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Breed-specific lipid-related gene expression in the subcutaneous fat of Large White and Erhualian pigs at weaning

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Abstract. The Erhualian (EHL) pig possesses significantly lower growth rates and higher adipose deposition compared with the Large White (LW) pig. To further understand the mechanism of breed lipid deposition difference at the early postnatal age, we employed an animal model of EHL and LW pigs at weaning age to compare the lipid metabolism differences in subcutaneous fat. The result showed that serum triglyceride in EHL was significantly higher ($P < 0.05$) than that of LW. Peroxisome proliferator-activated receptor-γ protein level in EHL was significantly higher ($P < 0.01$) though CCTTA enhancer-binding protein level demonstrated no change compared with LW pigs. Hormone sensitive lipase, adipose tissue triglyceride lipase mRNA expression and the lipase activity were significantly lower ($P < 0.05$) in EHL. Uncoupling protein-2 protein content was significantly lower ($P < 0.01$) in EHL than that in LW pigs. We first cloned the nucleotide sequence of Zinc-α2-glycoprotein (ZAG) with 1090 bp and found that both ZAG mRNA expression and protein level in EHL pigs was significantly lower ($P < 0.01$) than that of LW pigs. β3 adrenergic receptor mRNA expression in EHL pigs was significantly higher ($P < 0.01$) than that of LW pigs, though tumour necrosis factor α gene expression demonstrated no significant difference. Therefore, the significant breed lipid metabolism difference in subcutaneous fat exists at an early postnatal age between EHL and LW pigs, and this difference may originate from two causes including the increased lipid synthesis and reduced lipid mobilization in EHL pigs compared with LW pigs.

1   Introduction

In recent years, an epidemic of obesity has been observed around the world. Adipose metabolism has received much attention because substantial adipose deposition is related to many metabolic diseases such as diabetes, hypertension, etc. Furthermore, it was estimated that the genetic basis for obesity could approximately range from 40 to 70 % (Singla et al., 2010). The pig is one of the most important domestic species for meat production and is also an ideal model for metabolic studies including obesity (Spurlock et al., 2008). Concerning the breed specific adipose metabolism, though there have been some reports demonstrating the breed differences (Quintanilla et al., 2002; Li et al., 2008a, b; Nakajima et al., 2011; Wei et al., 2011), most of them were interested in the pigs' metabolism at the finishing period. Actually, early life is a critical period for body lipid deposition

(Wells, 2007). After birth, the adipose tissue expansion takes place rapidly as a result of increased fat cell size as well as an increase in fat cell number (Gregoire et al., 1998). Therefore, understanding the adipose metabolism at the early postnatal stage is also decisive for clarifying the breed difference of lipid deposition in pigs.

Fat metabolism mainly includes adipogenesis and lipolysis. CCTTA enhancer-binding protein (C/EBPβ) and peroxisome proliferator-activated receptor-γ (PPARγ) are well-established key transcription factors for adipogenesis. PPARγ is highly expressed and essentially required for the formation, differentiation and survival of adipose tissue in vivo (Koh et al., 2009). C/EBPβ is an important factor that causes adipogenesis and plays a dual role as a stimulator of cell determination and differentiation (Darlington et al., 1998). Lipolysis depends most on adipose tissue triglyceride lipase (ATGL) and hormone sensitive lipase (HSL). ATGL

could catalyse the initial step of the triglyceride's hydrolysis (Schweiger et al., 2006). HSL is highly expressed in adipocytes, and lipolysis is influenced by its protein content, activity and the distribution in the cells (Granneman et al., 2007). For the fat utilization, carnitine palmitoyl transferase-I (CPT1) is a limited enzyme of fatty acid β-oxidation and uncoupling protein-2 (UCP2) plays a critical role in the biological traits of animal body weight, basal metabolic rate and energy conversion (Li et al., 2007). Whether these lipid metabolism genes play a role in breed differences at early postnatal age in pigs is not clear.

