



## Expression levels of candidate genes for intramuscular fat deposition in two Banna mini-pig inbred lines divergently selected for fatness traits

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### Abstract

Intramuscular fat (IMF) content plays an important role in meat quality. Many genes involved in lipid and energy metabolism were identified as candidate genes for IMF deposition, since genetic polymorphisms within these genes were associated with IMF content. However, there is less information on the expression levels of these genes in the muscle tissue. This study aimed at investigating the expression levels of sterol regulating element binding protein-1c (SREBP-1c), diacylglycerol acyltransferase (DGAT-1), heart-fatty acids binding protein (H-FABP), leptin receptor (LEPR) and melanocortin 4 receptor (MC4R) genes and proteins in two divergent Banna mini-pig inbred lines (BMIL). A similar growth performance was found in both the fat and the lean BMIL. The fat meat and IMF content in the fat BMIL were significantly higher than in the lean BMIL, but the lean meat content was lower. The serum triacylglycerol (TAG) and free fatty acid (FFA) contents were significantly higher in the fat than in the lean BMIL. The expression levels of SREBP-1c, DGAT-1 and H-FABP genes and proteins in fat BMIL were increased compared to the lean BMIL. However, the expression levels of LEPR and MC4R genes and proteins were lower.

**Keywords:** intramuscular fat (IMF), expression levels, Banna mini-pig inbred line (BMIL).

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### Introduction

In pig production, intramuscular fat (IMF) content is an important determinant of meat quality characteristics such as tenderness, juiciness and flavor (Fernandez *et al.*, 1999). Because of the importance of IMF on the economics of pig production, it is important to elucidate the molecular mechanisms underlying intramuscular fat deposition (Wood *et al.*, 2008). Particularly, in the Chinese local pigs, the IMF content is higher than in other commercial pig breeds (Zhao *et al.*, 2009), which makes them an ideal model for elucidating the mechanism of IMF deposition.

During the past decades, many efforts have been made to appoint possible candidate genes for IMF deposition (Schwab *et al.*, 2009). Sterol regulating element binding protein-1c (SREBP-1c), acyl CoA:diacylglycerol acyltransferase (DGAT-1) (Mourot and Kouba, 1998; Ding *et al.*, 2000; Chen *et al.*, 2008; Switonski *et al.*, 2010) and the intracellular heart fatty acid binding protein (H-FABP) affect the IMF content in pigs (Gerbens *et al.*, 1997, 1998,

1999, 2000; Glatz *et al.*, 2003; Gardan *et al.*, 2007; Li *et al.*, 2007; Li *et al.*, 2010; Switonski *et al.*, 2010). Genetic variants of the leptin receptor (LEPR) and the melanocortin 4 receptor (MC4R) genes were reported to be associated with IMF deposition (Mackowski *et al.*, 2005; Ovilo *et al.*, 2005; Bruun *et al.*, 2006; Jokubka *et al.*, 2006; Kim *et al.*, 2006; Piórkowska *et al.*, 2010; Fan *et al.*, 2009, 2010; Li *et al.*, 2010; Switonski *et al.*, 2010). Although LEPR and MC4R are mainly studied in hypothalamus, some reports indicated that they could be expressed in muscle tissue as well (Stinckens *et al.*, 2009; Li *et al.*, 2010; Larkina *et al.*, 2011; Tyra *et al.*, 2011). Global genomics analyses also led to the hypothesis that IMF deposition could be affected by both lipid metabolism and energy metabolism (Liu *et al.*, 2009; Cánovas *et al.*, 2010). However, most of the studies only focused on lipogenic genes, ignoring energy metabolism genes (Mourot and Kouba, 1998; Zhao *et al.*, 2009). Moreover, these reports usually used different pig breeds as fat and lean models, which were fed diets according to their individual nutritional standard. These studies could not rule out the possibility that the different expressions of genes might be due to breed characteristics rather than to actual variations in IMF deposition.

