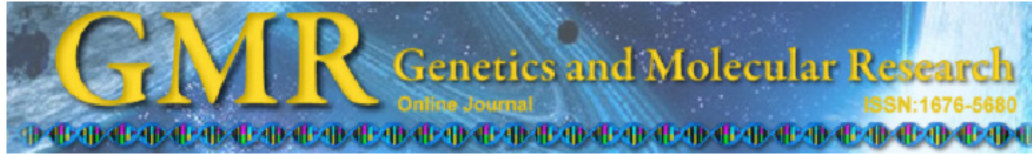

Methylation-sensitive amplification polymorphism analysis of fat and muscle tissues in pigs

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ABSTRACT. DNA methylation may be involved in regulating the expression of protein-coding genes, resulting in different fat and muscle phenotypes. Using a methylation-sensitive amplified polymorphism approach, we obtained 7423 bands by selective amplification of genomic DNA from six different fat depots and two heterogeneous muscle types from Duroc/Landrace/Yorkshire cross-bred pigs. The degrees of DNA methylation, determined by the percentages of hemi- and fully methylated sites relative to the total number of CCGG sites, were similar in male and female pigs for each specific tissue [χ^2 test; P (two-tailed) > 0.05]. Gender bias was therefore ignored. There were significant differences in the degree of DNA methylation among the eight tissue types [χ^2 test; P_{total} (two-tailed) = 0.009]. However, similar degrees of methylation were observed among the six fat depots [χ^2 test; P_{fat} (two-tailed) = 0.24 > 0.05] and between the two muscle types [χ^2 test; P_{muscle} (two-tailed) = 0.76 > 0.05]. We conclude that the degree of DNA methylation differs between porcine fat and muscle tissue, but that the methylation status of a particular tissue type is similar, despite being deposited at different body sites.

Key words: Pig; Fat; Muscle; DNA methylation; MSAP

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INTRODUCTION

DNA methylation at the fifth position of the pyrimidine ring of cytosines in the dinucleotide CpG sequence provides one of many layers of epigenetic mechanisms that control and modulate gene expression via the regulation of chromatin structure (Tost, 2010). Several epigenetic studies in recent decades have also revealed roles for DNA methylation in gene imprinting (McGrath and Solter, 1984; Amor and Halliday, 2008; Henckel and Arnaud, 2010), X-chromosome gene silencing (Avner and Heard, 2001; Huynh and Lee, 2005), miRNA expression (Lujambio et al., 2008), and long-lasting memory (Miller et al., 2010).

Pigs are of enormous agricultural significance, and pork, which consists of fat and muscle, is a major protein source for humans. Different fat depots have been suggested to be anatomically, functionally and metabolically distinct. Visceral fat has been more extensively studied than compartmental subcutaneous fat, because of its involvement in various metabolic syndromes. In terms of muscles, the longissimus dorsi and psoas major muscles are located at different body sites; they are composed of a wide variety of functionally diverse fiber types and exhibit distinct phenotypes. Previous studies have revealed numerous differences in gene expression profiles among different fat depots and phenotypically distinct muscles (Pan et al., 2005; Xiong and Liu, 2008; Liu and Xiong, 2009; Pan et al., 2010). These discoveries have implied that sophisticated epigenetic regulatory mechanisms, such as DNA methylation, may be responsible for this phenomenon.

To investigate the different degrees of DNA methylation in fat and muscle tissues taken from different deposits, we performed methylation-sensitive amplified polymorphism (MSAP) analysis of six different fat depots and two phenotypically distinct muscles from DLY (Duroc/Landrace/Yorkshire) cross-bred pigs.

MATERIAL AND METHODS

Tissues and DNA extraction

Three male and three female 180-day-old DLY pigs were used. Pigs were allowed free access to food and water under normal conditions, and were humanely sacrificed as necessary, to ameliorate suffering. Six different fat depots (greater omentum, mesenteric adipose, leaf fat, upper and inner layer of back fat, and intermuscular fat) and two phenotypically distinct muscles (longissimus dorsi and psoas major) were rapidly separated from each pig. Genomic DNA was extracted using a TIANamp Genomic DNA kit (Tiangen, China), following manufacturer instructions. RNase was used to degrade the residual RNA. Finally, the purified DNA templates were examined using 1% agarose gel electrophoresis and a NanoVue™ Plus spectrophotometer (General Electric Company, USA).

