Enhanced adipogenesis in Mashen pigs compared with Large White pigs

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\textbf{ABSTRACT}

Intramuscular fat (IMF) is critical pork meat quality; however, its content is relatively low in commercial pig breeds. The objective of this experiment was to investigate the difference in intramuscular adipogenesis between Mashen and Large White pigs. 12 male Mashen and Large White piglets (six in each breed) were used, and longissimus dorsi muscle was sampled for analysis. The results showed that back fat thickness (\(p<.01\)) and leaf fat weight (\(p<.05\)) were increased in Mashen pigs. Moreover, the IMF contents were greater in Mashen pigs (\(p<.01\)). With exception of sterol regulatory element-binding protein 1, mRNA expression of other adipogenic markers, including Zinc finger protein 423 (\(p<.05\)), platelet-derived growth factor receptor \(\alpha\) (\(p<.05\)), CCAAT enhancer binding protein \(\beta\) (C/EBP\(\beta\), \(p<.05\)), C/EBP\(\alpha\) (\(p<.05\)) and peroxisome proliferator-activated receptor \(\gamma\) (PPAR\(\gamma\), \(p<.01\)) were greater in Mashen pigs; consistently, protein abundance of both C/EBP\(\alpha\) (\(p<.01\)) and PPAR\(\gamma\) (\(p<.05\)) were also greater. Both mRNA expression of fatty acid synthase (FAS) (\(p<.01\)) and its activity (\(p<.05\)) were elevated in Mashen pigs, whereas no difference of acetyl-CoA carboxylase activity was observed. In addition, protein content of fatty-acid binding protein 4 (FABP4) was elevated in Mashen pigs. The malate dehydrogenase activity was greater (\(p<.05\)) and hormone-sensitive lipase activity was lower in Mashen pigs. In summary, IMF content was enhanced in Mashen pigs, which at least partly through enhancing intramuscular adipogenesis.

\textbf{Introduction}

Intramuscular fat (IMF), or marbling, is critical for meat quality of pork (Brewer et al. 2001). Over the past decades, the swine industry has been remarkably successful at reducing the adiposity of the carcass and enhancing the lean growth through intensive genetic selection (Faucitano et al. 2004). However, the high lean ratio of carcases has dramatically reduced IMF and impaired the meat quality (Lonergan et al. 2001). Recently, consumer demand for high-quality meat is increasing in most countries, which requires meat industry to produce pork with high IMF deposition. Therefore, many studies have been carried out to enhance IMF, the majority of which focus on the conversion of preadipocytes to adipocytes, lipid metabolism and adipocytes hypertrophy through nutritional management (Rosenvold & Andersen 2003; Hausman et al. 2009). A better understanding of molecular and cellular mechanisms regulating IMF development is needed.

The Large White pigs is a fast-growing pig breed originating from England, and is renowned for its lean carcase and high growth rate (Larzul et al. 1997). The Mashen pigs are indigenous breed that demonstrates strong adaptability to local conditions in Shanxi province, China (Zhao et al. 2015). Although the growth rate and feed efficiency are lower compared to the Western commercial pig breeds, including Large White, Landrace and Duroc, the Mashen pigs breed has genetic diversity and excellent reproductive ability (Zhao et al. 2015). We hypothesised that IMF content and adipocytes formation ability are the difference between Mashen and Large White pigs. The objective of the present study was to investigate the difference in intramuscular adipogenesis between the two breeds.

\textbf{Materials and methods}

\textit{Care and use of animals}

All animal procedures were approved by the Shanxi Agricultural University Animal Care and Ethics Committee. A total of 12 male Mashen and Large White piglets (six in each breed, castrated at 7 day)
were selected randomly from Datong Pig Breeding Farm (Shanxi, China). Here, we only use six piglets per breed because of the large IMF difference between two breeds, which provides sufficient power to discern breed effects. Piglets were weaned at 28 day of age, housed in breed groups and fed with same commercial starter diet (Dabeinong Technology Group Co., Ltd, Beijing, China) for 60 day. At the age of 90 day, piglets were changed to commercial fattening diet (Dabeinong Technology Group Co., Ltd, Beijing, China) until slaughter. Animals were allowed access to feed and water ad libitum under the same management conditions.