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The Banna Mini-pig Inbred Line (BMIL) was established in China in 1980 (Huo *et al.*, 2012). The original ancestors were a sow and her son. The further reproduction occurred by full-sibling or parent-offspring mating. Progressive inbreeding generated the fat and the lean BMIL. The body weight of mature pigs reaches about  $45.00 \pm 1.50$  kg. They are ideal experimental models for a better understanding of the genetic mechanisms leading to divergent fat deposition phenotypes, because they have the same or a very similar background genotype and were fed the same diets in the same environment (Guo *et al.*, 2008). The aim of this study was to compare the expression levels of the SREBP-1c, DGAT-1, H-FABP, LEPR and MC4R genes and proteins in fat and lean BMIL and to explain the phenotypic variations of IMF deposition in these two divergent BMILs.

## Materials and Methods

The animal experiments and protocols used in this study were approved by the Yunnan Agricultural University Institutional Animal Care and Use Committee. The fat and lean BMIL were fed in the Yunnan Key Laboratory of BMIL, Yunnan Agricultural University, China.

Fat and lean BMIL animals of the 25th generation were selected, with an inbreeding coefficient of 99.56%. A total of twelve healthy pigs were fed ad libitum the same corn-soybean diets (Table 1) in the same environmental

conditions for  $1.5-1.6 \pm 0.45$  years. Their body weights were determined every week, and their growth performances were calculated. Then the pigs were slaughtered by exsanguination after electrical stunning, and their hot carcass weight was measured. The fat-free lean and fat contents were calculated from the left side of the carcass, after a 20 h chill at 2 °C. The average backfat thickness of the first, tenth, last-rib and last-lumbar sites were calculated. Longissimus muscle samples from the last ribs were collected from each one of the animals, snap-frozen in liquid nitrogen, and stored at -80 °C prior to analysis.

### IMF content

The IMF content, expressed as weight percentage of dry muscle tissue, was evaluated 24 h after slaughter, using the Soxhlet petroleum-ether extraction method.

### Serum parameters

Blood samples were centrifuged at 600 x g for 15 min. Serum was obtained and stored at -20 °C for posterior analyses. Serum FFA and TG concentrations were determined using the respective commercial kit assays (Nanjing Jiancheng Biochemical Reagent Co., Nanjing, China) according to the protocols provided by the manufacturer.

### RNA extraction and Reverse transcription (RT)

Total RNA was extracted from the muscle tissues using Trizol reagent according to the manufacturer's protocol (Takala, Japan). Total RNA concentration was then quantified by measuring the absorbance at 260 nm in an Eppendorf Biospectrophotometer (Eppendorf AG, Hamburg, Germany). The absorption ratios (260/280 nm) ranged from 1.8 to 2.0. The integrity of the RNA was verified by 1.4% agarose-formaldehyde gel electrophoresis. Total RNA (2 µg) was then reverse-transcribed by incubation at 42 °C for 1 h in a 25 µL mixture consisting of MMLV (Promega Co., Shanghai, China), RNase inhibitor (Promega), and oligo dT. The reaction was terminated by heating to 95 °C for 5 min and quick cooling on *i.e.* cDNA was stored at -20 °C for PCR amplification.

### Real-Time Quantitative PCR (RT-qPCR)

RT-qPCR was performed to quantify the mRNA expression abundance of genes SREBP1c, H-FABP, DGAT1, LEPR and MC4R. β-actin was used as internal control. The 25 µL PCR mixture contained 12.5 µL of 2iQ SYBR Green Supermix, 0.5 µL (10 mM) of each primer (Table 2), and 1 µL of cDNA. Mixtures were incubated in an iCycler iQ Real-time Detection system (Bio-Rad Laboratories, Hercules, CA, USA). A melting curve analysis was performed to ensure that only a single PCR product was amplified. Control reactions were set for each sample. PCR amplification efficiencies were between 91% and 103%. The amount of specific target was calculated according to the following

