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Full Length Research Paper

Study for the expression of *Adiponectin*, fatty acid binding protein (FABP*)4*, stearoyl-CoA desaturase (SCD) genes and the methylation of *SCD* promoter in porcine muscle and fat tissues

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East Asian consumers such as Korean and Japanese prefer the roasted pork, because lots of the researches have been carried out for the genetic improvement of intramuscular fat contents in pig. The fatty acid binding protein (FABP*)4* and stearoyl-CoA desaturase *(*SCD*)*, related to lipid metabolism are critical genes responsible for the fat content trait in meat, and the *Adiponectin* is a gene involved in lipid metabolism as an adipocyte-secreting hormone. Here, we performed the expression profiling of three lipid metabolism-related genes, according to different pig breeds, and tissues, and also applied an epigenetic technique to obtain the epigenetic insights into their differential expressions. The *FABP4* expression level in muscle tissue increased at the young stage, but gradually decreased after adult stage. *Adiponectin* gene was expressed more significantly in fat tissues than in other tissues. Fat-specific *SCD* gene was much more expressed in the adipose tissue of Berkshire than that of Yorkshire at 80 kg stage. The CG methylation analysis of *SCD* promoter revealed that -58th, -471st and -540th cytosines from the transcription initiation site were more methylated in muscle tissues than in fat tissues and in contrast, -68, -96 and -266th cytosines were more methylated in fat tissues than in muscle tissues, which allows explaining the epigenetic mechanism of tissue-specific *SCD* expression. Taken together, our data may provide an important resource for the technical development of intramuscular fat-related molecular marker.

Key words: Adiponectin, DNA methylation, fatty acid binding protein (FABP4), pork, stearoyl-CoA desaturase.

INTRODUCTION

East Asians such as Korean and Japanese have been long been accustomed to eating roasted pork and thus, there is preference for pork parts with tenderness, flavor and meat soluble as to appropriate intramuscular fat and texture. For this reason, they enjoy black pigs (Berkshire breed) that have higher level of intramuscular fat compared to other species. Accordingly, we have carried out with molecular biological studies related to intramuscular fat and texture, among key elements of meat quality. Generally, lipid metabolism in muscle begins from the cellular influx of fatty acid by the FABP. Subsequently, the acetyl-CoA is converted to malonyl-CoA by acetyl-CoA carboxylase, and then, fatty acid synthase activates the palmitate synthesis from the malonyl-CoA. Finally, the palmitate is converted to

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unsaturated fatty acid by SCD and then is accumulated in muscle or fat tissues. For the muscular fat accumulation, FABP4 (Gerbens et al., 1998), *GLUT4* (Zorzano et al., 2005), *ACC* (Murray et al., 2000), *ACL* (Smith and Prior, 1981), *LPL* (Eckel, 1989) and *SCD* (Taniguchi et al., 2004) are major genes. In addition, *Adiponectin* encodes a hormone secreted by adipocytes and is known to regulate the energy and fat metabolism in humans and animals (Scherer et al., 1995). Among them, SCD (Taniguchi et al., 2004), FABP4 (Gerbens et al., 1998) and *Adiponectin* (Hu et al., 1996) specially are involved in the fat synthesis and accumulation and its regulation. Function of these three genes in more detail is as follows. SCD in higher animals is a key enzyme that regulates the synthesis of unsaturated fatty acids. SCD synthesizes palmitoleic acid (16:1) and oleic acid (18:1) by using palmitic acid (16:0) and stearic acid (18:0) as a substrate.

