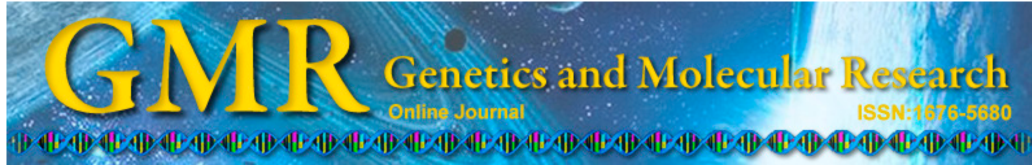

Promoter and first exon methylation regulate porcine *FASN* gene expression

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ABSTRACT. DNA methylation is a stable epigenetic mark mediating gene expression. Methylation is crucial for diverse biological processes, including aging and embryo development. *FASN* (fatty acid synthase) plays an important role in *de novo* lipogenesis, through catalyzing the reductive synthesis of long-chain fatty acids. In this study, we investigated the *FASN* gene expression pattern and corresponding DNA methylation status in the inner layer of backfat from Jinhua pigs at different developmental stages. Our results showed that *FASN* gene expression increases with age and is positively associated with adipocyte volume ($r = 0.98$, $P < 0.01$). In addition, the DNA methylation level for the first exon (0.11, CGI 3) of the *FASN* gene is approximately 8-fold lower than levels for its promoter (0.94, CGI 1&2) (two-way ANOVA, $P_{CGI} < 0.01$). The association analysis revealed that both promoter ($r = -0.944$, $P < 0.01$) and first exon methylation ($r = -0.774$, $P < 0.01$) are significantly and negatively correlated with *FASN* gene expression. Our results will benefit future investigations of the epigenetic mechanism underlying *FASN* gene expression.

Key words: Pig; FASN; DNA methylation; MassArray; Adipocyte

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INTRODUCTION

FASN (fatty acid synthase), responsible for the reductive synthesis of long-chain fatty acids from acetyl-CoA, malonyl-CoA, and nicotinamide adenine dinucleotide, plays a central role in *de novo* lipogenesis in mammals (Wakil, 1989; Semenkovich, 1997; Yan et al., 2002; Smith et al., 2003). Previous studies indicated that FASN contributes largely to energy homeostasis and regulation of body weight. Thus, the *FASN* gene has been implicated in various health problems, including the development of obesity, association with insulin resistance, type 2 diabetes, and some cancers. In turkeys, *FASN* gene polymorphisms are associated with increased fat mass (Sourdioux et al., 1999). Similarly, in studies on Pima Indians and German children, a novel missense mutation in the *FASN* gene caused changes to body fat and BMI (Kovacs et al., 2004; Körner et al., 2007). In human adipose tissue, higher *FASN* expression and FASN activity are mediators that link increased body fat mass to the metabolic consequences of caloric excess, such as insulin resistance, dyslipidemia, and altered adipokine serum profile (Berndt et al., 2007). Furthermore, inhibition of *FASN* expression results in reduced food intake and substantial weight loss in both mice (Loftus et al., 2000) and chicken (Loftus et al., 2000; Dridi et al., 2006).

DNA methylation is an epigenetic modification involving the transfer of a methyl group onto the C5 position of cytosine to form 5-methylcytosine (Razin and Riggs, 1980). Differential DNA methylation is involved in a number of biological processes, including embryo development, aging, and X inactivation (Cotton et al., 2011; Hinoue et al., 2012; Numata et al., 2012; Smith et al., 2012). Thus, disruptions to the methylation process have also been implicated in cancer. Recently, gene-, tissue-, and age-specific variation in DNA methylation levels have been observed and are receiving intensive attention. Genome-wide studies of methylome have emphasized that the position of methylation in the transcript unit is closely related to gene regulation (Laird, 2003; Brenet et al., 2011; Jones, 2012). Thus, DNA methylation may be a major mechanism behind differential *FASN* expression and key to understanding FASN function.

