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Association of DNA Methylation Levels with Tissue-specific Expression of Adipogenic and Lipogenic Genes in *Longissimus dorsi* Muscle of Korean Cattle

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ABSTRACT: Epigenetic factors, such as DNA methylation status, may regulate adipogenesis and lipogenesis, thus affecting intramuscular fat (IMF) deposition in *longissimus dorsi* muscle (LM) of beef cattle. In Korean cattle steers, the LM consists mainly of muscle tissue. However, the LM tissue also contains IMF. We compared the gene expression levels between the IMF and muscle portions of the LM after tissue separation. Real-time polymerase chain reaction analysis showed that the mRNA levels of both adipogenic peroxisome proliferator-activated receptor gamma isoform 1 (*PPARG1*) and lipogenic fatty acid binding protein 4 (*FABP4*) were higher ($p<0.01$) in the IMF than in the muscle portion of the LM. We determined DNA methylation levels of regulatory regions of the *PPARG1* and *FABP4* genes by pyrosequencing of genomic DNA. DNA methylation levels of two of three CpG sites in the *PPARG1* gene promoter region were lower ($p<0.05$) in the IMF than in the muscle portion of the LM. DNA methylation levels of all five CpG sites from the *FABP4* gene promoter region were also lower ($p<0.001$) in the IMF than in the muscle portion. Thus, mRNA levels of both *PPARG1* and *FABP4* genes were inversely correlated with DNA methylation levels in regulatory regions of CpG sites of the corresponding gene. Our findings suggest that DNA methylation status regulates tissue-specific expression of adipogenic and lipogenic genes in the IMF and muscle portions of LM tissue in Korean cattle. (**Key Words:** Adipogenesis, DNA Methylation, Intramuscular Fat, Korean Cattle)

INTRODUCTION

Beef production in Korea mainly focuses on the meat quality, particularly the degree of intramuscular fat (IMF) deposition; adipogenesis and lipogenesis are important processes for IMF deposition. Adipogenic/lipogenic transcriptional networks regulate IMF deposition in beef cattle (Moisa et al., 2014). Japanese Wagyu cattle are well known for their extremely high marbling (Duarte et al., 2013) indicated that intramuscular adipogenesis was enhanced in Wagyu compared with Angus muscle. Adipogenic peroxisome proliferator activated receptor gamma (*PPARG*), which is a nuclear receptor and transcription factor, regulates lipid metabolism by controlling the expression of various lipogenic genes, such as adipocyte fatty acid binding protein 4 (*FABP4*), lipoprotein lipase, acyl-CoA synthase, and fatty acid transport protein (Sarjeant and Stephens, 2012). Our recent studies showed that *PPARG* and *FABP4* gene expression is associated with IMF deposition in Korean cattle (Jeong et al., 2013; Ahn et al., 2014).

DNA methylation is an important epigenetic marker of the transcriptionally repressed state of the genes (Jones and Takai, 2001). Epigenetic regulatory mechanisms including DNA methylation are reportedly involved in the transcriptional activation of *PPARG* during adipogenesis

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(Musri et al., 2007). Studies have demonstrated the contribution of *PPARG* promoter DNA methylation to its expression in adipocyte cell culture systems (Noer et al., 2006; Fujiki et al., 2009). DNA methylation status may be one of mechanisms regulating adipogenic/lipogenic gene expression during IMF deposition. However, involvement of DNA methylation in regulation of adipogenesis and lipogenesis in cattle is unknown. DNA methylation status may be one of mechanisms regulating adipogenic/lipogenic gene expression during IMF deposition. In this study, association of DNA methylation levels and *PPARG1* and *FABP4* gene expression levels were examined in the IMF and muscle portions of the *longissimus dorsi* muscle (LM) tissues in Korean cattle steers.

MATERIALS AND METHODS

All experimental procedures involving animals were approved by the Chonnam National University Institutional Animal Use and Care Committee (CNUIAUCC), Republic of Korea. The experiments were conducted in accordance with the Animal Experimental Guidelines provided by CNUIAUCC.

Animals and tissue samples

In this study, we used steer LM tissue samples from previous work (Bong et al., 2012). Slaughter age was 846±30 days, and carcass weight was 398±10 kg. We separated the muscle and IMF portion from the intact LM tissues to determine tissue-specific DNA methylation pattern, as previously described (Bong et al., 2012).