In the final stage of the synthesis of fat, SCD converts long-chain fatty acids into monounsaturated fatty acids, which are accumulated as triglycerides in muscle and fat tissues (Liu et al., 2009). For the fatty acid metabolism, FABPs in membrane regulates the flow of fatty acids and are known to exist as 9 families. Particularly, FABP4 is involved in the accumulation of intracellular fat and fatty acid oxidation by interacting fatty acid during the differentiation of adipocytes (Mercade et al., 2006). Adiponectin (ACRP30 or AdipoQ), a hormone secreted by adipocytes, is known to be involved in the energy regulation and glucose and fat metabolism in humans and animals(Hu et al., 1996; Scherer et al., 1995). Human *Adiponectin* encoded by APM1 (adipos most abundant transcript 1) is located in 3q27 associated with diabetes (Yamauchi et al., 2001). This gene is 16 kb in size and encodes protein consisting of 244 amino acids. In addition, there is no promoter area, characterized by the TATA box (Schaffler et al., 1999).This gene increases the effect of glucose and fatty acid oxidation by inducing muscle AMPK (AMP-activated protein kinase) phosphorylation (Yamauchi et al., 2002). Through this effect of APM1 in the glucose regulation, regulates the intramuscular fat production, leading to the meat texture, especially the pH and water holding capacity.

Altogether, these genes are associated with the fat-related phenotypes, but studies for fat-related traits of meat have been concentrated into the SNP analysis and the *in vivo* studies using pig tissues to identify gene expression patterns is still lacking. Moreover, recently there have been a few epigenetic researches changing gene expression patterns without sequence variations (Henderson and Jacobsen, 2007; Martin and Zhang, 2007). This epigenetic change includes acetylation, methylation, phosphorylation and ubiquitinylation, which regulate the gene expression pattern, and affect the gene expressions, involved in fat synthesis and accumulation and even its regulation. Previously, we constructed a complementary deoxyribonucleic acid (cDNA) chip, composed of 4,434 ESTs, using total Ribonucleic acid (RNAs) from Berkshire pork muscle tissues, and microarray analysis, using the chip with RNAs from fat and muscle tissues, revealed that various genes are expressed differentially between muscle and fat tissues (KIM et al., 2005). Among the differentially expressed genes, particularly, Adiponectin, FABP4 and SCD showed a significant difference between fat and muscle. In this study, we assessed the gene expression profiling of Adiponectin, FABP4 and SCD according to breeds, growth stage and tissue. In addition, the CpG methylation analysis of SCD promoter was conducted to obtain epigenetic insights into the SCD differential expression. Altogether, these results may provide an important resource for the genetic improvement of intramuscular fat contents in pig.

MATERIALS AND METHODS

Animal and sample collection

Berkshire and Yorkshire (from Sungchuk Farm, the lineage formed with the pigs from Kagoshima) female pigs whose body weight reached 60, 80 and 110 kg were butchered three times, and their tissues were immediately taken, soaked into liquid nitrogen and kept in a freezer at -80°C until total RNA isolation.

RNA extraction and cDNA preparation

Total RNA was isolated from collected samples using Trizol reagent according to the manufacturer's instruction (Life Technologies, Invitrogen). 2 ml of Trizol reagent was added to 0.1 ~ 0.2 g of tissue grounded and mixed well with homogenizer, and a 1 ml aliquot of the mixture was transferred into a 1.5 ml e-tube. Then, the mixture was left alone at Real-Time (RT) for 10 min and centrifuged at 12,000 rpm for 10 min, and the solution excluding the cell debris was saved into a new tube. 200 µl of chloroform was added to the supernatant, and then 500 µl isopropanol was added to precipitate the RNA. The RNA was washed with 70% ethanol and dried at RT. The extracted RNA was dissolved in water and quantified spectrophotometrically at 260 nm. For each sample, an RNA aliquot was subjected to electrophoresis in 1.5% agarose gel to verify its integrityand.

Semi-quantitative real-time polymerase chain reaction (RT PCR)

For RT-PCR, the first strand cDNA was synthesized by using Superscript II Reverse Transcriptase, according to the manufacture's protocol (Invitrogen, USA). Briefly, a 5 of extracted RNA was added to a reaction mixture consisting of 4 µl of 5X First strand Buffer (Invitrogen, Carlsbad, CA), 1 µl of 10 mM Deoxynucleotide Triphosphates (dNTPs) (Promega, USA) dissolved DEPC-water, 2 µl of 0.1M Dithiothreitol (DTT) (Invitrogen, USA), 1 µl (200 U/µl) of SuperScript ReverseTranscriptase II (Invitrogen, USA), 1 µl (0.5 µg/µl) of oligo-d(T) 12 to 18 primer (Invitrogen,USA), 1 µl of RNase Inhibitor (Invitrogen, USA), and RNase-free water. Then, the RT step was carried out at 42°C for 1 h, followed by heating at 70°C for 15 min and adding 1 µl RNase H at 37°C for 20 min before storage at 4°C. A set of negative control was also included except for the reverse transcription reaction. For internal control for assessing the relative amount of target gene from different tissue samples, the GAPDH gene was used for comparison

Table 1. The primers used in the present study.