Pigs (*Sus scrofa*) are a recognized model for studying human obesity, as they share many physiological characteristics with humans, such as similar metabolic features, cardiovascular systems, proportional organ sizes, and adipose distribution (Lunney, 2007; Spurlock and Gabler, 2008). They are therefore ideal subjects to examine the physiological effects of *FASN* expression and how methylation affects that process. Here, we investigated the gene expression levels and corresponding DNA methylation status of the porcine *FASN* gene in the inner layer of backfat (ILB) of pigs from different developmental stages. Our results highlight the function of the *FASN* gene in lipogenesis and underscore the role of DNA methylation in *FASN* mRNA expression.

MATERIAL AND METHODS

Animals and tissue collection

All animal studies were performed in compliance with the Institutional Animal Care and Use Committee in the College of Animal Science and Technology, Sichuan Agricultural University, Sichuan, China under permit No. DKY-B20121406. The inner layer of backfat was collected from Jinhua pigs in three developmental stages (0, 30, 180 days, three pigs in each stage). All nine adipose samples were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction.

Adipose measurements

Formalin-fixed tissues were embedded in paraffin using a TP1020 semi-enclosed tissue processor (Leica, Germany). Thin tissue sections (6 μm) were obtained using a RM2135 rotary microtome (Leica) for histomorphometric evaluation. After being stained with hematoxylin and eosin, digital images were captured with a TE2000 fluorescence microscope (Nikon, Japan). Approximately 100 adipocytes per sample were randomly selected using the Image Pro-Plus 6.0 software (Media-Cybernetics, USA) to measure adipocyte cell volume, as described previously (Li et al., 2012).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from the frozen adipose tissues using TRIzol regent (Invitrogen, Carlsbad, CA, USA), and the PrimeScript RT Master Mix Kit (TaKaRa, Dalian, China) was used to reverse transcribe total RNA into cDNA. Primer sequences are shown in Table 1. We performed qRT-PCR using a CFX96 Real-Time PCR detection system (Bio-Rad, Richmond, CA) with the SYBR Premix Ex Taq Kit (TaKaRa). All experiments contained a negative control and each sample was analyzed in triplicate. Relative expression levels of the target mRNAs were calculated by employing the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Table 1. List of gene-specific primers used for qRT-PCR.

Genes	GeneBank ID	Primer sequence (5' to 3')	Amplicon (bp)
FASN	NC_010454.3	F: CTCCAAGCAGGCGAACACG R: CGAAGGGAAGCAGGGTTGAT	99
*ACTB**	DQ178122.1	F: TCTGGCACCACACCTTCT R: TGATCTGGGTCATCTTCTCAC	114
*TBP**	DQ178129.1	F: GATGGACGTTCGGTTTAGG R: AGCAGCACAGTACGAGCAA	124
*TOP2B**	AF222921.1	F: AACTGGATGATGCTAATGATGCT R: TGGAAAAACTCCGTATCTGTCTC	137

**ACTB* (β actin), *TBP* (TATA box binding protein), and *TOP2B* (topoisomerase II β) are the endogenous control genes. F and R indicate forward and reverse primers, respectively.

CpG island prediction

The *FASN* gene sequence [2000 bp upstream of transcription start site (TSS) to 2000 bp downstream of transcription termination site] was downloaded from the Ensembl Genome Browser (Sscrofa 10.2) and scanned for the distribution of CpG islands using the CpG island searcher online software (http://cpgislands.usc.edu/). We found 14 CpG islands based on the following criteria: regions of DNA greater than 200 bp, with a GC content above 50%, and an observed/expected CpG ratio greater than 60%. The promoter region was defined as 2000 bp upstream of TSS.

MassARRAY

DNA extraction

For ILB in each developmental stage, we pooled equal quantities (5 μg) of genomic

DNA, isolated from three female Jinhua pigs with the Blood and Tissue DNA Kit (QIAGEN, Dusseldorf, Germany). The purified DNA templates were tested for quality and purity using a NanoDrop ND-1000 spectrophotometer (NanoDrop, DE, USA), then validated with 1% agarose gel electrophoresis.

