Epigenetic DNA methylation in the promoters of *Peroxisome Proliferator-Activated Receptor* γ in chicken lines divergently selected for fatness

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Epigenetic DNA methylation in the promoters of *Peroxisome Proliferator-Activated Receptor γ* in chicken lines divergently selected for fatness[1]

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ABSTRACT: Peroxisome proliferator-activated receptor γ is a master regulator of adipocyte differentiation and function. Expression of *PPARγ* in mammals is regulated by DNA methylation; however, it is currently unknown whether chicken *PPARγ* expression is regulated by DNA methylation. To enhance our understanding of molecular mechanisms underlying chicken adipose tissue development and adipogenesis, we investigated the promoter methylation status and gene expression of *PPARγ* gene in Northeast Agricultural University broiler lines divergently selected for abdominal fat content (NEAUHLF). Deoxyribonucleic acid methylation was analyzed by bisulfite sequencing method, and mRNA expression was detected by real-time quantitative real time reverse-transcription polymerase chain reaction (RT-PCR). The analyzed region located from –1,175 to –301 bp upstream of the translation start codon ATG contains 6 CpG dinucleotides, which are located at positions –1,014, –796, –625, –548, –435, and –383 bp, respectively. The results revealed that the 3 CpGs at positions –548, –435, and –383 bp showed differential methylation between the lean and fat chicken lines, but the other 3 CpG sites at positions –1,014, –796, and –625 bp did not. *PPARγ* gene promoter methylation in both chicken lines decreased with age, and *PPARγ* promoter methylation levels were significantly higher in lean than fat broilers at 2 wk of age (79.9 to 64.5%; $P < 0.0001$), at 3 wk of age (66.7 to 58.3%; $P < 0.0001$), and at 7 wk of age (50.0 to 42.7%; $P = 0.0004$). Real-time quantitative RT-PCR analysis showed that, negatively correlated with DNA methylation (Pearson's $r = –0.653$, $P = 0.0057$), *PPARγ* expression was increased with age and significantly lower in lean than fat chicken lines at 2, 3, and 7 wk of age ($P < 0.0001$). In conclusion, our findings suggest that chicken *PPARγ* is regulated by DNA methylation during adipose tissue development.

Key words: adipose tissue, chicken, DNA methylation, peroxisome proliferator-activated receptor

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INTRODUCTION

Peroxisome proliferator-activated receptor γ is the master regulator of adipogenesis in mammals and birds (Tontonoz et al., 1995; Gerhold et al., 2002; Tontonoz and Spiegelman, 2008; Wang et al., 2008) and is a candidate gene for common obesity in adipose tissue. Recent evidence has suggested that DNA methylation regulates the expression of *PPARγ* in mammalian adipogenesis (Fujiki et al., 2009). Due to the difference in adipogenesis and lipogenesis between mammals and birds, so far, little is known about the DNA methylation status of *PPARγ* gene and its role in chicken adipose development. To enhance our understanding of molecular mechanisms underlying chicken adipose tissue development and adipogenesis, it is essential to investigate DNA methylation status and its effect in chicken adipose development.

The Northeast Agricultural University broiler lines divergently selected for abdominal fat content (**NEAUHLF**) have been established by long-term divergent selection on abdominal fat percentage (**AFP**) and plasma very low density lipoprotein concentration

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since 1996 (Liu et al., 2007). The G0 generation of the NEAUHLF both derived from the commercial Arbour Acres grandsire line, which was then divided into 2 lines according to plasma very low density lipoprotein concentration at 7 wk of age. After 14 generations of selection, the AFP of the fat chicken line was 4.5-fold greater than that of the lean chicken line. Peroxisome proliferator-activated receptor γ is the master regulator of chicken adipogeneis, so we hypothesized that the chicken *PPARγ* gene is differentially methylated in the lean and fat chicken lines during adipose tissue development.

The aim of the study was to investigate the promoter methylation status and mRNA expression of chicken *PPARγ* gene in the abdominal adipose of the lean and fat chicken lines of the 14th generation of NEAUHLF at 2, 3, and 7 wk of age. Our findings suggest that chicken *PPARγ* is regulated by DNA methylation during adipose tissue development.