At weaning, muscle biopsy sites were shaved and aseptically cleaned with betadine and 70% ethanol, and were locally anesthetised using 2% lidocaine (Shandong Hualu Pharmaceutical Co., Ltd, Shandong, China). Biopsy samples from *longissimus dorsi* (LD) muscle were obtained using sterile biopsy punch (5mm, Miltex Inc., York, PA) for early adipogenic gene expression analysis. At 180 day of age, the body weights (BW) were recorded, and all pigs were anaesthetised by inhaled CO2 and slaughtered. Leaf (kidney) fats were removed on both sides of the carcase and weighed. One piece of LD muscle from the left side was sampled at the anatomic centre, minced and snap-frozen in liquid nitrogen, and second piece was fixed in 4% fresh paraformaldehyde (PFA) for paraffin embedding. Carcases were chilled for 24 h at 4°C and then the left side of each carcase was ribbed between the 10th and 11th rib interface. Tenth-rib backfat thickness was determined by measuring the fat thickness at the 10th rib using a ruler, three-fourths of the lateral length of the loin muscle perpendicular to the outer skin surface.

**Intramuscular fat content analysis**

Intramuscular fat content was determined using the Soxhlet petroleum-ether extraction method according to a previous report (Zhang et al. 2014). Briefly, LD muscles obtained during animal harvest were lyophilised and then subjected to ether extraction using Soxhlet apparatus. After 8 h extraction, samples were removed, air-dried and reweighed to determine fat loss.

**Masson trichrome stain**

Unless stated otherwise, all chemicals are of analytical grade. Masson Trichrome stain was performed as previously described (Foidart et al. 1981). Briefly, LD muscle fixed by 4% PFA (pH 7.4) was serially dehydrated in ethanol and xylene, and embedded in paraffin. Following embedding, blocks were sectioned at 7 μm using a microtome (Leica, German). Then, sections were dewaxed and rehydrated serially by incubations with xylene and different concentrations of ethanol. After that, sections were subjected to trichrome staining for histological examination, which stained muscle fibre red, nuclei black and collagen blue. Images were taken at a 100 × magnification under a DMI8 microscope (Leica, Germany) and digitally analysed for intramuscular adipocytes area using Image J software (NIH, Bethesda, MD). 10 fields of each sample were traced per animal in a blinded fashion for observation.

**Real-time quantitative PCR (RT-PCR)**

Total RNA in LD muscle sample was extracted using Trizol reagent (Sigma, Saint Louis, MO) followed by DNase I (#M0303s, New England Biolabs, Ipswich, MA) digestion. Concentration and the integrity of RNA samples were determined by both NanoDrop instrument (ND-2000, Nanodrop Instruments, Thermo Scientific, Rockfork, IL) and electrophoresis with 2% agarose gel. RNAs with A260:280 nm ratios greater than 1.9 and A260:230 nm ratios greater than 1.8 were used. A total of 1μg RNA was used for cDNAs synthesis using a reverse transcription kit (TAKARA Co., Ltd. Dalian, China), and then RT-PCR was performed using the CFX RT-PCR detection system (Bio-Rad, Hercules, CA) and an SYBR Green RT-PCR kit (TAKARA Co., Ltd. Dalian, China). The primer sets used are listed in Table 1. Three replicates for each sample were used, and both the non-template negative control and no reverse transcriptase negative control were used. The standard curves were derived from the 10-fold serial dilutions of cDNA (5 points), and PCR efficiencies were calculated from the slopes of the standard curves. The PCR cycle parameters were as follows: 36 3-step cycles of 95°C, 20 s; 55°C, 20 s and 72°C, 20 s. After amplification, a melting curve (0.01°C/sec) was carried out for confirmation of product purity and agarose gel electrophoresis was used to confirm the targeted size. Relative mRNA content was normalised to 18S rRNA content (Zhang et al. 2015), and 2-ΔΔCt method was used to analyse the relative changes in gene expression.

**Western blotting**

Muscle samples (100 mg) were homogenised in 500 μl of ice-cold lysis buffer containing 20 mM Tris-HCl (pH 7.4 at 4°C), 2% SDS, 1% Triton X-100, 5.0 mM EDTA, 5.0 mM EGTA, 1 mM DTT, 100 mM NaF, 2 mM sodium...
vanadate, 0.5 mM phenylmethylsulfonyl fluorid. Homogenates were centrifuged for 15 min at 12,000 g, for soluble protein separation, and protein concentration was determined using a BCA Protein Assay Kit (Sangon Biotech Co., Ltd, Shanghai, China). Then, supernatant was mixed with an equal amount of sample loading buffer containing 150 mM Tris-HCl (pH 6.8), 20% glycerol, 2 mM 2-mercaptoethanol, 0.004% (wt/vol) bromophenol blue and boiled for 3 min for electrophoresis. Proteins separated by SDS-PAGE were transferred to nitrocellulose (NC) membranes and blocked with blocking buffer (Sangon Biotech Co., Ltd, Shanghai, China) for 1 h. The NC membrane was incubated with primary antibodies in a 1:1000 dilution (vol/vol) at 4°C overnight. After that, NC membranes were washed with phosphate-buffered saline Tween-20 (PBST) four times for 5 min each and incubated with a secondary antibody for 1 h. After washing, NC membrane was subjected to Western blot analysis using an EasyBlot ECL kit (Sangon Biotech Co., Ltd, Shanghai, China) and scanned with Gel Doc™ XR+ (Bio-Rad, Hercules, CA). Band density was normalised to β-actin content.