In the past 20 years, it has been recognized that adipose tissue produces a large number of cytokines which are termed "adipokines". These adipokines have an autocrine function on adipose tissue homeostasis and they are secreted into the peripheral circulation with a systemic effect on metabolic events (Guerre-Millo, 2004; Fantuzzi et al., 2005). Recently, observations demonstrated that Zinc-α2-glycoprotein (ZAG) exists in both white and brown adipose tissue (Bing et al., 2004; Mracek et al., 2010) and the expression of ZAG is inversely related to the mass of white adipose tissue (Dahlman et al., 2005; Rolli et al., 2007). It has been found that ZAG could bound to β3 adrenergic receptors (β3-AR) and could activate GTP-dependent adenylate cyclase to elevate intracellular cAMP level and HSL activity (Russell et al., 2002, 2004). Additionally, tumour necrosis factor α (TNFα) causes a dose-dependent decrease of ZAG expression (Bao et al., 2005). However, until now, studies on ZAG have been restricted to humans and rats, and no results have been observed in pigs. Whether it is expressed in adipose tissue of pigs and whether it is differently expressed in the two breeds is unknown.

Erhualian (EHL), a famous Chinese indigenous pig breed, belongs to the same family as Meishan, which is characterized by high reproductive rates and superior meat quality. Compared with Large White pigs (LW), EHL pigs possess significantly lower growth rate and higher adipose deposition. Though previous studies have shown the cellular difference of adipocyte in subcutaneous fat (Nakajima et al., 2011) and the quantitative trait locus (QTL) analysis of growth and fatness data between growing Landrace and Meishan pigs (Quintanilla et al., 2002; Wei et al., 2011), the adipose metabolism difference at the early postnatal stage is still largely unclear between these two breeds.

Therefore in the present study we employed LW and EHL pigs as animal models and detected the expression of lipogenesis-related genes, including PPARγ and C/EBPβ, lipolysis-related genes, including HSL and ATGL, fatty acid oxidation-related genes, including CPT1 and UCP2 and the adipokine ZAG, to compare the difference in subcutaneous fat between LW and EHL pigs at weaning age. The results will shed new light on the difference in lipid metabolism of different pig breeds.

2   Material and methods

Six male piglets from three litters (two from each litter) of purebred LW and EHL sows from two neighbouring pig breeding farms in Changzhou, Jiangsu Province, China, were slaughtered at 25 days of age just before weaning. The subcutaneous fat was taken and immediately placed into liquid nitrogen and stored at $-80\,^\circ$C. The slaughter and sampling procedures complied with the "Regulation regarding the Management and Treatment of Experimental Animals" (2008) no. 45 set by Jiangsu Provincial People's Government. The experiment protocol was specifically approved by the Animal Ethics Committee of Nanjing Agricultural University.

2.1   RNA extraction and reverse transcription (RT)

Total RNA was extracted using Trizol Plus RNA Purification Kit (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA). Concentration of the extracted RNA was measured using NanoDrop 1000 Spectrophotometer. Ratios of absorption (260/280 nm) of all preparations were between 1.8 and 2.0. RNA integrity was confirmed by agarose electrophoresis and DNA contamination was examined by PCR. Reverse transcription was performed using the total RNA (2 µg) in a final volume of 25 µL containing $1 \times$ RT-buffer, 100 U Moloney Murine Leukemia Virus reverse transcriptase (M-MLV) (Promega Corp., Fitchburg, WI, USA), 8 U RNase inhibitor (Promega), 5.3 µmol L^{-1} random hexamer primers and 0.8 µmol L^{-1} dNTP (TaKaRa Bio Inc., Otsu, Shiga, Japan).

2.2   PCR

PCR was performed in Gene Amp PCR system (PerkinElmer Inc., Waltham, MA, USA). 2 µL RT product was used for general PCR in a final volume of 50 µL containing 2.5 U LA Taq (TaKaRa, Japan), 5 µL $10 \times$ LA PCR Buffer, 400 µM dNTPs, 36.5 µL ddH$_2$O and 0.2 µM forward and reverse primers for ZAG (forward primes: 5'-CCCAGCCATTCCCATAACTA-3', reverse primers: 5'-CCCAGTGCTGGCACTCTTCT-3'), using a program of 35 cycles (30 s at 95 $^\circ$C, 30 s at 58 $^\circ$C, 30 s at 72 $^\circ$C) and a final step of 10 min at 72 $^\circ$C. The obtained PCR products were analysed on 2 % agarose gel and purified with an AxyPrep DNA Gel Extraction Kit (Axygen Scientific, Inc., Hangzhou, China) and then sequenced.