Methylation analysis

We treated the inspected DNA with bisulphite using an EZ DNA methylation-Gold Kit (ZYMO Research, Irvine, CA, USA), following manufacturer protocols. We then performed quantitative methylation analysis on the treated DNA using the Sequenom MassARRAY platform (CapitalBio, Beijing, China), as described previously (Li et al., 2012). This platform uses matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, in combination with RNA base-specific cleavage. PCR primers were designed with EpiDesigner software (Sequenom, San Diego, CA, USA). The spectra methylation ratios were analyzed with the EpiTy-per software version 1.0 (Sequenom). The amplified fragments and PCR primers are given in Table 2.

Table 2. Primer sequences for quantitative analysis of DNA methylation using the MassARRAY system.

Name	Primer sequence (5' to 3')	Amplicon		
		CpG island ordinal	Size (bp)	No. of CpGs covered
FASN-1	F: aggaagagagGGTTTTGTAGGTGGGGGAGT	CGI1	215	15
	R: cagtaatacgactcactatagggagaaggctAAAACCTCAATCTCCTTTACACAAA			
FASN -2	F: aggaagagagGGGGTATATATAGGGTTTGGGG	CGI2	385	13
	R: cagtaatacgactcactatagggagaaggctAACATATAAACAACCCTCAAAAATCC			
FASN -3	F: aggaagagagGGATTTAGAAAATAGTTTTGTATAAGAGTG	CGI3	490	36
	R: cagtaatacgactcactatagggagaaggctAAATCCAAATCTCTAACTCCCAAAA			
	R: cagtaatacgactcactatagggagaaggctATCCCACAAATAAACAAACCTCTTT			

Statistical analysis

All statistical analyses were performed in in IBM SPSS Statistics 19 (SPSS 19.0; IBM Corporation, 2010, Armonk, NY, USA). Pearson's correlations were used to examine the relationship between *FASN* expression and adipocyte volume, as well as methylation levels. Comparisons between groups were performed using one-way and two-way ANOVAs.

RESULTS

Developmental patterns of adipocyte cell volume

As shown in Figure 1, the results of our one-way ANOVA revealed that adipocyte volumes and *FASN* expression of porcine ILB showed significant age-dependent patterns, increasing with porcine postnatal development (Figure 1A and B). Indeed, a significant positive correlation was observed between adipocyte volume and *FASN* mRNA expression (Figure 1C, $r = 0.98$, $P < 0.01$), consistent with the role of *FASN* in *de novo* fatty acid synthesis and adipose tissue development.

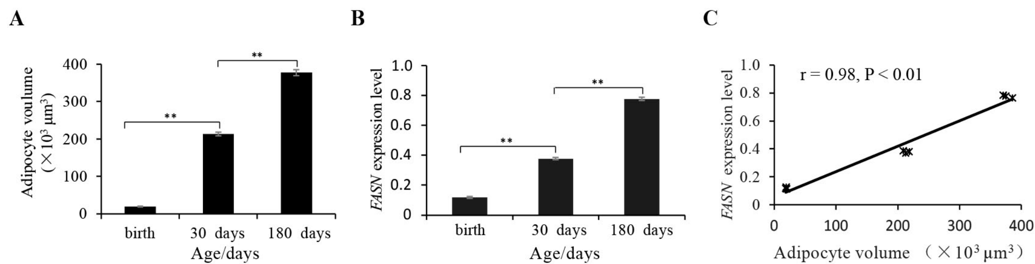

Figure 1. Adipocyte volumes and expression levels of porcine *FASN* in ILB during porcine postnatal development. **A.** Mean volumes of ILB adipocytes at day 0, 30, and 180 (N = 3). **B.** *FASN* expression levels in ILB at day 0, 30, and 180 (N = 3). **C.** Relationship between adipocyte volumes and *FASN* expression levels. All data were analyzed using one-way ANOVA. All data are reported as mean ± SD, $^{**}P < 0.01$.