Antibodies

Antibodies against ACC (no. 3662) and β-actin (no. 4970) were purchased from Cell Signalling (Danvers, MA). PPARγ (sc-7196), C/EBPα (sc-9314) and FABP4 (sc-18661) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Donkey anti-rabbit secondary antibody (no. 10056) was purchased from Sangon Biotech Co., Ltd. (Shanghai, China).

Enzyme activity analysis

*Longissimus dorsi* muscle powder (500 mg) was weighed out and homogenised on ice in 4.5 mL of 0.9% saline, and then centrifuged at 2500 × g for 10 min at 4°C. The supernatant was used for analysing the enzyme activity. The enzyme activities of MDH, LPL, HSL, FAS and ACC were measured using commercial ELISA kits (Jianchen Biotech Co., Ltd, Nanjing, China) by following the manufacturer’s instructions.

Statistical analysis

Statistical analysis was performed using Graphpad Prism 6 software package (Monrovia, CA). Six animals were used in each group and each animal was considered as an experimental unit. Data were expressed as mean ± standard error of mean (SEM) and analysed using Student’s t-test. Statistical significance was considered at p < .05 for all data.

Results

Body weight, back fat thickness and leaf fat weight of Mashen and Large White pigs

Figure 1(A) shows the appearance of both Mashen pigs and Large White pigs. At 180 day of age, BW of Mashen pigs was lower than that of Large White pigs (Figure 1(B), 68.33 ± 3.28 kg vs 98.02 ± 2.33 kg). The back-fat thickness (Figure 1(C), 29.67 ± 2.03 mm vs 14.07 ± 0.96 mm) and the leaf fat weight (Figure1(D), 0.76 ± 0.06 kg vs 0.49 ± 0.04 kg) in Mashen pigs were greater than that of Large White pigs.

Intramuscular fat content of Mashen and Large White pigs

Masson Trichrome staining results showed that there were more intramuscular adipocytes in LD muscle of Mashen pigs compared with Large White pigs (Figure 1(B)). The size of intramuscular adipocytes was increased in Mashen pigs (Figure 1(C)). Moreover, Mashen pigs possessed greater IMF content compared with Large White pigs (Figure 1(D), 0.76 ± 0.06 kg vs 0.49 ± 0.04 kg) in Mashen pigs were greater than that of Large White pigs.

Table 1. Primer sequences for real-time PCR.

| Name   | Sequence (5′-3′) | Length, bp |
|--------|-----------------|------------|
| PPARγ  | AACATTTCACAAGGTTGACCA CAGCTTCGGGAATGGGATG 190 |
| C/EBPα | CTCCACCGTCGAGCTTAC TCTCTATTTGGGCAGGAGA 98 |
| C/EBPβ | GCAAGGACGAGATACAAAGA ACCTTATGCTGCTGCTGGGA 102 |
| Zfp423 | GACGACCCACAACTCCTCGG AGGACTTGTCGCAGAACTGG 154 |
| PDGFRa | CCTCTACACCAACTGAGGG AATTCCACCACATGCGGAGGG 98 |
| SREBP1 | GCCGATGATGGCACGATCTCC TCCCCATCCAGAAGAAGACG 156 |
| FAS    | GAACTTCTGGAGTCTGGGCC ATCTGTTCTGGCTGCTGGGA 155 |
| ACC    | CTGAAATCGGTGTTTTGTTG GCTACCTCCTGCAACTGGGA 174 |
| FABP4  | AGGCCTTCTTCTCTACACGTTGA AGGCCACTCACCACGTTCTTC 128 |
| 18 S   | CCCACGGATGAGAAGAGTTGGACGCAGAAAGG 122 |
Expression of adipogenic markers in LD muscles of Mashen and Large White pigs

Real-time PCR results suggested that mRNA expression of Zinc finger protein 423 (Zfp423) and platelet-derived growth factor receptor α (PDGFRα) in LD muscle were greater in Mashen pigs than that of Large White pigs (Figure 3(A,B), 28 day of age). At 180 day after birth, transcriptional factors, including C/EBPα, C/EBPβ and PPARγ were observed greater in Mashen pigs compared with Large White pigs (Figure 3(C–E)), whereas there was no difference in SREBP1 mRNA content detected (Figure 3(F)). In agreement with mRNA expression, the protein contents of both C/EBPα and PPARγ were greater in LD muscle of Mashen pigs compared with Large White pigs (Figure 4(A,B)).