2.3   Quantitative PCR

Quantitative PCR was performed with Mx3000P (Stratagene, Agilent Technologies, Inc., Santa Clara, CA, USA). Mock RT and no template controls (NTC) were set to monitor the possible contamination of genomic DNA both at RT and PCR. The pooled sample made by mixing equal quantities of total RT products (cDNA) from all samples was used to

optimize PCR conditions and tailor the standard curves for each target gene. Melting curves were performed to ensure a single specific PCR product for each gene. 2 μL of the eight-fold dilution of RT product were used for RT-PCR in a final volume of 25 μL containing 12.5 μL SYBR Green Real-time PCR Master Mix (TaKaRa, Japan) and 0.4 μM of each forward and reverse primers for ZAG, HSL, PPARγ, C/EBPβ, TNFα, β3-AR, ATGL, UCP2 and CPT1 genes (sequences of primers were shown in Table 1). All samples were normalized with the reference gene 18S rRNA. The PCR products for each gene were sent for sequencing to verify accuracy (Haojia Biotech, Ltd., Shanghai, China). The reported sequences exactly matched those published in GenBank. The method of 2^{-Ct} was used to analyse the real-time PCR data. All samples were included in the same run of RT-PCR and repeated in triplicates.

2.4 Protein extraction and western blot analysis

Total protein was extracted from 500 mg of frozen subcutaneous fat in 1 mL lysis buffer (15 0 mmol L^{-1} NaCl, 10 mmol L^{-1} Tris-HCl, 5 mmol L^{-1} EDTA, 1 % Triton X-100 and 0.1 % SDS (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). The protease inhibitor cocktail (Roche Applied Science) was added according to the manufacturer's instructions. The protein concentration was measured with the BCA Protein Assay Kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Thirty micrograms of protein extract were used for electrophoresis on a 12 % SDS-PAGE gel. western blot analyses for ZAG (13399-1-AP, Proteintech Group, USA, diluted 1 : 5000), C/EBPβ (sc-150x, Santa Cruz, USA, diluted 1 : 2000), UCP-2 (11081-1-AP, Proteintech group, USA, diluted 1 : 5000) and PPARγ (BS1587, Bioworld, China, diluted 1 : 1000) were carried out according to the recommended protocols provided by the manufacturers. β-actin (AP0060, Bioworld, USA, diluted 1 : 6000) was used as a reference in the western blot analyses.

2.5 Lipase (including HSL and ATGL) activity assay

500 mg frozen subcutaneous fat in 1 mL homogenization buffer (0.1 mmol L^{-1} K$^+$-PBS containing 1 mmol L^{-1} MgCl$_2$, 1 mmol L^{-1} DTT and 1 mmol L^{-1} EDTA) was homogenized in ice for 30 min and then centrifuged for 10 min at 12 000 × g at 4 °C. The protein concentration of supernatants was measured with the BCA Protein Assay Kit (Pierce Chemical Corp., Rockford, USA). Triolein without glycerin was used as substrate which can be hydrolysed to glycerol by the two enzymes, HSL and ATGL. The supernatants together with prepared triolein were incubated for 1 h at 37 °C. The lipases in supernatants activate the lipolytic degradation of the triolein emulsion. The released glycerol was measured with a commercial kit (Applygen, China). To fit the activities of the enzymes to the linear range of stan-

Table 1. The primer sequences of the target genes.

Target genes	PCR products, bp	Primer sequences
ZAG	106	F: 5'-gggcaggtttggctgtgag-3' R: 5'-ggctgggatttctttgttg-3'
PPARγ	210	F: 5'-gcccttcaccactgttgatt-3' R: 5'-gagttggaaggctcttcgtg-3'
TNFα	160	F: 5'-ccacgctcttctgcctactgc-3' R: 5'-tcggctttgacattggctacaa-3'
β3-AR	149	F: 5'-gctggttgcccttctttgtgg-3' R: 5'-gcgaaagtctgggctgtggc-3'
HSL	84	F: 5'-accctcggctgtcaacttctt-3' R: 5'-tcctccttggtgctaatctcgt-3'
FAS	206	F: 5'-gtcctgctgaagcctaactc-3' R: 5'-tccttggaaccgtctgtg-3'
ATGL	153	F: 5'-acctgtccaacctgctgc-3' R: 5'-gcctgtctgctcctttatcca-3'
C/EBPβ	158	F: 5'-gacaagcacagcgacgagta-3' R: 5'-agctgctccaccttcttctg-3'
UCP2	111	F: 5'- tcacccaatgtcgctcgta-3' R: 5'-gcagggaaggtcatctgtca-3'
CPT1	126	F: 5'- actgagaccgtgcgttcct-3' R: 5'-cagatgctggtgcttttcg-3'
18S	122	F: 5'-cccacggaatcgagaaagag-3' R: 5'-ttgacggaagggcacca-3'

dard curves constructed with pure enzymes, all samples were measured in duplicates at appropriate dilutions. The activity of lipases is expressed as nanomoles released glycerol per milligram protein per hour.