MSAP analysis of DNA methylation

The different degrees of methylation in each tissue were measured by MSAP. The different cytosine methylation statuses of CCGG sites could be detected using the methylation-sensitive isoschizomers, *Hpa*II and *Msp*I, which recognize the same sequence (CCGG) but display different sensitivities to DNA methylation. *Hpa*II cleaves sequences with hemi-

methylated external cytosines (mCCGG), whereas, *Msp*I can only cut sequences that are fully methylated at internal cytosines (CmCGG) (Xu et al., 2000). Hemimethylation of external cytosines, full methylation of internal cytosines, and non-methylation thus represent the three major cytosine methylation states that can be identified as bands in MSAP electropherograms (Figure 1).

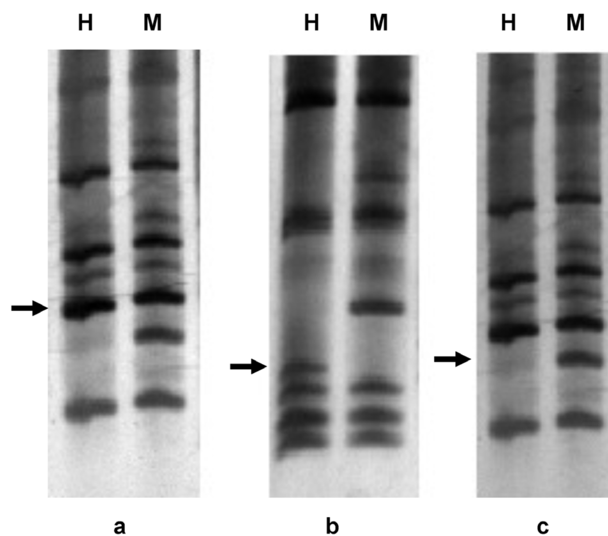

Figure 1. Three methylation status reflected by three types of MSAP bands. H and M indicate the enzyme combinations of *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I, respectively. Type **a** indicates unmethylation of both cytosines, which are bands present in both *Hpa*II and *Msp*I digest. Type **b** indicates hemi-methylation of the external cytosines, which are bands present in *Hpa*II but absent from the corresponding *Msp*I digest. Type **c** indicates full methylation of the internal cytosines, which are bands absent from *Hpa*II but present in the corresponding *Msp*I digest.

Briefly, MSAP involved four steps: digestion, ligation, pre-amplification, and selective amplification. The protocols used in this study were in accordance with those of Xu et al. (2000), with minor modifications. The restriction enzymes *Eco*RI, *Hpa*II, and *Msp*I were purchased from New England Biolabs Inc. (USA). The adapters and primers used are listed in Table 1.

RESULTS AND DISCUSSION

Table 2 lists the distributions of the MSAP bands produced following selective amplification of genomic DNA from the eight tissues surveyed, using 20 pairs of selective primer combinations (Table 1). As expected, that largest proportion of MSAP bands (7432) represented nonmethylated sites (5989, 80.58%), reflecting the intrinsic bias of the MSAP method, which preferentially amplifies unmethylated bands (Xu et al., 2000; Lu et al., 2008). Fully methylated (821, 11.05%) and hemimethylated sites (622, 8.37%) accounted for smaller proportions of bands. In accordance with previous reports (Ohgane et al., 2005; Sha et al., 2005; Xu et al., 2007; Yang et al., 2007; Lu et al., 2008), we found more hemimethylated than fully methylated sites (approximately 1.3-fold).

Table 1. Adapter and primer sequences.