Gene	Primers	Usage	Size (bp)
Adiponectin	F 5'-TGCTGTTGTTGGGAGCTGTTC-3' R 5'-AGGAAGCCTGTGAAGATGGAG-3'	RT-PCR	691
A-FABP	F 5'-CTGAGATTGCCTTCAAATTG-3' R 5'-GGTGGTTGTCTTTCCATCCC -3'	RT-PCR	229
SCD-1	F 5'-TGCCTCACTCGAAAGGGAAC -3' F 5'-AGTAGGTGCTTGGGTCTGGG -3'	RT-PCR	448
SCD-2 (gDNA)	F: 5'- CCCACCCCCCTACTGAGTCCTCTTC-3' R: 5'- TCTCCTCTTGCAGCAAGTGGGCC-3'	Bisulfite sequencing PCR	580
SCD-3(bDNA)	F: 5'-TTTATTTTTTTATTGAGTTTTTT-3' R: 5'-TCTCCTCTTACAACAAATAAACC-3'	Bisulfite sequencing PCR	587
GAPDH	F 5'-GTCGTGGAGTCCACTGGTGT-3' R 5'-CCCATCACAAACATGGGGGC-3'	RT-PCR	119

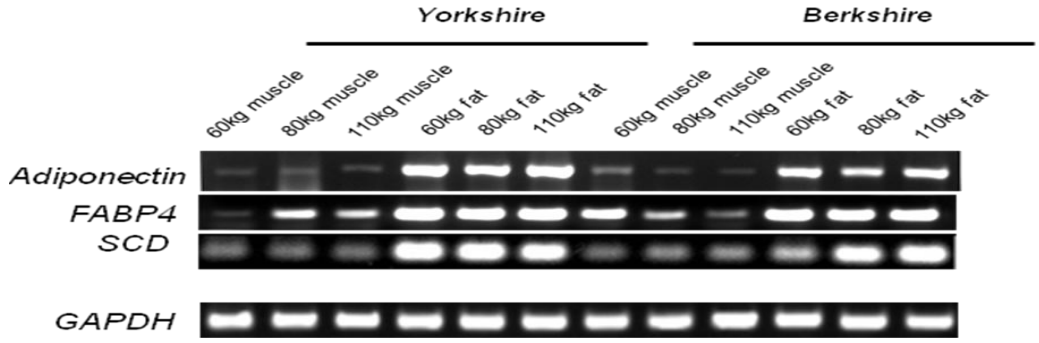

Figure 1. Expression levels of adiponectin, FABP4 and SCD in the muscle (tenderloin) and fat (backfat) tissues of Berkshire and Yorkshire pigs at various body weights.

(Table 1). RT-PCR products of the four genes were separated on a 2% tris–acetate– ethylene diamine tetraacetic acid (TAE) agarose gel and visualized by ultraviolet (UV) after ethidium bromide staining.

DNA bisulfite sequencing analysis

Bisulfite sequencing was performed according to the EpiTect® Bisulfite Kit (QIAGEN, Valencia, CA)'s protocol, using 2 μg of genomic DNA. For bisulfite sequencing of amplified SCD promoters, the bisulfite-treated gDNA was used as template. The PCR products of amplified promoters were cloned into either pGEM-T easy vector (Promega, Madison, WI) and at least 13 individual clones were sequenced for each sample.