2.6 Statistical analyses

All data are presented as mean ± standard error. Statistical analyses were carried out with SPSS 13.0 for Windows. The differences were analysed using a t test for independent samples. A P value of less than 0.05 was considered to be significant.

3 Results

3.1 Body weight and serum triglyceride concentration between LW and EHL pigs

The body weight of LW and EHL pigs at weaning age was 8.10 ± 0.34 and 4.08 ± 0.25 kg, respectively. The serum triglyceride content of LW and EHL pigs was 0.29 ± 0.03 and 0.62 ± 0.12 mmol L^{-1}, respectively. Both the body
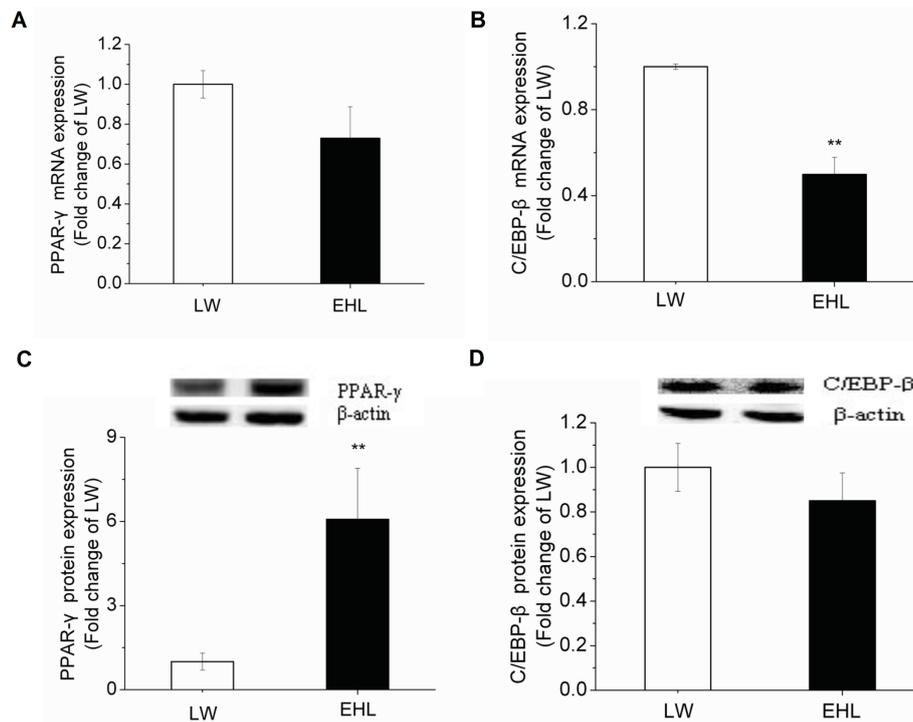
Figure 1. mRNA and protein expression of genes involved in adipogenesis in the subcutaneous fat of LW and EHL pigs. **(a)** PPARγ mRNA expression, **(b)** C/EBPβ mRNA expression, **(c)** PPARγ protein expression, **(d)** C/EBPβ protein expression. The values shown represent the mean $\pm$ standard error, $n = 6$; **$P < 0.01$ vs. LW.

weight and serum triglyceride concentration demonstrated a significant breed difference ($P < 0.05$).

3.2 Lipogenesis- and lipolysis-related gene expression in the subcutaneous fat between LW and EHL pigs

Though C/EBPβ mRNA expression was significantly lower ($P < 0.01$) in the subcutaneous fat of EHL than in that of LW pigs; the protein expression demonstrated no difference. PPARγ mRNA expression showed no difference between the two breeds, while its protein level was significantly higher ($P < 0.01$) in the subcutaneous fat of EHL than in that of LW pigs (Fig. 1).