RNA extraction and quantitative real-time polymerase chain reaction

To detect expression levels of *PPARG1* and *FABP4* genes, total RNA was isolated as previously described (Jeong et al., 2013) using TRIzol reagent (Molecular Research Center, Cincinnati, OH, USA). Total RNA was quantified by absorbance at 260 nm, and the integrity of total RNA was verified through agarose gel electrophoresis and ethidium bromide staining of the 28S and 18S bands. Total RNA (0.5 μg) was reverse-transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA).

Real-time polymerase chain reaction (PCR) was performed as previously described (Jeong et al., 2013) using the QuantiTect SYBR Green RT-PCR Master Mix (Qiagen, Valencia, CA, USA) and an Opticon Sequence Detection system (MJ Research, Waltham, MA, USA) with gene-specific primers (Table 1). The ΔΔCT method was used to determine the fold change in mRNA expression relative to the housekeeping gene, ribosomal protein, large, P0.

DNA methylation determination by pyrosequencing of bisulfite-treated genomic DNA

Genomic DNA extraction and analysis of DNA methylation were performed by the DisGene Company (Daejon, Republic of Korea). Briefly, tissue genomic DNA was purified using a NucleoSpin Tissue column (Macherey-Nagel GmbH & Co., Duren, Germany).

To determine DNA methylation levels, target regions of *PPARG1* (Figure 1a) and *FABP4* (Figure 2a) genes were selected from the CpG islands, which were searched using CpG Island Searcher (USC Norris Comprehensive Cancer Center, USA; http://www.uscnorris.com/cpgislands2/cpg.aspx). Transcription factor binding sites (Figures 1a and 2a) were determined using TFSEARCH (Computational Biology Research Center, National Institute of Advanced Industrial Science and Technology, Japan; http://www.cbrc.jp/research/db/TFSEARCH.html). We tried to find promoter regions of the genes in which important transcription factor binding sites were located that may regulate gene expression.

Bisulfite treatment of genomic DNA was performed using the EpiTect Bisulfite Kit (Qiagen, USA). The bisulfite-treated DNA was amplified by PCR using primers indicated in Table 2. Pyrosequencing of PCR products was done using primers shown in Table 2. The degree of methylation at each CpG site was determined from the ratio of thymine (T) and cytosine (C) by the following equation:

$$\% \text{ methylation} = (\text{C peak height})\times 100/(\text{C peak height}+\text{T peak height}).$$

Statistical analysis

Data are expressed as means±standard errors.

Table 1. Primer sequences used in real-time polymerase chain reaction

Gene name (Symbol)	Accession number	Primer	Sequence	Length (bp)
Fatty acid binding protein 4 (*FABP4*)	BT10868	Forward	gctgcacttctttctcacct	140
		Reverse	ttcctggtagcaaagcccac	
Peroxisome proliferator- activated receptor gamma1 (*PPARG1*)	NM_181024	Forward	tgatcagaagcctgcgtctc	116
		Reverse	ttacggaaacgtccctcttg	
Ribosomal protein, large, P0 (*RPLP0*)[1]	BT19086	Forward	cgcatctgtaccccattctatc	85
		Reverse	agcaagtgggaaggtgtaatc	

[1] House keeping gene.