Expression patterns of lipid metabolism markers in LD muscle of Mashen and Large White pigs

As shown in Figure 5(A), compared with Large White pigs, mRNA content of fatty acid synthase (FAS) was greater in Mashen pigs. Both the mRNA and protein abundance of acetyl-CoA carboxylase (ACC) did not differ between two breeds (Figure 5(B,C)). Although there was no difference in fatty acid binding protein 4 (FABP4) mRNA content (Figure 5(D)), the protein abundance was greater in LD muscle of Mashen pigs (Figure 5(E)).

Lipid metabolic enzyme activities in LD muscle of Mashen and Large White pigs

Activity of FAS was greater in Mashen pigs (Figure 6(A)), whereas ACC activity did not vary between the two breeds (Figure 6(B)). Compared with Large White pigs, the malate dehydrogenase (MDH) enzyme activity was greater (Figure 6(C)) and hormone-sensitive lipase (HSL) activity was lower (Figure 6(E)) in Mashen pigs. There was no difference in lipoprotein lipase (LPL) activity between Mashen and Large White pigs (Figure 6(D)).

Discussion

In animals, body fat tissues can be divided into four main categories, including subcutaneous, visceral, intermuscular and intramuscular fat (Monziols et al. 2007). The subcutaneous and visceral fats have low commercial value in pigs reared for fresh meat consumption, and excessive fat deposition in these sites affect the production efficiency, profitability and marketability. Thus, reduction of fat deposition is desirable for both producers and consumers, and pigs have been genetically selected for increased lean growth. However, due to the negative relationship between high lean growth and IMF accumulation, genetic selection reduces IMF content and the quality of pork (Hausman et al. 2014).
Figure 2. Intramuscular fat (IMF) content and adipocytes size between Mashen (■) and Large White pigs (□) at 180 day of age. (A) Masson trichrome staining of longissimus dorsi (LD) muscle, arrows indicated appearance of adipocytes. (B,C) The number and size distribution of intramuscular adipocytes. (D) IMF content. **p < .01, *p < .05 (Mean ± SEM; n = 6).

Figure 3. mRNA expression of adipogenic markers in LD muscle of Mashen (■) and Large White pigs (□) at 28 and 180 days of age. (A,B) Zinc finger protein 423 (Zfp423) and Platelet-derived growth factor receptor α (PDGFRα) mRNA expression in LD muscle of pigs at 28 days of age. (C–F) mRNA expression of CCAAT enhancer binding protein β (C/EBPβ), C/EBPα, peroxisome proliferator-activated receptor γ (PPARγ) and sterol regulatory element-binding protein 1 (SREB1) in LD muscle of pigs at 180 days of age. Relative mRNA expression was normalised to 18S rRNA content. **p < .01, *p < .05. (Mean ± SEM; n = 6).
Figure 4. Protein abundance of C/EBPα and PPARγ in LD muscle of both Mashen (■) and Large White pigs (□) at 180 day of age. (A) Western blot band of C/EBPα. Ns refers to non-specific band. (B) Bands of PPARγ, two bands refer to 54 kDa and 57 kDa isoforms, respectively. Band density was normalised to β-actin content. **p < .01, *p < .05. (Mean ± SEM; n = 6).

Figure 5. FAS, acetyl-CoA carboxylase (ACC) and fatty acid-FABP4 abundance in LD muscle of Mashen (■) and Large White pigs (□) at 180 day of age. (A) mRNA expression of FAS. (B) mRNA expression of ACC. (C) ACC protein abundance. (D,E) mRNA and protein abundance of FABP4. Relative mRNA expression was normalised to 18S rRNA content, and band density was normalised to β-actin content in western blotting analysis. **p < .01. (Mean ± SEM; n = 6).
In the present study, we found that Mashen pigs have a lower final BW, greater backfat thickness and leaf fat content compared with the Large White pigs. In beef cattle, a certain degree of IMF contributes to tenderness, taste and juiciness (Grunert et al. 2004), and an IMF content of 2–3% in the LD muscle is considered optimal for the palatability of pork (Altmann & Pliquett 2006). We found that Mashen pigs had a greater IMF content than Large White pigs, and whether the pork tenderness and palatability were different remains to be further explored.