The mRNA expression of HSL and ATGL was significantly lower ($P < 0.01$) in the subcutaneous fat of EHL than in that of LW pigs (Fig. 2). The lipases (including HSL and ATGL) activity was also significantly lower ($P < 0.05$) in the subcutaneous fat of EHL than in that LW pigs (Fig. 2). mRNA expression of CPT1 and UCP2, two key genes involved in fatty acid oxidation, was significantly lower ($P < 0.05$) in the subcutaneous fat tissue of EHL than that of LW pigs. Furthermore, UCP2 protein level was also significantly lower ($P < 0.01$) in the subcutaneous fat of EHL than that of LW pigs (Fig. 3).

3.3 Porcine ZAG gene sequencing and expression

In the present study, we cloned the nucleotide sequence containing the complete open reading frame (ORF) of porcine ZAG with 1090 bp. The ORF sequences are conservative between species. MegAlign software (DNASTAR, Inc., Madison, WI, USA) was used to compare the homology of ZAG. The results showed that porcine ZAG gene nucleotide sequence possesses 82.2 % homology with cattle and 69.5 % homology with mice, respectively. Both the mRNA and protein expression of ZAG in the subcutaneous fat of EHL was significantly lower ($P < 0.01$) than that of LW pigs (Fig. 4).

3.4 TNFα, β3-AR mRNA expression in the subcutaneous fat between LW and EHL pigs

The expression of β3-AR mRNA in subcutaneous fat of EHL pigs was significantly higher ($P < 0.01$) than that of LW pigs. No difference was observed in the expression of TNFα between the two breeds of pigs (Fig. 5).

4 Discussion

The EHL pig is a famous Chinese indigenous pig breed with high reproductive traits and meat quality, similar to its close relative the Meishan (MS) pig, whereas the LW pig is a lean type of pig. Concerning the fat deposition between these
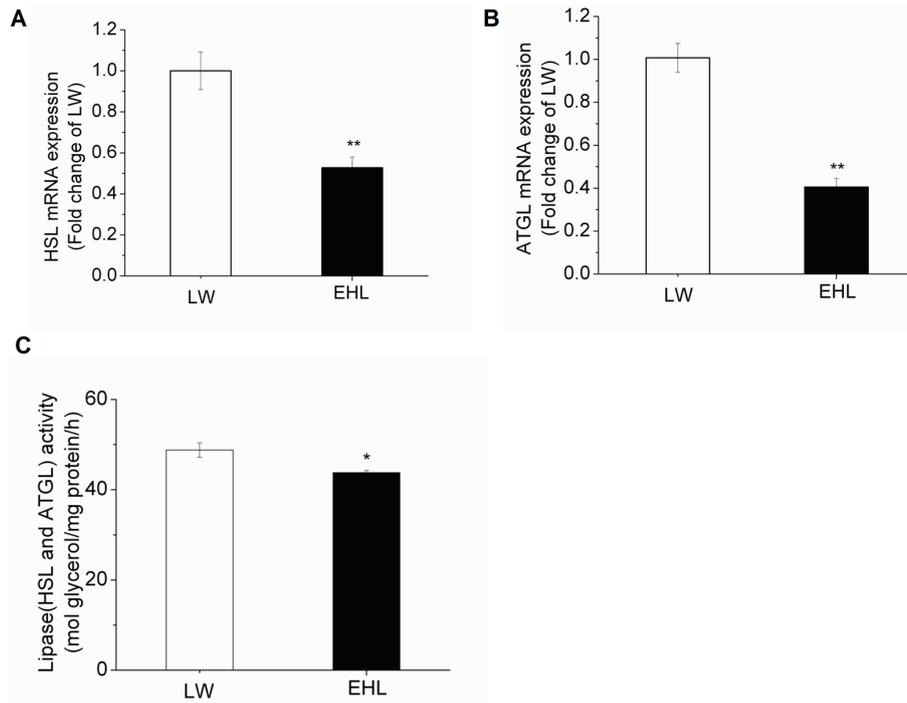
Figure 2. mRNA and protein expression of genes involved in lipolysis and lipases activity in the subcutaneous fat of LW and EHL pigs The values shown represent the mean ± standard error, $n = 6$; *$P < 0.05$ vs. LW; **$P < 0.01$ vs. LW. **(a)** HSL mRNA expression, **(b)** ATGL mRNA expression and **(c)** Lipases activity (HSL and ATGL).
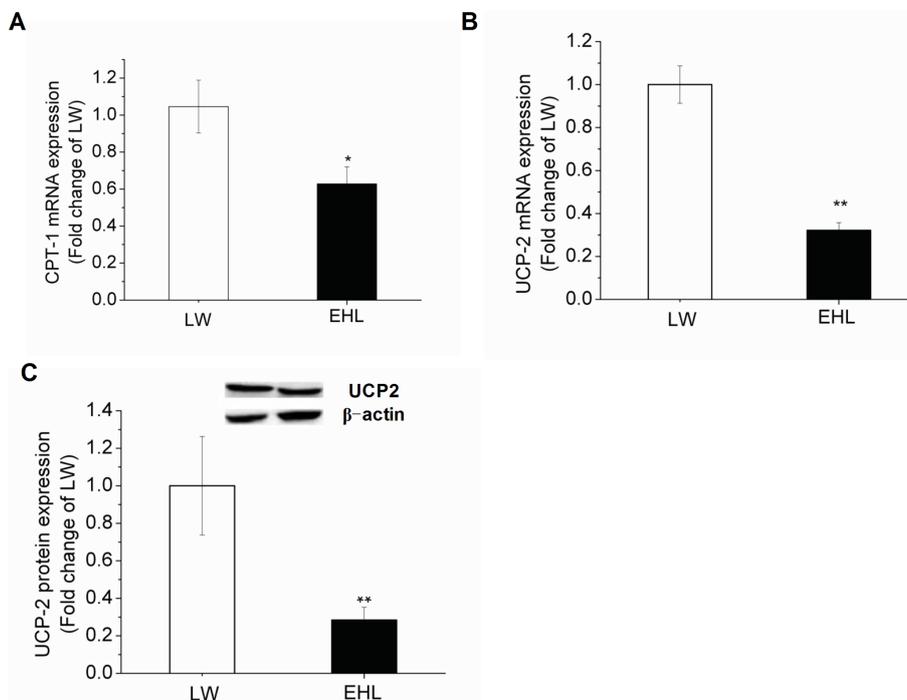