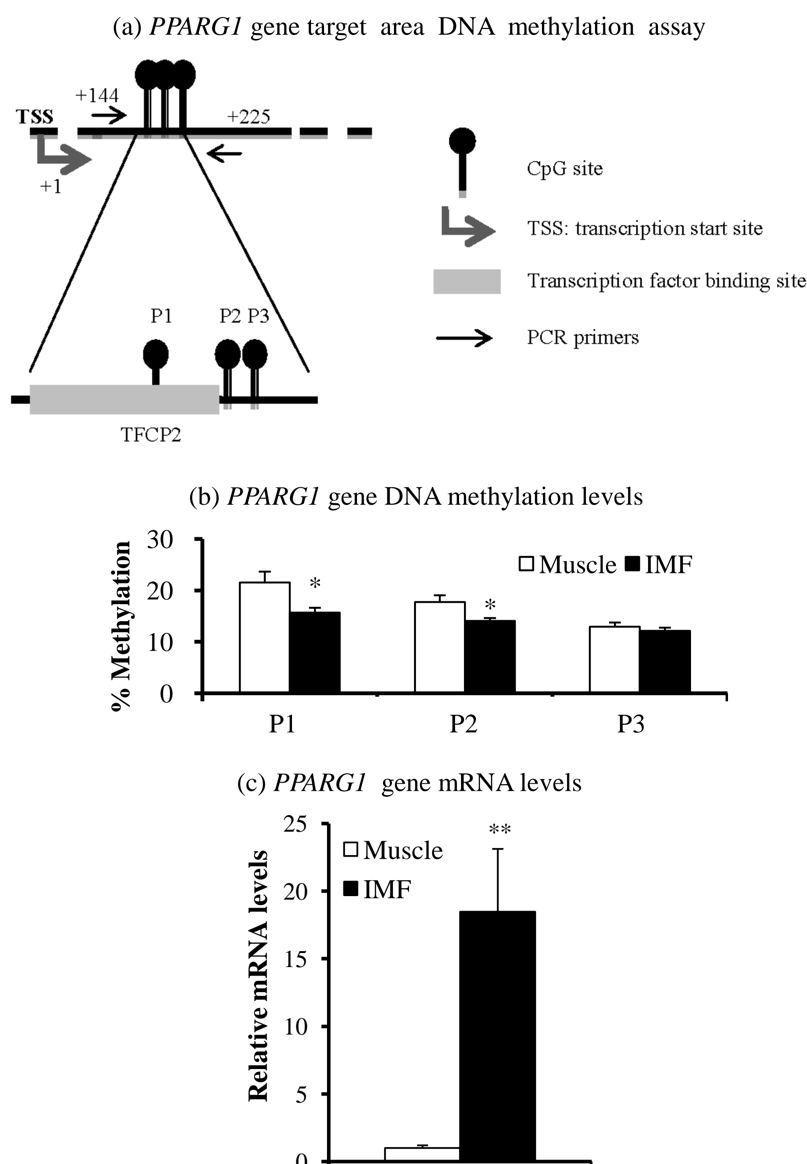

Figure 1. Target area (a), DNA methylation levels (b), and mRNA levels (c) of the peroxisome proliferator activated receptor gamma 1 (*PPARG1*) gene in intramuscular fat (IMF) and muscle portion of Korean cattle steer *longissimus dorsi* muscle tissue. (a) DNA methylation assay target area of the *PPARG1* gene promoter region and transcription factor binding sites. TFCP2, transcription factor CP2. (b) DNA methylation levels were determined by pyrosequencing of bisulfite-treated DNA. Values are the mean+SE (n = 5). (c) The mRNA levels were determined by real-time PCR and normalized against a housekeeping gene. Muscle portion data were normalized to 1.0. Values are the mean+SE (n = 10). * p<0.05; ** p<0.01. PCR, polymerase chain reaction; SE, standard errors.

Differences between muscle and IMF portions of the LM tissues were analyzed by the general linear model procedure in SAS (SAS Inst. Inc., Cary, NC, USA), and model included tissue type and a random error.

RESULTS AND DISCUSSION

Real-time PCR analysis showed that mRNA levels of both *PPARG1* (Figure 1b; p<0.01) and *FABP4* (Figure 2b; p<0.001) genes were higher in the IMF portion than in the muscle portion of the LM. Our recent study also showed higher *PPARG1* (Jeong et al., 2013) and *FABP4* (Ahn et al., 2014) mRNA levels in IMF than in the muscle portion of the LM.

Next, we determined DNA methylation levels within CpG island promoter regions of the *PPARG1* and *FABP4* genes. DNA methylation levels of two of three CpG sites from the *PPARG1* gene regulatory region (+144 to +225) were lower (p<0.05) in the IMF portion than in the muscle portion of the LM (Figure 1c). DNA methylation levels of all five CpG sites from promoter regions (–9,664 to –9,469) of the *FABP4* gene were lower (p<0.001) in the IMF portion