Type	ID	Sequence (5'→3')
Adapters[1]	HMA1	CGAGCAGGACTCATGA
	HMA2	GATCATGAGTCCTGCT
	EA1	CTCGTAGACTGCGTACC
	EA2	AATTGGTACGCAGTCTAC
Pre-amplification primers	HM+0	ATCCATGAGTCCTGCTCGG
	E+0	GACTGCGTACCAATTC
Selective primers[2]	HM+CTGA(HM+4)	ATCCATGAGTCCTGCTCGGCTGA
	HM+CTGT(HM+4)	ATCCATGAGTCCTGCTCGGCTGT
	E+GCT(E+3)	GACTGCGTACCAATTCGCT
	E+TGT(E+3)	GACTGCGTACCAATTCTGT
	E+AAC(E+3)	GACTGCGTACCAATTCAAC
	E+ACA(E+3)	GACTGCGTACCAATTCACA
	E+AGT(E+3)	GACTGCGTACCAATTCAGT
	E+AGA(E+3)	GACTGCGTACCAATTCAGA
	E+AAG(E+3)	GACTGCGTACCAATTCAAG
	E+ACT(E+3)	GACTGCGTACCAATTCACT
	E+CGT(E+3)	GACTGCGTACCAATTCCGT
	E+CTG(E+3)	GACTGCGTACCAATTCCTG

[1]HMA and EA represent *Hpa*II-*Msp*I adapter and *Eco*RI adapter, respectively; [2]combinations of E+3 and HM+4 were used to generate the methylation-sensitive polymorphic fragments.

Table 2. Distribution of MSAP bands produced by selective amplification.

Tissue type	Gender	N	Total band number	Non-methylated CCGG sites (%)	Methylated CCGG sites		
					Hemi-methylated sites (%); external C	Fully methylated sites (%); internal C	Total (%)
Leaf fat	♀	3	466	350 (75.11)	51 (10.94)	65 (13.95)	116 (24.89)
	♂	3	463	363 (78.40)	42 (9.07)	58 (12.53)	100 (21.60)
	♀+♂	6	929	713 (76.75)	93 (10.01)	123 (13.24)	216 (23.25)
Greater omentum	♀	3	459	375 (81.7)	37 (8.06)	47 (10.24)	84 (18.30)
	♂	3	451	365 (80.93)	38 (8.43)	48 (10.64)	86 (19.07)
	♀+♂	6	910	740 (81.32)	75 (8.24)	95 (10.44)	170 (18.68)
Mesenteric adipose	♀	3	495	404 (81.62)	38 (7.68)	53 (10.71)	91 (18.38)
	♂	3	484	398 (82.23)	35 (7.23)	51 (10.54)	86 (17.77)
	♀+♂	6	979	802 (81.92)	73 (7.46)	104 (10.62)	177 (18.08)
Intramuscular fat	♀	3	463	361 (77.97)	45 (9.72)	57 (12.31)	102 (22.03)
	♂	3	453	362 (79.91)	41 (9.05)	50 (11.04)	91 (20.09)
	♀+♂	6	916	723 (78.93)	86 (9.39)	107 (11.68)	193 (21.07)
Upper layer of back fat	♀	3	480	382 (79.58)	41 (8.54)	57 (11.88)	98 (20.42)
	♂	3	397	299 (75.31)	41 (10.33)	57 (14.36)	98 (24.69)
	♀+♂	6	877	681 (77.65)	82 (9.35)	114 (13.00)	196 (22.35)
Inner layer of back fat	♀	3	438	356 (81.28)	37 (8.45)	45 (10.27)	82 (18.72)
	♂	3	494	393 (79.55)	41 (8.30)	60 (12.15)	101 (20.45)
	♀+♂	6	932	749 (80.36)	78 (8.37)	105 (11.27)	183 (19.64)
Longissimus dorsi muscle	♀	3	495	424 (85.66)	32 (6.46)	39 (7.88)	71 (14.34)
	♂	3	412	341 (82.77)	30 (7.28)	41 (9.95)	71 (17.23)
	♀+♂	6	907	765 (84.34)	62 (6.84)	80 (8.82)	142 (15.66)
Psoas major muscle	♀	3	519	436 (84.01)	37 (7.13)	46 (8.86)	83 (15.99)
	♂	3	463	380 (82.07)	36 (7.78)	47 (10.15)	83 (17.93)
	♀+♂	6	982	816 (83.1)	73 (7.43)	93 (9.47)	166 (16.90)
Total		48	7432	5989 (80.58)	622 (8.37)	821 (11.05)	1443 (19.42)

There was no significant variation in degree of DNA methylation between male and female pigs for any tissue [χ^2 test; P (two-tailed) > 0.05] (Figure 2A). Consistently, Weber et al. (2005) reported similar degrees of methylation in primary non-transformed human fibroblasts in males and females (r = 0.88), using the more advanced MeDIP-Chip approach. It was

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therefore possible to ignore any effects of gender bias in subsequent analyses.