Figure 3. mRNA and protein expression of genes related to energy metabolism in the subcutaneous fat of LW and EHL pigs. **(a)** CPT1 mRNA expression, **(b)** UCP2 mRNA expression and **(c)** UCP2 protein expression. The values shown represent the mean ± standard error, $n = 6$; *$P < 0.05$ vs. LW; **$P < 0.01$ vs. LW.
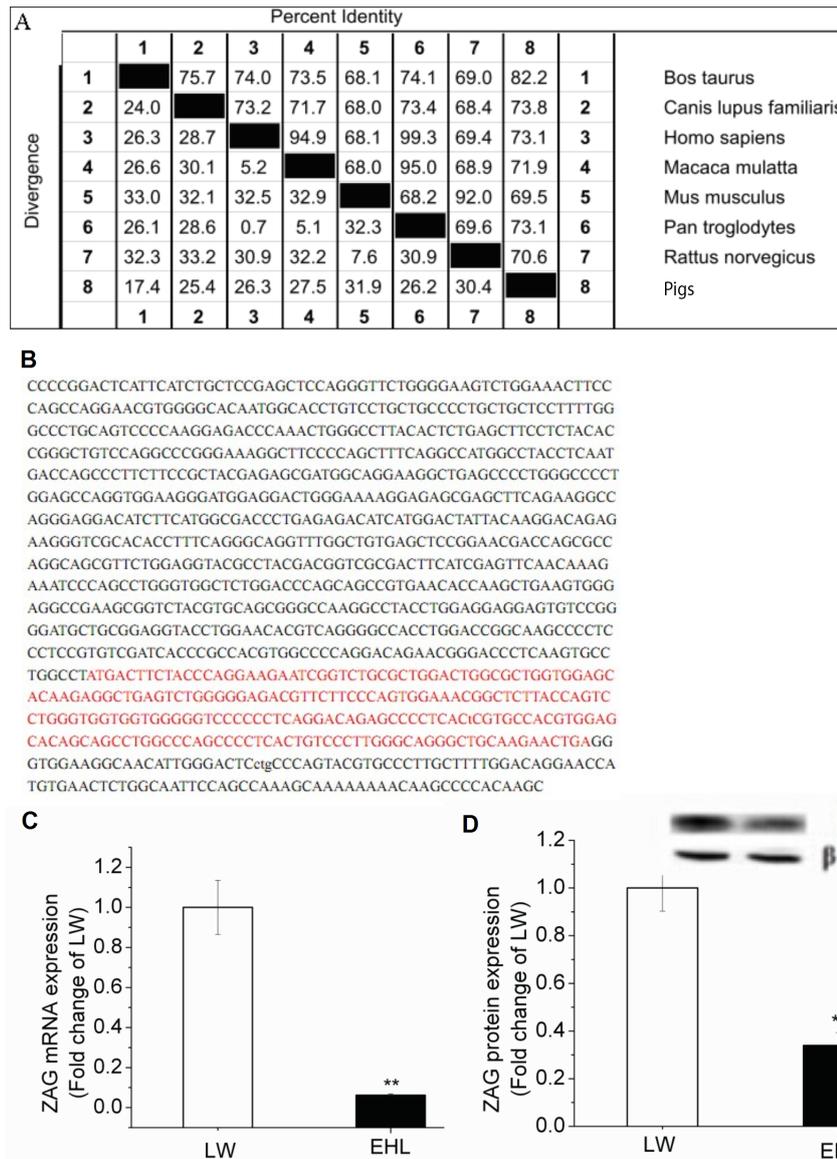
Figure 4. Porcine ZAG gene sequencing and its mRNA and protein expression between LW and EHL pigs. **(a)** Homology analysis, **(b)** porcine ZAG cDNA sequence, **(c)** ZAG mRNA expression and **(d)** ZAG protein expression. The values shown represent the mean $\pm$ standard error, $n = 6$; $**P < 0.01$ vs. LW.