RESULTS AND DISCUSSION

Characterization of *Adiponetictin*, FABP4 and SCD gene expressions according to pig breeds

As shown in Figure 1, Adiponectin, FABP4 and SCD gene expressions were analyzed using RNAs from two breeds such as the Yorkshire and Berkshire breeds. The result revealed that *Adiponectin* was expressed higher in adipose tissue of Yorkshire than that of Berkshire. According to Daniele's report, *Adiponectin* was expressed higher in large white, meat type, than in casertana pig, fat type (Daniele et al., 2008), which is similar with our result. Because Adiponectin, a hormone secreted by adipocytes, regulate adipogenesis, the differential expression of *Adiponectin* can be considered as critical genes determining the meat or fat classification types. FABP4 gene was expressed more rapidly in the Berkshire 60 kg muscle tissue than Yorkshire 80 kg one (Figure 1). FABP4, is known to be involved in fat accumulation in the cells, and was expressed more quickly and significantly in Berkshire than in Yorkshire, which supported that Berkshire has more excellent marbling than Yorkshire does (Mercade et al., 2006). SCD gene was expressed later in Berkshire fat tissue than in Yorkshire; its expression increased gradually at

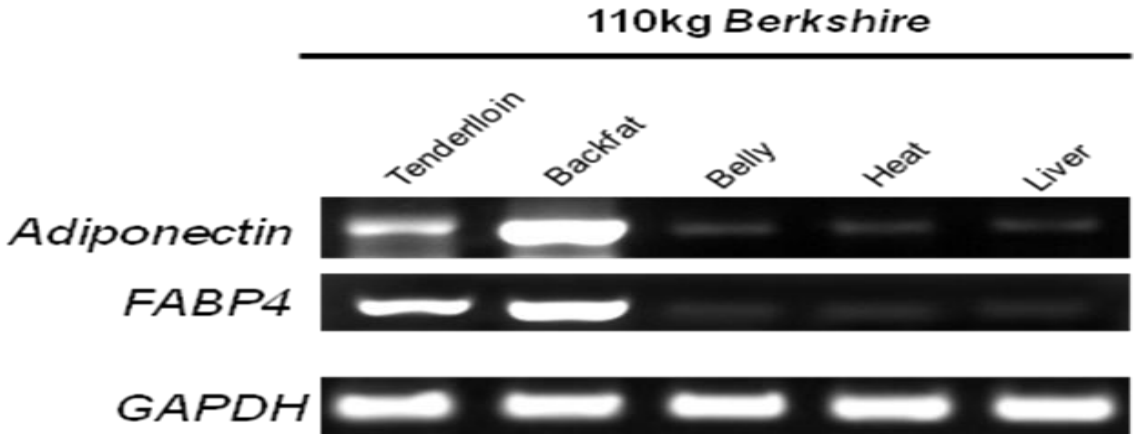

Figure 2. Expression levels of *adiponectin* and *FABP4* in various tissues of *Berkshire* pig at the growth stage of 110 kg body weight.

80 kg stage of Berkshire. SCD plays an important role in fatty acid synthesis because it is involved in the fatty acid desaturation in the fatty acid metabolism (Liu et al., 2009). The late expression of SCD gene in Berkshire can explain that Berkshire accumulates extra energy as fatty acids in adipose tissue later than Yorkshire does. If the differences of three gene expression patterns are further analyzed correctly, these will be available as basic data explaining differences of fat-related traits in each species.

Characterization of *Adiponetictin*, FABP4 and SCD gene expressions according to growth stage

To identify gene expressions according to growth stage, we investigated whether there are any differences in the muscle and fat at 60, 80 and 110 kg stages. As shown in Figure 1, *Adiponectin* and SCD showed no differences in the growth stages. According to Ramsay's report, it was expressed higher in young pig with high birth weight than one with low birth weight, indicating that *Adiponectin* has association with pig growth (Ramsay et al., 2010). However, Adipnectin has no significant relationship with the pig growth stages in this study. The expression of FABP4 increased at 60 to 80 kg stages but decreased at 110 kg. Gardan et al. (2007) reported that the expression levels of FABP4 increases in subcutaneous fat of 80 day old pigs but decreases in subcutaneous fat of 210 day-old pigs (Gardan et al., 2007), which is similar with our result. The FABP4 is known to be responsible for the transport of fatty acid outside the cell and has been reported to be associated with marbling score, back fat thickness and growth (Gerbens et al., 1998). From these facts, the sudden expression of FABP4 in the rapid growth may be caused by that FABP4 supplies fatty acid from outside to inside of muscle cells pig muscle tissue because a large amount of energy is required for pig muscle cells at 60 to 80 kg stage. *Adiponetictin*, FABP4 and SCD genes are expressed mainly in adipose tissue of mostly all the growth phase and is expected to be involved in lipid metabolism. However, FABP4 increases in muscle tissue of 60 to 80 kg pigs, which suggest that it is involved in fat metabolism of muscle tissue during this period.