The intramuscular adipocytes are derived from pluripotent mesenchymal stem cell (MSCs), which are abundant in the foetal and neonatal skeletal muscle, and have the capacity to develop into several cell types, i.e. adipocytes, myocytes, chondrocytes and osteocytes (Du et al. 2013). Adipogenesis of MSCs can be separated into two steps, adipogenic commitment and preadipocyte differentiation into mature adipocytes (Tang & Lane 2012). Platelet-derived growth factor receptor α is a marker of mesenchymal progenitors that contribute to fat cell formation in skeletal muscle (Uezumi et al. 2010). The increased PDGFRα mRNA content in LD muscle of Mashen Pigs suggested greater number of mesenchymal progenitors in Mashen pigs. The adipogenic commitment is initiated by Zfp423 while preadipocyte differentiation is regulated by PPARγ and C/EBPα (Gupta et al. 2012). The enhanced IMF content and increased number of intramuscular adipocytes observed in Mashen pigs might be due to enhanced adipogenic commitment of MSCs during skeletal muscle development. Consistently, Zfp423 mRNA content was greater in Mashen pigs than Large White pigs, suggesting greater adipogenic commitment.

During adipogenic differentiation, C/EBPβ is first expressed, which induces the expression of C/EBPα and PPARγ (Rosen & MacDougald 2006). C/EBPα directly binds to the PPARγ promoter to induce its expression. Activation of PPARγ initiates the expression of adipocyte specific genes and terminal differentiation to become mature adipocytes (Siersbæk et al. 2012). In the present study, the mRNA expression of C/EBPα, C/EBPγ and PPARγ were all elevated in Mashen pigs compared with Large White pigs. Consistently, protein abundance of both C/EBPα and PPARγ were increased in Mashen pigs. Therefore, the observed greater IMF in Mashen pigs may be due to the enhanced adipogenic terminal differentiation.

As a basic helix-loop-helix-leucine zipper protein, SREBP1 is another important adipogenic regulator downstream of C/EBPα and PPARγ (Kim & Spiegelman 1996). SREBP1 promotes the transcription of enzymes involved in lipid synthesis, which provide ligands for PPARγ to further enhance its transcription activity (Kim et al. 1998; Yellaturu et al. 2009). In the present study, we did not observe difference in SREBP1 mRNA expression between two breeds, indicating that SREBP1 was unlikely responsible for the difference of IMF.
In the present study, greater intramuscular adipocytes size was observed in Mashen pigs. To test whether fatty acid synthesis was altered between two breeds, we measured lipogenic markers. Both ACC and FAS are enzymes involved in fatty acid synthesis (Cai et al. 2016). Acetyl-CoA carboxylase is a biotin-dependent enzyme that catalyses the carboxylation of acetyl-CoA to produce malonyl-CoA (Zu et al. 2013), while FAS catalyses the synthesis of palmitate from acetyl-CoA and malonyl-CoA (Kawano & Cohen 2013). We found that FAS mRNA content was elevated, and accordingly, its enzyme activity was greater in Mashen pigs. For ACC, both the protein abundance and enzyme activity remained unchanged between two breeds. Apart from acetyl-CoA, nicotinamide adenine dinucleotide phosphate (NADPH) is also essential for de novo fatty acid synthesis. In non-ruminant animals, MDH is responsible for the production of more NADPH molecules from cytosolic citrate (Shingfield et al. 2010). As expected, the MDH activity was greater in Mashen Pigs than that of the Large White pigs. Besides the de novo synthesis, fatty acids are also absorbed from blood circulation via the activity of LPL (Zaidi et al. 2013). In present study, the LPL enzyme activity was similar between two breeds. FABP4 is a carrier protein required for transporting free fatty acids (Iso et al. 2013) and its abundance was greater in Mashen pigs, indicating greater ability for triglyceride accumulation in Mashen LD muscle. To assess whether triglycerides degradation differs between two breeds, we analysed HSL activity. Our results showed that HSL activity was greater in Large White pigs. Because HSL is one of the key enzymes responsible for lipolysis (Carmen & Víctor 2006), the greater HSL activity suggests a greater lipid mobilisation in Large White pigs.

Conclusions
The IMF content is greater in Mashen pigs compared with Large White pigs, which accompanied with greater abundance of progenitor cells and enhanced adipogenic terminal differentiation.

Disclosure statement
The authors report no conflicts of interest.

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