two breeds, previous studies suggested that adipose tissue development occurs much earlier in MS than in LW pigs (Mourot et al., 1996), and preadipocyte proliferation also demonstrated the breed difference in vitro (Gerfault et al., 1999). Recent studies have shown that the quantitative trait locus (QTL) for the lipid metabolic characteristics is in both two breeds located on chromosome 7 (Demars et al., 2007; Gondret et al., 2012). In the present study, we demonstrated that though the body weight of the EHL pig is only half of LW pigs, the serum triglyceride level in EHL pigs is significantly higher compared with LW pigs. Additionally, a significant difference was also observed in both adipogenesis-

and lipolysis-related gene expressions between EHL and LW piglets at weaning age.

Regarding the adipogenesis, C/EBPβ and PPARγ are well-established key transcription factors. PPARγ could stimulate the differentiation from preadipocytes into mature adipocytes and is closely associated with fat formation. PPARγ-deficient mice exhibited smaller adipocytes and decreased fat mass compared with wild mice (Kubota et al., 1994). C/EBPβ is also known to directly influence adipocytes' development (Rosen et al., 2000). It could lead preadipocytes to the proliferative stage and activates PPARγ to promote the differentiation of adipocytes (Devine et al., 1999). C/EBPβ also could make other cells turn into
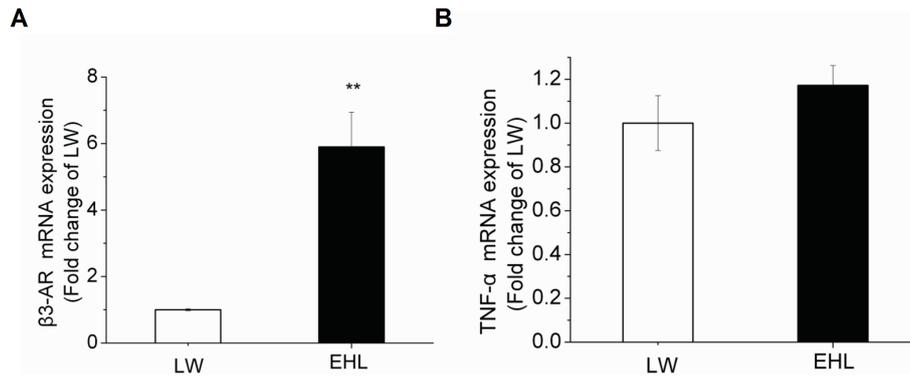
Figure 5. mRNA expression of TNFα and β3-AR in subcutaneous fat of LW and EHL pigs. **(a)** TNFα mRNA expression and **(b)** β3-AR mRNA expression. The values shown represent the mean $\pm$ standard error, $n = 6$; ** $P < 0.01$ vs. LW.

adipocytes (Wu et al., 1995). In the present study, though the mRNA expression of C/EBPβ decreased, the protein concentration demonstrated no significant difference. The protein level of PPARγ was significantly higher in EHL than in LW pigs. PPARγ could regulate the expression of lipid metabolism-related genes to promote the synthesis of triglycerides in adipocytes and could cause the cell volume increase (Vidal-Puig et al., 1997). Additionally, PPARγ could also inhibit the expression of genes involved in lipolysis and fatty acid release (Prusty et al., 2002). From our study we infer that EHL pigs may possess greater capability for adipogenesis.