Characterization of *Adiponetictin*, FABP4 and SCD gene expressions according to tissues

To investigate adipose-specific expression, we identified the *Adiponectin* and FABP4 expressions using RNAs from the muscle, fat, belly, heart and liver. As shown in Figure 2, *Adiponectin* and FABP4 showed more significant expression in adipose tissue than in different tissues. According to Dai's report, *Adiponectin* was expressed only in adipose tissue, when they investigated its expression in liver, kidney, muscle, fat, heart, pancreas and brain (Dai et al., 2006; Ding et al., 2004; Lord et al., 2005), which was quite consistent with our result. In addition, Liu et al. (2009) reported that FABP4 is expressed in group with excellent marbling, indicating that it may be associated with the fat content of muscle. The SCD gene is known to be specially expressed in adipose tissue of pigs and to be very important to determine the phenotype of fat accumulation (Taniguchi et al., 2004). Therefore, three genes play an important role in fat metabolism occurring in adipose tissue of pigs and are important genes to determine the fat traits in meat quality.

Identification of SCD promoter methylation patterns that regulate fat-specific expression

We identified adipose-specific genes, which have been known to be important in fat metabolism. To identify the epigenome factors controlling the expression patterns in

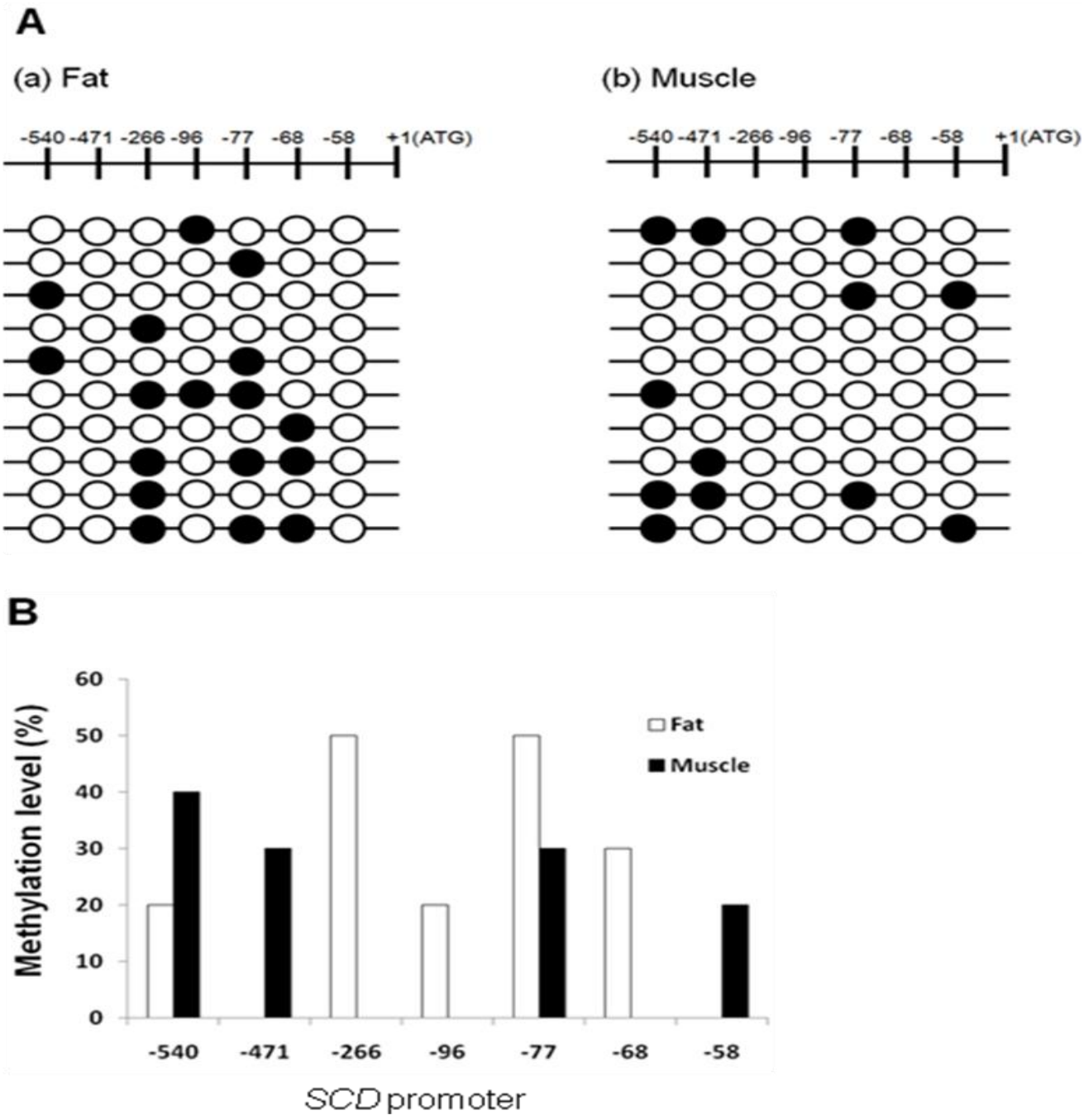

Figure 3. Bisulfite sequencing analysis of SCD promoter; **A** Genomic DNA is isolated from muscle and fat tissue (**a** and **b**) and then, subjected to the bisulfite conversion. Finally, the genomic DNA is amplified by PCR using SCD-specific primers, and subsequently, the cytosine methylation differences were identified. The +1 represents a transcription start site. Open and filled circles represent unmethylated and methylated cytokines; **B** Comparison of CpG methylation level between muscle and fat tissue. Black and white bars indicate the methylation levels of muscle and fat tissue, respectively.