When we ignored the gender bias, there were no significant differences in degrees of DNA methylation between the six fat depots [χ^2 test; P_{fat} (two-tailed) = 0.24] or between the two muscle tissues (P_{muscle} = 0.76) (Figure 2B). Leaf fat (23.25%) exhibited the highest degree of methylation among the six fat depots, followed by the upper layer of back fat (22.35%), intermuscular fat (21.07%), inner layer of back fat (19.64%), greater omentum (18.68%), and mesenteric adipose (18.08%). In addition, both muscle types exhibited lower degrees of methylation compared to the fat tissues (15.66 and 16.90% for the longissimus dorsi and psoas major muscles, respectively). However, there was significant variation in degree of DNA methylation among the eight tissue types [χ^2 test; P_{total} (two-tailed) = 0.009], which highlighted the significantly different degrees of DNA methylation between fat and muscle tissues. Tissue-specific methylation is known to be a common feature (Azhikina and Sverdlov, 2005; Byun et al., 2009; Ali and Seker, 2010), and the results of this study tentatively suggest that tissues/cell types involved in the same biological processes exhibit similar degrees of methylation, despite being deposited at different body sites.

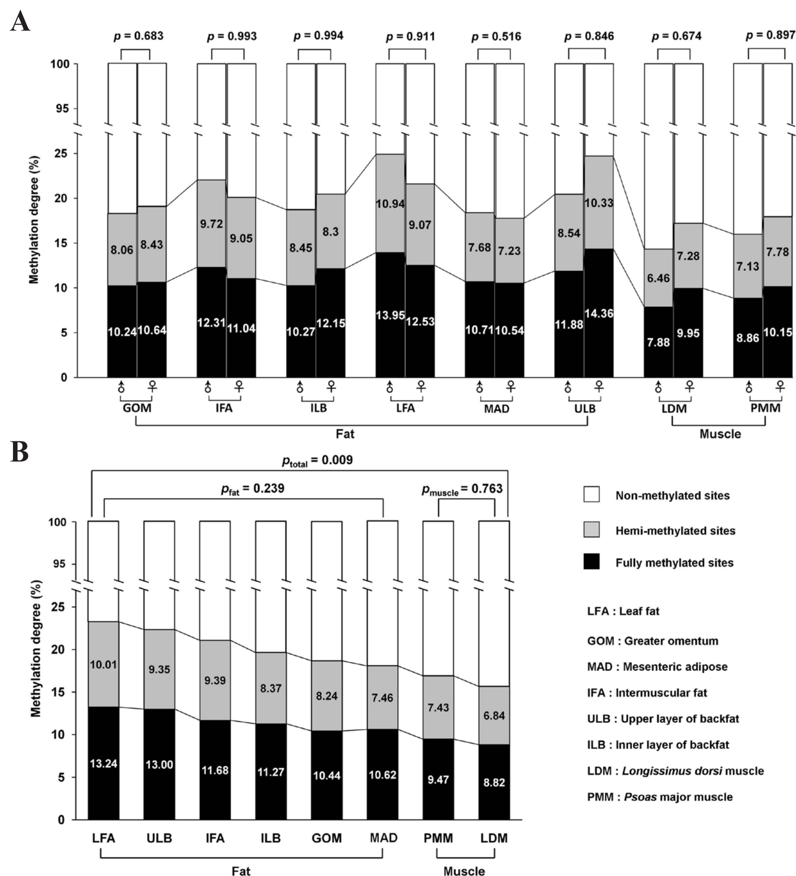

Figure 2. Variations in degree of DNA methylation across surveyed tissues. The χ^2 test was used to compare the percentages of hemi-, full- and non-methylated sites (**A**) between males and females for each tissue, and (**B**) across eight tissue types (P_{total}), across six fat tissues (P_{fat}), and between two heterogeneous muscle types (P_{muscle}). Gender bias was ignored, because it was not statistically significant (χ^2 test; P > 0.05).

Based on the results of this study, we conclude that the differences in DNA methylation between fat and muscle tissues are likely to be associated with cell differentiation and their distinct biological functions, but we need further studies for confirmation.

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