For the lipolysis, the mobilization of triglyceride in fat cells is mainly dependent on HSL and ATGL. ATGL could hydrolyse triglycerides and HSL could hydrolyse both triglycerides and diglycerides. Both of them are the limited enzymes of triglyceride hydrolysis process. The present study showed that EHL pigs had significantly lower HSL and ATGL mRNA expression than LW pigs, and the lipase activity of the EHL pig was also lower than that of the LW pigs. The results showed that EHL pigs lesser capability for lipolysis than LW pigs. CPT is a limited enzyme of fatty acid β-oxidation. Intensive research has revealed a critical role for UCP in the control of mitochondrial reactive oxygen species production and in various physiological events such as obesity and diabetes (Rosen et al., 2000; Zhang et al., 2001). Elevated UCP expression could promote the utilization of the free fatty acids and could translate their energy into heat production (Ceperuelo-Mallafré et al., 2009). In the present study, the mRNA expressions of CPT1 and UCP2 were significantly lower in the subcutaneous fat of EHL than in that of LW pigs. The level of UCP2 protein decreased significantly in EHL compared with LW pigs. It seems that the breed difference of lipid deposition has existed at early postnatal age. Similar to breed lipid metabolism differences at the finishing stage (Li et al., 2008a, b), the two breeds demonstrated a big difference in lipid metabolism at weaning age. EHL pigs possess significantly greater capability for adipogenesis and the

lesser capability for lipolysis and utilization compared with LW pigs.

ZAG is newly defined as a kind of adipokine. A previous study has demonstrated that elevated ZAG could decrease blood glucose, triglyceride and free fatty acid levels in mice compared with the control group (Ceperuelo-Mallafré et al., 2009). Furthermore, there is a clear correlation between ZAG content and body weight reduction (Todorov et al., 1998). Both body weight and epididymal fat were reduced in ZAG overexpressive mice (Gong et al., 2009). An injection of human ZAG could increase over-fed mice's utilization of sugar intake, the rate of glucose metabolism and lipid oxidation in heart, brain and brown fat tissue (Russell and Tisdale, 2002). There is growing evidence pointing to ZAG as a potential therapeutic target for obesity (Gong et al., 2009; Gao et al., 2010). However, until now, there has been no report on ZAG in pigs. In the present study, we first cloned a part of porcine ZAG cDNA sequence including ORF, and the nucleotide sequence homology analysis showed that porcine ZAG gene possesses the highest homology with cattle (82.2 %). Furthermore, both mRNA expression (almost 10 times higher) and protein level (2 times higher) of ZAG were significantly lower in EHL compared with LW pigs. The present study suggests that ZAG expressed in porcine adipose tissue, and its expression existed great breed difference at the early postnatal age. It may directly correlate with the fat deposition in pigs.

It has been demonstrated that ZAG bounds to β3-AR and activated GTP-dependent adenylate cyclase in the membrane as well as intracellular cAMP level and HSL activity (Russell et al., 2002, 2004). The effect of ZAG can be blocked by β3-AR antagonist SR59230 (Russell et al., 2002). In the present study, β3-AR mRNA expression in white adipose tissue of EHL was significantly higher than that of LW pigs. This may be due to the low content of ZAG. It has been shown that the proinflammatory cytokine TNFα decreased in a dose-dependent manner after ZAG expression (Bao et al., 2005), whereas in the present study TNFα gene expres-

sion demonstrated no significant difference between the two breeds. Regardless, it needs further study to better understand the pathway of ZAG in porcine lipid metabolism.

In conclusion, EHL and LW pigs demonstrated a significant breed difference in lipid metabolism at early postnatal age. EHL pigs showed greater capability for lipid synthesis and lower lipid mobilization compared with LW pigs. ZAG may be an important adipokine involved in the formation of breed-specific fat deposition.

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