Methylation pattern of the porcine *FASN* gene

Given that the promoters and the first exon are important elements for gene expression (Rollins et al., 2006), we selected and amplified the first three CpG islands covering the *FASN* promoter and first exon (Figure 2A). Of the 78 CpG sites identified, 59 were considered informative and suitable for further analysis.

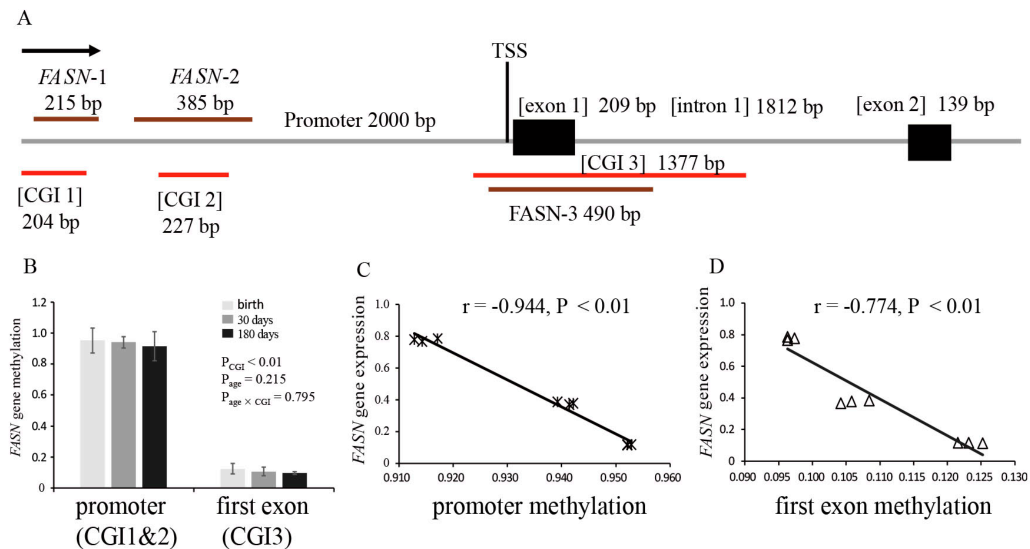

Figure 2. DNA methylation analysis of the porcine *FASN* gene. **A.** Structural diagram of CpG islands and amplified fragments in promoter and first exon regions. The black boxes indicate the first two exons, while the red and dark redlines represent the predicted CpG islands and the corresponding amplified fragments, respectively. **B.** Methylation status of CpG islands (N = 3). **C.** Pearson's correlation between promoter methylation and *FASN* gene expression in porcine ILB. **D.** Pearson's correlation between first exon methylation and *FASN* gene expression in porcine ILB. All data were analyzed using two-way ANOVA. All results are reported as means ± SD.

Compared with the promoter (0.94, CGI1 & 2), the first exon (0.11, CGI3) is significantly hypomethylated, by approximately 8-fold (two-way ANOVA, $P_{CGI} < 0.01$, N = 9; Figure 2B). Thus, to activate gene expression, the requirements for hypomethylation are less stringent for the first exon compared to the promoter, similar to previous findings (Brenet et al., 2011). The promoter and the first exon showed no difference in DNA methylation levels between developmental periods, although there was a slight decrease with age in methylation levels for both elements (two-way ANOVA, $P_{age} = 0.215$, N = 3; Figure 2B). Our results thus demonstrate significant location-specific methylation patterns in porcine *FASN*.

Promoter and first exon methylation negatively correlate with gene expression

Pearson's correlations were employed to further investigate the relationship between *FASN* gene expression levels and methylation status. *FASN* gene expression was found to be negatively correlated with overall methylation levels of both the promoter (r = -0.944, P < 0.01; Figure 2C) and the first exon (r = -0.774, P < 0.01; Figure 2D). When we examined individual CpG sites, we found that 44 and 50% of CpG sites in the promoter and first exon regions, respectively, exhibited a significant negative correlation with gene transcription (Table 3). Our results highlight the essential roles of promoter and first exon methylation in regulating gene transcription. We therefore corroborated previous studies showing that first exon methylation is associated with gene silencing and promoter methylation is inversely correlated with gene expression (Brenet et al., 2011).