**Table 1** - Composition of diets.

	Percentage (%)
Corn	68.98
Wheat bran	22.53
Soybean meal	5.62
Fish meal	0.50
Limestone	0.97
Monocalcium phosphate	0.10
Salt	0.30
Premix <sup>1</sup>	1.00
Calculated nutritional composition	
Crude protein	10.42
Total Lysine	0.69
Digestive energy (MJ/kg)	13.11
Calcium	0.46
Total phosphorus	0.37
Available phosphorus	0.14

<sup>1</sup>Vitamin premix contents per kilogram of diet: vitamin A - 8,267 IU; vitamin D2 - 2,480 IU; vitamin E - 66 IU; menadione (as menadione pyrimidinol bisulfite complex) - 6.2 mg; riboflavin - 10 mg; Ca d-pantothenic acid - 37 mg; niacin - 66 mg; vitamin B12 - 45 µg; d-biotin - 331 µg; folic acid - 2.5 mg; pyridoxine - 3.31 mg; thiamine - 3.31 mg; vitamin C - 83 µg; Trace mineral premix contents per kilogram of diet: Zn - 127 mg; Fe - 127 mg; Mn - 20 mg; Cu - 12.7 mg; I - 0.80 mg, in the form of zinc sulfate, ferrous sulfate, manganese sulfate, copper sulfate, ethylenediamine dihydride, respectively, with calcium carbonate as carrier, besides 0.3 mg Se per kilogram of diet.

**Table 2** - Specific primers used for real-time quantitative PCR (RT-qPCR)

Gene name	Sequence	Product size	Position	Accession number
β-actin	F: 5'-GCCGCACCACTGGCATTGTC-3'	399 bp	Exon 2	DQ845171
	R: 5'-AGGTAGTTTCGTGGATGCCGCAG-3'		Exon 3	
SREBP-1c	F: 5'-GCGACGGTGCCTCTGGTAGT-3'	218 bp	Exon 1	AY496867
	R: 5'-CGCAAGACGGCGGATTTA-3'		Exon 2	
H-FABP	F: 5'-CCTGGAAGCTAGTGGACAGC-3'	227 bp	Exon 1	AJ416019
	R: 5'-TGCCTCTTCTCGTAAGTGC-3'		Exon 2	
DGAT-1	F: 5'-AAGGACGGACACGACGATG-3'	289 bp	Exon 1	AY093657
	R: 5'-GGAACGCAGTCACAGCAAAG-3'		Exon 5	
LEPR	F: 5'-GTGATAACTGCATTTGACTTGGC-3'	285 bp	Exon 1	NM001024587
	R: 5'-CTGCAATGTGTCTGCATGTACAG-3'		Exon 1	
MC4R	F: 5'-TGGAGAAAATCGCTGAGGCTACC-3'	632 bp	Exon1	NM214173
	R: 5'-ATGATGAACAAAACCCCGACACC-3'		Exon2	

formula:  $\text{ratio} = 2^{-\Delta C_T \text{Target (sample - calibrator)}} / 2^{-\Delta C_T \beta\text{-actin (sample - calibrator)}}$ . All primers used were designed by Primer Premier 5 and synthesized by Shanghai Shengong Biological Company (Shanghai, China).

### Western blotting

Muscle samples were collected and homogenized on ice in 700 μL buffer A [50 mM Tris-HCl (pH 7.5), 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 10% glycerol] containing 1% Triton X-100, 5 μM aprotinin, leupeptin and pepstatin. The lysates were centrifuged at 6000 g for 20 min at 4 °C to remove insoluble material. Thereafter, supernatant extracts were collected and protein concentration determined, using the method described by Bradford (1976). Then the extracts were frozen at -80 °C until the western blot analyses were performed.