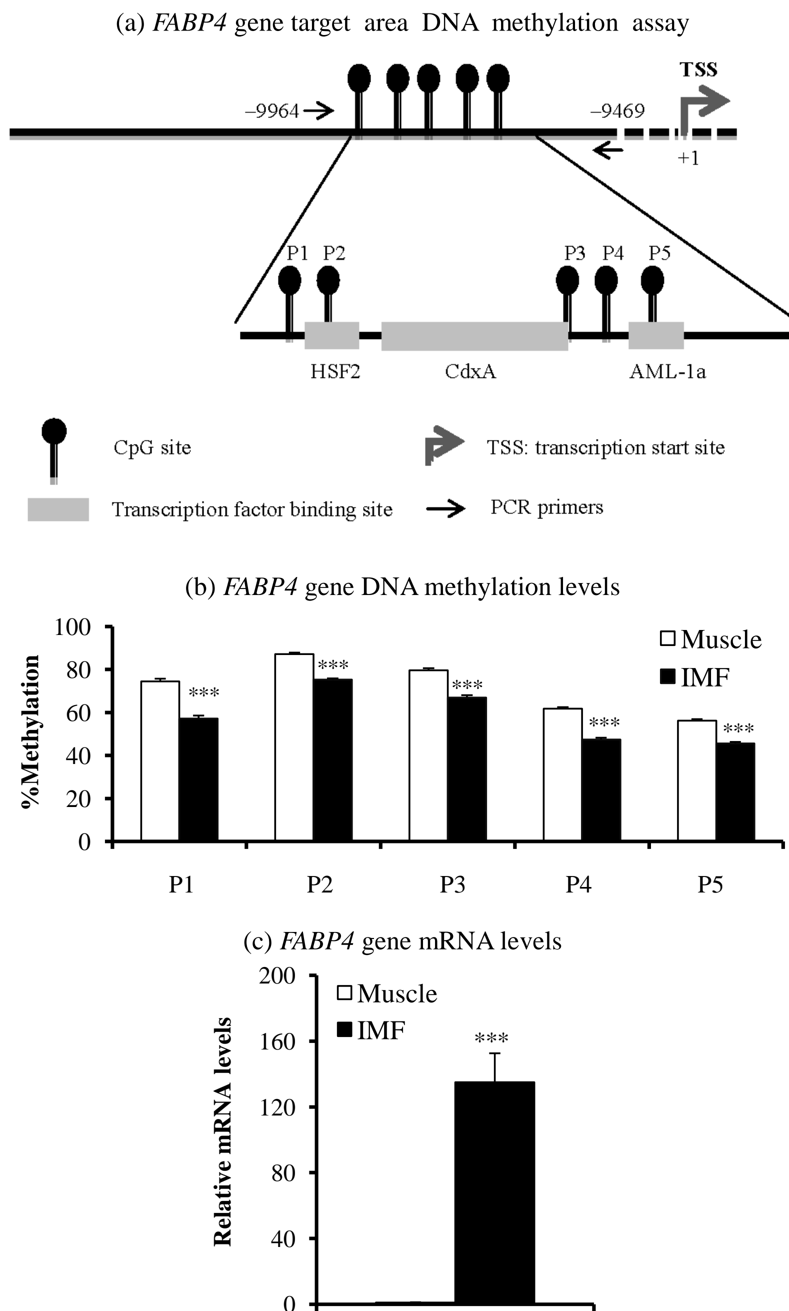

Figure 2. Target area (a), DNA methylation levels (b), and mRNA levels (c) of the fatty acid binding protein 4 (*FABP4*) gene in intramuscular fat (IMF) and the muscle portion of Korean cattle steer *longissimus dorsi* muscle tissue. (a) DNA methylation assay target area in the *FABP4* gene promoter region and transcription factor binding sites. HSF2, heat shock factor 2; CdxA, caudal-related homeobox A; AML-1a, acute myeloid leukemia-1a. (b) DNA methylation levels were determined by pyrosequencing bisulfite-treated DNA. Values are the mean+SE (n = 5). (c) The mRNA levels were determined by real-time PCR and normalized with a housekeeping gene. Muscle portion data were normalized to 1.0. Values are the mean+SE (n = 10). *** p<0.001. PCR, polymerase chain reaction; SE, standard errors.

than in the muscle portion (Figure 2c). Thus, transcription levels of both *PPARG1* and *FABP4* genes were inversely correlated with DNA methylation levels of regulatory regions of CpG sites of the corresponding gene.