Table 3. Pearson's correlations of all amplified CpG sites.

Gene element	Location	CpG sites	Methylation			r value	P value
			0 day	30 days	180 days		
Promoter	CGI 1	*FASN*-1_CpG_1	0.94	0.9	0.87	-0.94**	<0.01
		FASN-1_CpG_2.3	0.98	0.95	0.93	-0.91**	<0.01
		FASN-1_CpG_4	0.94	0.94	0.91	-0.95**	<0.01
		FASN-1_CpG_9.10.11	1	0.92	1	0.12	0.76
		FASN-1_CpG_13.14	0.99	1	0.98	-0.52	0.15
	CGI 2	*FASN*-2_CpG_1.2	1	0.97	0.64	-0.95**	<0.01
		FASN-2_CpG_3.4	1	0.91	0.95	-0.43	0.25
		FASN-2_CpG_8.9	1	0.89	1	0.12	0.76
		FASN-2_CpG_10.11	1	1	0.97	-0.88**	<0.01
		FASN-2_CpG_12	0.73	0.91	0.81	0.32	0.4
		FASN-2_CpG_13.14	0.81	0.94	0.89	0.48	0.19
		FASN-2_CpG_20	0.94	0.92	0.88	-0.95**	<0.01
		*FASN*2_CpG_21.22	1	1	0.98	-0.9**	<0.01
		FASN-2_CpG_23.24	1	0.92	1	0.12	0.76
First exon	CGI 3	*FASN*-3_CpG_2.3.4	0.79	0.55	0.55	-0.81**	0.01
		FASN-3_CpG_5	0.02	0	0	-0.78**	0.01
		FASN-3_CpG_6.7.8	0.07	0.05	0.02	-0.93**	<0.01
		FASN-3_CpG_9.10.11	0.12	0.05	0.07	-0.56	0.12
		FASN-3_CpG_12	0.36	0.29	0.18	-1.00**	<0.01
		FASN-3_CpG_19	0.08	0.08	0.09	0.57	0.11
		FASN-3_CpG_20.21.22	0.28	0.25	0.23	-0.95**	<0.01
		FASN-3_CpG_23.24	0.04	0.2	0.06	0.01	0.98
		FASN-3_CpG_26	0.05	0.08	0.04	-0.34	0.36
		FASN-3_CpG_27	0.06	0.08	0.08	0.69	0.04
		FASN-3_CpG_28.29.30	0.06	0.01	0.01	-0.82**	0.01
		FASN-3_CpG_31.32	0.15	0.12	0.2	0.66	0.05
		FASN-3_CpG_33	0.13	0.03	0.06	-0.57	0.11
		FASN-3_CpG_35	0.05	0.08	0.15	0.99**	<0.01
		FASN-3_CpG_37.38	0	0.04	0.02	0.42	0.26

*P < 0.05; **P < 0.01.

DISCUSSION

In this study, we demonstrated that the *FASN* gene is highly but differentially expressed in porcine adipose tissues at distinct development periods. We also showed that *FASN* expression is significantly and positively correlated with adipocyte volume. These results demonstrate the important role of *FASN* in porcine adipogenesis, as with previous investigations in human. Although a large amount of studies have identified the important roles of methylation in regulating gene expression, few investigations focused on the roles of methylation in *FASN* gene. To further investigate the potential epigenetic mechanism underlying differential *FASN* expression, we examined the methylation status of CpG islands in the promoter and first exon regions for corresponding samples. Our results indicated that DNA methylation in porcine *FASN* is location specific and that there was a significant negative correlation between *FASN* gene expression and its methylation levels in the promoter and first exon.

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