To measure SREBP-1c, DGAT-1, H-FABP, LEPR and MC4R protein expression, 50 μg of total whole-cell protein extract were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% resolving gel), transferred to a nitrocellulose membrane and probed overnight, with rabbit polyclonal anti-SREBP-1c, anti-DGAT-1, anti-H-FABP, anti-LEPR, and anti-MC4R antibodies (Sigma, USA) at 1:500, 1:500, 1:1000, 1:1000 and 1:2000 dilutions, respectively. The membranes were then probed with a goat anti-rabbit IgG-horseradish peroxidase conjugate (1:20,000 dilution) (Sigma, USA) for 1 h at room temperature. Blots were developed using the SuperSignal West Pico Chemiluminescent Substrate system (Bio-Rad, Hercules, CA, USA) and imaged on X-film, for image analysis and densitometry. Signal intensity was quantified using the Quantity One 1-D analysis software (Bio-Rad, Hercules, CA, USA).

### Statistical analysis

Data regarding growth performance, sera parameters and mRNA expression were analyzed using paired *t*-tests with the Statistical Packages for Social Science (SPSS) 12.0 software. All data were presented as mean ± S.E.M. Differences were considered statistically significant if  $p < 0.05$ .

### Results

#### Growth performance, carcass traits and serum parameters

Growth performance, carcass traits and serum parameters are presented in Table 3. There was no significant difference in growth performance between the fat and lean BMIL ( $p > 0.05$ ). A significant reduction in fat percentage was found in the fat BMIL compared to the lean BMIL ( $p < 0.05$ ). A significant increase of lean meat was observed in the fat BMIL ( $p < 0.05$ ). The serum concentrations of TG and FFA as well as the IMF content were significantly higher in the fat BMIL than in the lean BMIL ( $p < 0.05$ ).

#### Expression levels of genes and proteins

Figures 1 and 2 show the expression levels of the SREBP1c, H-FABP, DGAT1, LEPR and MC4R genes and proteins in muscle tissue of fat and lean BMIL animals. The expression levels of the H-FABP, SREBP-1c and DGAT1 genes and proteins were significantly higher in the fat BMIL than in the lean BMIL ( $p < 0.05$ ). The mRNA and protein abundance of MC4R and LEPR was lower in the fat BMIL compared to the lean BMIL. The difference in LEPR gene expression between the fat and lean BMIL was significant ( $p < 0.05$ ).