Figures 1 and 2, we chose SCD promoter only because its promoter sequence information is known in National Center for Biotechnology Information (NCBI) database. DNA methylation patterns in the seven CpG sites of SCD promoter were investigated by bisulfate sequencing. The CG methylation analysis of SCD promoter revealed that -58th, -471st and -540th cytosines from the transcription initiation site were more methylated in muscle tissues than in fat tissues and in contrast, -68th, -96th and -266th cytosines were more methylated in fat tissues than in muscle tissues (Figure 3). Previous studies have highlighted potential mechanisms underlying epigenetic

modification of tissue function, suggesting that direct epigenetic modification of insulin action in muscle may be achieved through altered SCD 1 expression (Holness and Sugden, 2006). It was also suggested that elevated expression of SCD1 in skeletal muscle contributes to abnormal lipid metabolism and progression of obesity (Hulver et al., 2005). In this study, we first identified the porcine SCD promoter methylation profiles between muscle and fat tissues, and the results may allow explaining the epigenetic mechanism of tissue-specific SCD expression.

In conclusion, here we identified the expressions of three genes such as FABP4, Adiponectin and SCD, related to pork meat quality traits. The results revealed that three genes were expressed adipose-specifically, and especially, FABP4 increases in muscle tissue of 60 to 80 kg pigs, which suggest that it is involved in fat metabolism of muscle tissue during this period. In addition, the SCD expression increased in Berkshire adipose of 80 kg stage and its promoter methylation was first assessed by bisulfite sequencing. The CG methylation analysis of SCD promoter revealed that -58th, -471st and -540th cytosines from the transcription initiation site were more methylated in muscle tissues than in fat tissues and in contrast, -68, -96 and -266th cytosines were more methylated in fat tissues than in muscle tissues. Taken collectively, three genes are implied to be involved in the fat-related traits such as the pork back fat thickness, marbling, fat content and shear force and can be resources to understand the molecular biological mechanism in the fat-related traits. The DNA methylation results also may allow explaining the epigenetic mechanism of tissue-specific SCD expression and may provide an important resource for the technical development of molecular marker in the genetic improvement of pig intramuscular fat contents. For this, CpG methylation pattern analysis according to growth stages further is required.

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