Two types of PPARG splice variants, *PPARG1* and *PPARG2*, have been identified in several species, including mouse (Zhu et al., 1993), human (Elbrecht et al., 1996), and bovine species (Sundvold et al., 1997). Differential expression of two types of *PPARG* has been reported in several studies: *PPARG1* mRNA was expressed to a higher levels compared to *PPARG2* mRNA in human adipose tissues (Vidal-Puig et al., 1997; Yanase et al., 1997). We

Table 2. Primer sequences used in bisulfite pyrosequencing

Gene name (Symbol)	Accession number	Primer	PCR primer sequence (5'-3')	Sequencing primer (5'-3')	Target region: relative to transcription start site	Number of CpG sites checked
Peroxisome proliferator-activated receptor gamma isoform 1 (*PPARG1*)	NM_181024	Forward	tgaggtttgtggtgatgattattt	aacccaaataaataaaattct	+144 to +225	3
		Reverse	aacacaatttccccaaccatta			
Fatty acid binding protein 4 (*FABP4*)	BT10868	Forward	tttaattttttttgttaggaattgggttat	gttaggaattgggttatatagta	–9,664 to –9,469	5
		Reverse	aaaaacatacaacctaaatcccttaca			

PCR, polymerase chain reaction.

found that *PPARG1* mRNA levels in the IMF portions were 18-fold higher than muscle portion of the LM, whereas *PPARG2* mRNA levels in the IMF were only 2 fold higher than muscle portion (unpublished data), although similar mRNA levels of both transcripts were detected in bovine fat tissues in other study (Sundvold et al., 1997). Thus, we have chosen *PPARG1* rather than *PPARG2* for DNA methylation analysis. In a previous study, the *PPARG2* gene promoter in 3T3-L1 preadipocytes was hypermethylated, but was also progressively demethylated upon the induction of differentiation, which was accompanied by an increase in mRNA expression (Fujiki et al., 2009). They showed that *PPARG* gene expression was inhibited by methylation of its promoter region. The CpGs within the *FABP4* promoter were methylated in muscle progenitor cells, whereas CpGs were relatively unmethylated in adipose stem cells (Sorensen et al., 2010). A recent study also showed that treatment with 5-aza-29-deoxycytidine, a demethylating agent, decreased adipocyte differentiation, resulting in the downregulation of *PPARG2* and *FABP4* gene expression (Zych et al., 2013).

Methylation at specific CpG positions could influence the affinity for specific transcription factors toward DNA molecules (Deaton and Bird 2011). We found that DNA methylation levels of the first two *PPARG1* promoter CpG sites (+187, +203) were lower in the IMF portion than those in the muscle portion of steers. These regions (+144 to +225) are located on the first exon of *PPARG1* and contains a CP2 transcription factor binding site (TFCP2). Decreased DNA methylation on specific CpG sites could permit the induction of *PPARG1* gene transcription. Thus, DNA methylation status may alter TFCP2 binding activity at CpG sites, regulating *PPARG1* gene transcription. Association of DNA methylation status with transcriptional control of the *PPARG1* gene via the TFCP2 has not been reported.

We also found that the IMF portion had lower DNA methylation levels in all five CpG sites of the *FABP4* gene upstream region (–9,664 to –9469) compared to the muscle portion, whereas the *FABP4* gene mRNA level was higher in IMF than in the muscle portion of intact LM in the current study as well as in our recent study (Ahn et al., 2014). The upstream region of the *FABP4* gene in which we measured DNA methylation status contains transcription factor binding sites for several transcription factors: heat shock transcription factor 2 (HSF2) on CpG site 2, caudal-related homeobox A (CdxA) on CpG site 3, and acute myeloid leukemia-1a (AML-1a) on CpG site 5. Thus, DNA methylation status may alter transcription factor binding activities at these CpG sites, regulating transcription of the *FABP4* gene. Whether DNA methylation status alters binding activity of these transcription factors on the *FABP4* gene promoter is unknown.

In this study, the differences of DNA methylation levels between IMF and muscle portion were about 10%. In contrast, the differences of *PPARG1* and *FABP4* gene expression levels between IMF and muscle portion were about 10 to 100 times (Figure 1c and Figure 2c). Our study suggests that minor difference of DNA methylation status of *PPARG2* and *FABP4* promoter may profoundly affect gene expression levels. Similarly, 5'-aza-cytideine, an inhibitor of DNA methylation, increased *PPARG2* mRNA levels over 20 times, although methylation levels were decreased about 2 times from 40% to 20% (Fujiki et al., 2009). They suggest that promoter demethylation is not the only factor controlling *PPARG2* expression.

In conclusion, DNA methylation status may regulate tissue-specific differential expression of *PPARG1* and *FABP4* genes in the IMF and muscle portion of LM tissues.

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