## Discussion

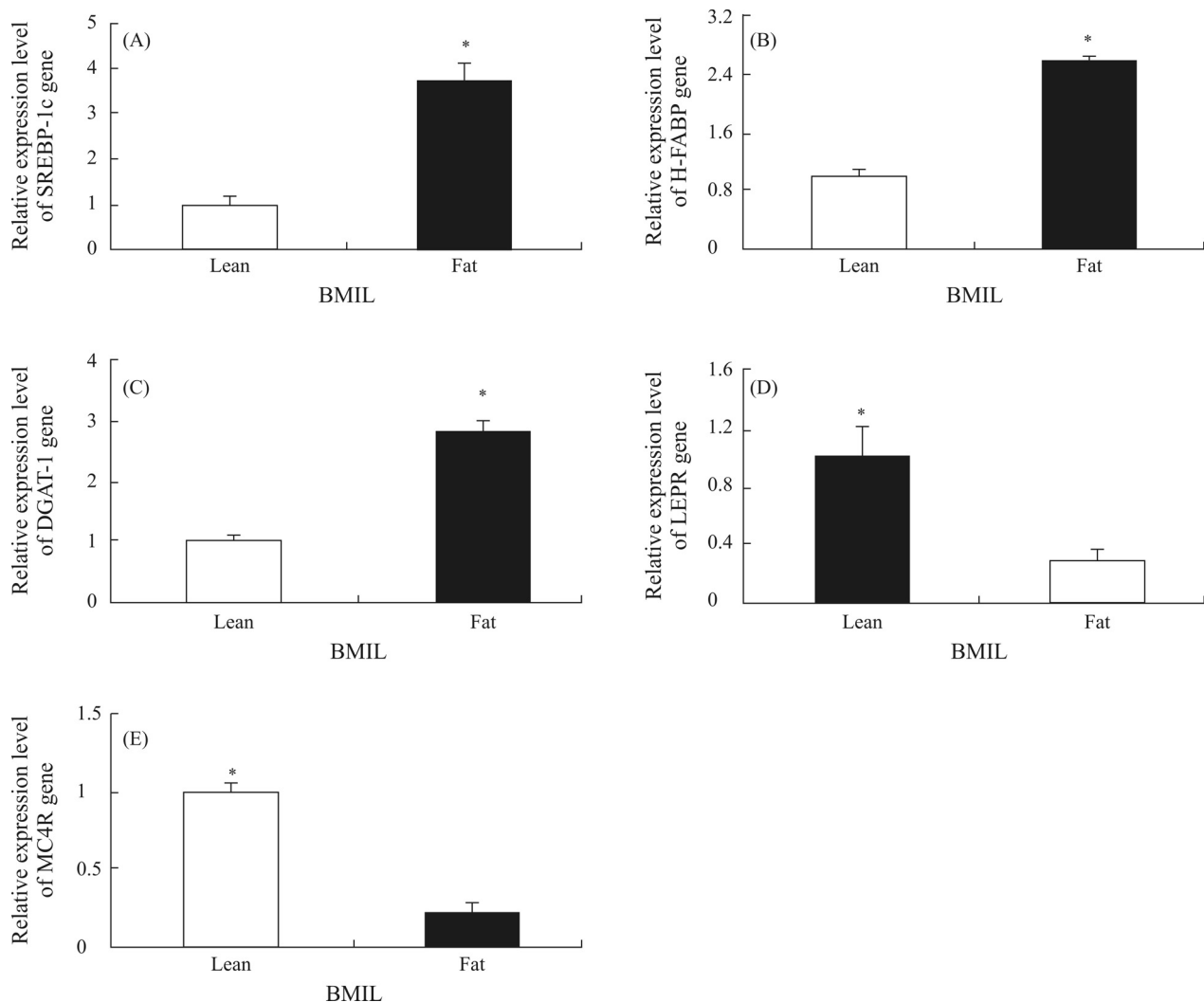
In this study, the growth performance of fat and lean BMIL animals showed no significant difference. However,

an increase in fat meat as well as in IMF content and reduced lean meat content in the fat BMIL compared to the lean BMIL was found. As a rule, animal obesity models

