Monziols, Bonneau, Davenel & Kouba, 2007).

3.3 Correlation analysis between tissue fat content and the expression level of ATGL gene

The correlation coefficients between ATGL expression level and fat content obtained from different kind of tissues during development were –0.94 for adipose tissue ($n=9$, $P<0.01$), –0.69 for muscle ($n=9$, $P<0.05$), –0.34 for liver ($n=9$, $P<0.36$), and –0.47 for kidney ($n=9$, $P<0.20$) respectively. We failed to find out any obvious relationship between ATGL expression level and fat content in heart samples during development in this study. These results indicate that the expression level of ATGL in a given tissue may be negatively correlated to tissue fat content. In HEK 293 cells, it was found that over-expression of ATGL decreased intracellular triglyceride levels (Lake et al., 2005). ATGL knockout mice accumulated TG in multiple tissues (Haemmerle et al., 2006). ATGL mRNA was down-regulated in db/db and ob/ob mice, indicating that ATGL may contribute to the development of obese mice by increasing TG accumulation (Villena et al., 2004). Most recently, ATGL was reported to be down-regulated in skeletal muscle of obese, insulin resistant mice and negatively correlated with intramuscular TG levels (Watt et al., 2008). Thus, to a specific tissue during development, ATGL expression is most likely to have a negative effect on the fat content. Although, we failed to find out any obvious relationships between ATGL expression level and fat content in heart samples during pig development, and we didn't get significant correlation from liver and kidney in this study. It may be the limited sample numbers or limited amount of these tissues from individuals.

IV. CONCLUSION

ATGL gene of Guizhou mini-pig was highly expressed in adipose tissue but poorly expressed in muscle, liver, heart and kidney in three developmental stages. The expression pattern of ATGL gene in the selected time points displayed an increase at first but a decrease later in adipose tissue, muscle, liver and heart during pig development. In addition, we found that the expression level of ATGL gene in the appointed time points during development displayed a significant but negative correlation to tissue fat content in adipose tissue and *Longissimus dorsi* muscle in present study. Our studies suggest that ATGL is most probably a key enzyme responsible for fat deposition in swine, and it may have great impact on meat quality by regulating intramuscular fat content during development.

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Table 1 Average fat content (g/100 g) of multiple tissues in different developmental stages of Guizhou mini-pig

	90d	180d	270d
Adipose tissue	75.9466 ±1.7190 ^{A}	69.5205 ±1.3962 ^{B}	83.4155 ±0.7029 ^{C}
Muscle	1.4138 ±0.0943 ^{A}	1.0743 ±0.0886 ^{B}	2.2143 ±0.1074 ^{C}
Liver	3.6703 ±0.0620 ^{a}	3.4304 ±0.1117 ^{a}	3.7827 ±0.0682 ^{b}
Heart	3.0640 ±0.1907 ^{a}	2.5071 ±0.0531 ^{b}	2.6258 ±0.0527 ^{b}
Kidney	3.2498 ±0.0991 ^{a}	3.1372 ±0.1615 ^{a}	2.9624 ±0.1882 ^{a}

Data are presented as means ± SD and represent independent experiments from individuals. Data with different superscript within a row represent significant difference at $P<0.01$(capital letter), or at $P<0.05$ (lowercase letter).

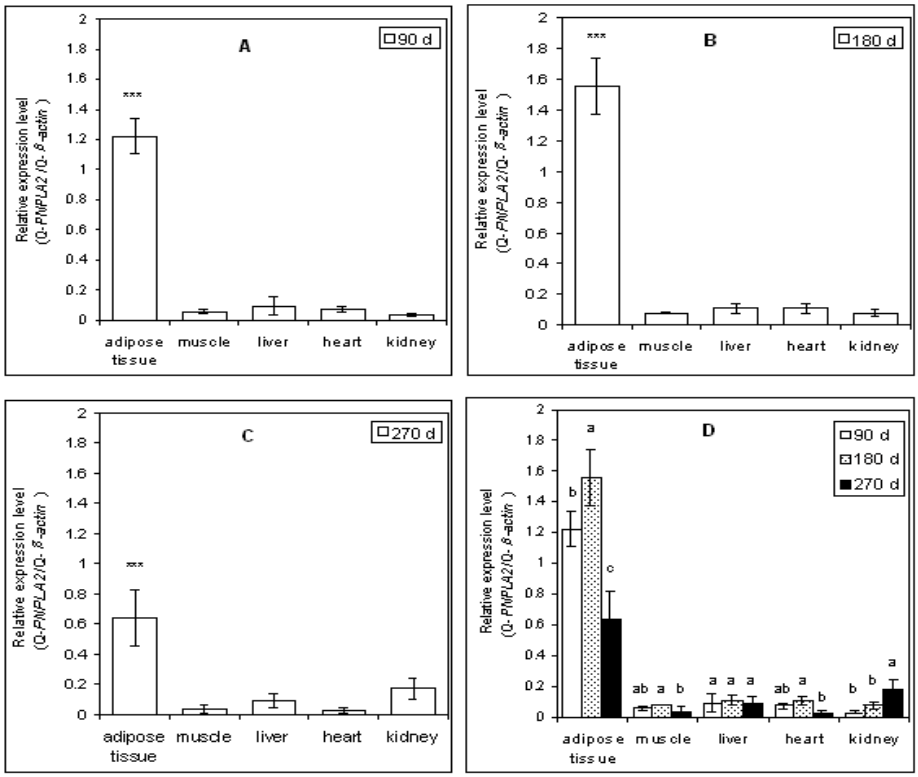

Fig. 1 mRNA expression level of pig ATGL gene (PNPLA2) in different stages and in various tissue samples. mRNA expression level of pig PNPLA2 was obtained by real-time fluorescent quantitative PCR (Q-PCR) using SYBR green as the dye. β-actin was used as the housekeeping gene in Q-PCR reaction. Q-PNPLA2/Q-β-actin denotes the relative quantity of PNPLA2. 90 d, 180 d and 270 d show the developmental stages when pigs were in 90 d, 180 d and 270 d old respectively. Data are presented as means ± SD and represent three independent experiments. Data with stars above each bar (as'***') represent significant difference at P<0.001 (panel A, B, and C). Data with different letters above each bar within a tissue group represents significant difference at P<0.05 (panel D).