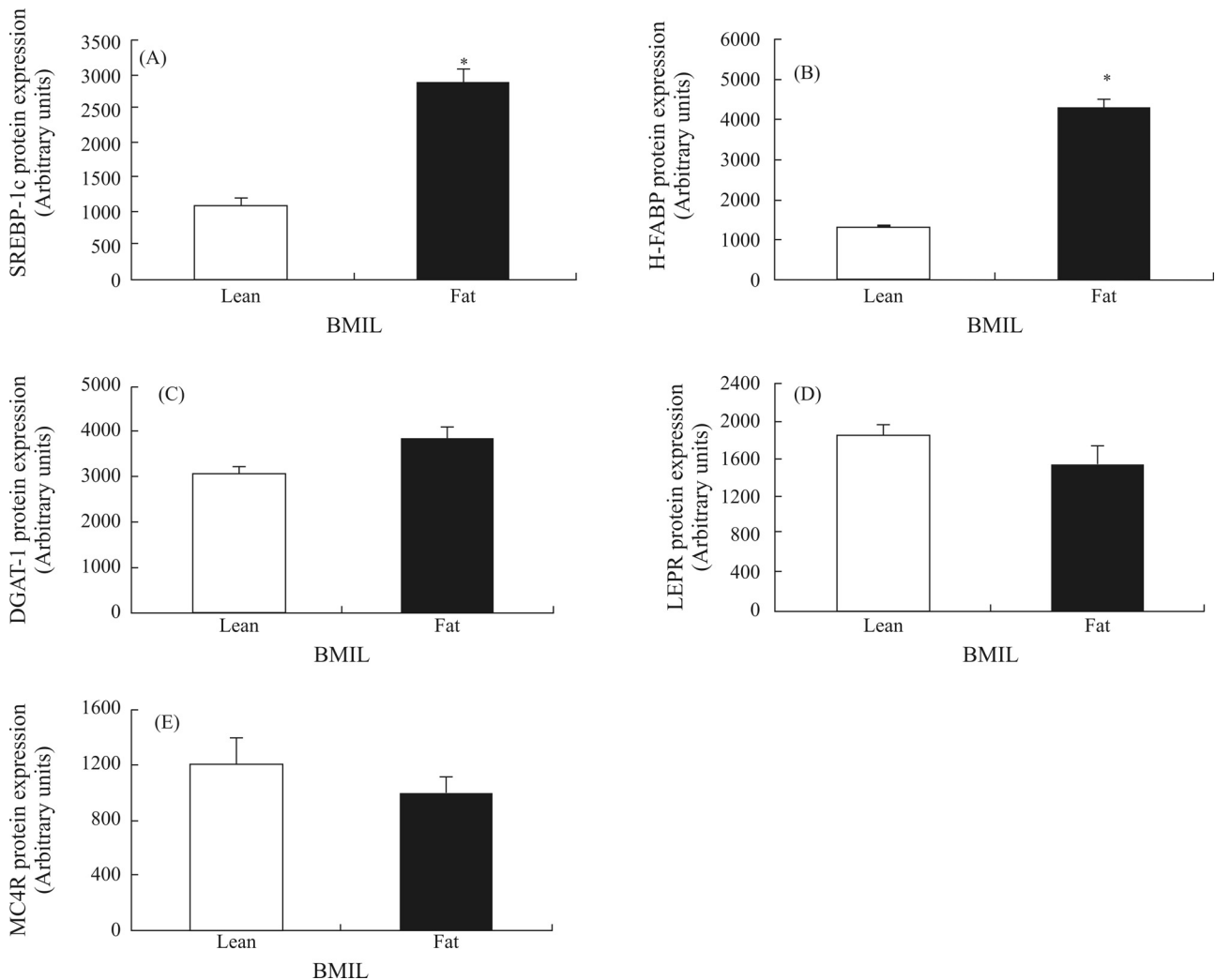
**Table 3** - Growth performance, carcass traits and serum parameters

	Indexes	Lean BMIL	Fat BMIL
Growth performance	Daily feed intake (g/d)	22.57 ± 0.06	28.67 ± 0.01
	Daily gain (g/d)	122.00 ± 24.98	121.00 ± 25.02
	Gain: feed ratio	4.11 ± 0.01	4.20 ± 0.01
Carcass traits	Lean meat percentage (%)	41.75 ± 1.01*	31.00 ± 0.77
	Fat meat percentage (%)	34.33 ± 1.68	53.10 ± 0.62*
	Average backfat thickness (cm)	1.31 ± 0.20	4.61 ± 0.13*
	IMF(%)	4.51 ± 0.10	7.82 ± 0.08*
Serum parameters	Triacylglycerol (mg/100 mL)	52.63 ± 1.77	61.97 ± 2.01*
	EFFA (μEq/L)	211.34 ± 19.99	266.99 ± 20.17*

Values are expressed as means ± SE; values in the same row with asterisk are significantly different at  $p < 0.05$ .



**Figure 1** - mRNA expression levels of SREBP-1c, H-FABP, DGAT1, LEPR and MC4R (A, B, C, D and E, respectively) in the fat and lean BMIL. Means ± SE with asterisk indicate a significant difference between the two groups ( $p < 0.05$ ).



**Figure 2** - Expression levels of proteins SREBP-1c, H-FABP, DGAT1, LEPR and MC4R (A, B, C, D and E, respectively) in the fat and lean BMIL. Means  $\pm$  SE with asterisk indicate a significant difference between the two groups ( $p < 0.05$ ).

weigh more than their lean counterparts. However, a similar feed intake was also reported in the lean Yorkshire and fat Ossabaw pigs (Wangsness *et al.*, 1980). This may suggest that the different fat deposition in the fat and lean BMIL pigs does not result from their feed intake, but rather from their genotypes for fatness traits.

TAG, the major component of IMF in muscles, is stored within intramuscular adipocytes (Gao and Zhao, 2009). It could be synthesized using fatty acids which were *de novo* synthesized or originated from feed nutrients. Hauser *et al.* (1997) reported that the plasma of fat pigs had a higher percentage of triglycerides than the plasma of lean pigs. Our results also showed that the fat BMIL had higher serum TAG and FFA concentrations as well as IMF content compared with the lean BMIL. These data indicate that more TAG or FFA from feed nutrients may be allotted to the muscle tissue of fat BMIL, leading to a higher IMF deposition than in the lean BMIL.

In general, an increase in IMF content is mainly due to an increase in TG contents (Fernandez *et al.*, 1999). Con-

sequently, the TG metabolism in muscle tissue should be a target for identification of genes involved in IMF deposition, especially the TAG synthesis metabolism. SREBP-1c can stimulate the transcription of genes encoding acetyl-CoA carboxylase (ACC) (Zhuang *et al.*, 2003) and fatty acid synthase (FAS) enzymes (Magana *et al.*, 2000). H-FABP plays a role in transporting the fatty acids, and has been regarded as a candidate gene for IMF deposition in pigs (Gerbens *et al.*, 1998; Glatz *et al.*, 2003; Chmurnyńska, 2006; Damon *et al.*, 2006; Gardan *et al.*, 2007). DGAT are microsomal enzymes which catalyze the final step of the triglyceride synthesis pathway (Yen *et al.*, 2008). Overexpression of DGAT enzymes in transgenic mice is sufficient to increase cellular TAG storage in skeletal muscle (Koliwad *et al.*, 2010). Our results show that the expression levels of the SREBP-1c, H-FABP and DGAT-1 genes and proteins in the fat BMIL pigs were higher than in the lean BMIL pigs. However, the difference in DGAT-1 protein expression was not significant. The expression of the DGAT-1 gene and protein was positively correlated



(Coleman and Lee, 2004). The results suggest that, in the fat BMIL, more fatty acids would be synthesized *de novo* and transported to store TAG.

Intramuscular TG is a major form of energy storage and represents a significant fuel for cellular metabolism (Gao and Zhao, 2009). MC4R and LEPR play an important role in the control of energy homeostasis. Many reports have shown that the genetic variants of MC4R and LEPR are associated with porcine fat deposition (Mackowski *et al.*, 2005; Oviló *et al.*, 2005; Bruun *et al.*, 2006; Jokubka *et al.*, 2006; Kim *et al.*, 2006; Fan *et al.*, 2009, 2010; Li *et al.*, 2010; Piórkowska *et al.*, 2010; Switonski *et al.*, 2010). The LEPR gene mediates the regulation of leptin effects (Halaas and Friedman, 1997; Houseknecht and Portocarrero, 1998; Li *et al.*, 2010). The MC4R gene has been most closely linked to controlling the energy balance in rodents. The interaction between melanocortins and their receptors in the hypothalamus is one of the main neuroendocrine pathways controlling energy balance (Yeo *et al.*, 2000; Wardlaw, 2001). However, some studies have indicated that these genes could also be expressed in the muscle tissue (Stinckens *et al.*, 2009; Li *et al.*, 2010; Larkina *et al.*, 2011; Tyra *et al.*, 2011). Our data show that the mRNA and protein abundance of LEPR and MC4R in the fat BMIL was lower than in the lean BMIL. However, the difference in MC4R protein expression was not significant. This may be due to the fact that the main function of MC4R is to regulate the food intake (Kim *et al.*, 2006). Since in the present study the feed intake by the fat and the lean BMIL pigs was similar, this may suggest that the IMF deposition could be affected by energy metabolism-related genes.

In conclusion, fat and lean BMIL with divergent phenotypes for IMF content did not result from feeding differences, but rather were influenced by feed nutrients TAG and FFA and the expression levels of genes and proteins which participate in the TAG synthesis process and the energy metabolism.

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