MATERIALS AND METHODS

Animals and Tissues

All animal work was conducted according to the guidelines for experimental animal studies, which was established by the Ministry of Science and Technology of the People's Republic of China (approval number: 2006-398) and was approved by the Laboratory Animal Management Committee of Northeast Agricultural University. Chickens from generation 14 of NEAUHLF were used. All birds were kept in similar environmental conditions and had free access to feed and water. The same feed was used for the divergently selected chicken lines. Commercial corn–soybean-based diets that meet all NRC requirements (NRC, 1994) were provided in the present study. From hatch to 3 wk of age, all birds received a starter feed (3,100 kcal of ME/kg and 210 g/kg of CP) and from 4 to 7 wk of age, all birds were fed a grower diet (3,000 kcal of ME/kg and 190 g/kg of CP). In total, 30 male birds (5 birds per line per time point) were slaughtered at 2, 3, and 7 wk of age, and the abdominal fat tissue was collected, snap-frozen, and stored in liquid nitrogen until the extraction of genomic DNA and total RNA.

Deoxyribonucleic Acid Extraction and Bisulfite Modification

Genomic DNA was extracted from the abdominal fat tissue of the divergently selected chicken lines at age 2, 3, and 7 wk using Trizol (Invitrogen, Carlsbad, CA) following the manufacturer's protocol and treated with EZ DNA Methylation-Gold kit (Zymo Research, Irvine, CA) according to manufacturer's recommendations. Genomic DNA quantification was performed on a NanoVue Spectrophotometer (GE LifeSciences, Piscataway, NJ, USA). The basic principle of bisulfite modification of DNA is that all unmethylated cytosines are deaminated and sulfonated, converting them to thymines, whereas methylated cytosines (5-methyl-cytosines) remain unaltered in the bisulfite reaction (Lorente et al., 2008).

Bisulfite Sequencing PCR

The 5′-flanking region (875 bp) from –1,175 to –301 bp upstream of the translation start site of the *PPARγ* gene (GenBank accession no. AB045597) was amplified from bisulfite modified chicken genomic DNA by bisulfite sequencing PCR (**BSP**). The primer (forward primer, 5′-TAAATGTTAAAAGTTATTTAT-3′, and reverse primer, 5′-ATTAACATCCAATACTCAAA-3′) designed using Methyl Primer Express Software v1. 0 (Applied Biosystems Inc., Foster City, CA) encompassed the –1,175 to –301 bp promoter region of chicken *PPARγ* gene (relative to the translation start codon ATG), which harbors no CpG island but 6 CpG sites. The PCR reactions were hot-started at 94°C for 10 min followed by 40 cycles of 94°C for 1 min, 58.4°C for 50 s, and 72°C for 30 s and finally extended at 72°C for 2 min. The amplified PCR products were purified, ligated into pEASY-T1-vector (Trans, Beijing, China), and transformed into Trans-T1competent *Escherichia coli* cells (Trans, Beijing, China). Ten ampicillin-resistant colonies per sample were subcultured for plasmid extraction and sequencing (Invitrogen, Shanghai, China; Sun et al., 2012).

Real Time Reverse-transcription Polymerase Chain Reaction (RT-PCR)

Total RNAs were isolated from the same samples used for BSP, using Trizol (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Ribonucleic acid quantification was performed using a NanoVue Spectrophotometer (GE LifeSciences, Piscataway, NJ, USA), and RNA integrity was verified by using denaturing agarose gel electrophoresis and comparing the 28S and 18S ribosomal RNA bands. The 28S/18S intensity ratio around 2 is considered to be good quality RNA. Reverse transcription was performed using 1 μg of total RNA, an oligo (dT) anchor primer, and ImProm-II reverse transcriptase (Promega Corp., Madison, WI). Reverse transcription conditions for each cDNA amplification were 25°C for 5 min, 42°C for 60 min, and 70°C for 15 min. The SYBR Green quantitative PCR was performed using the ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA). The primer sequences for chicken *PPARγ* were as follows: forward primer, 5′-GGGCGATCTT-

GACAGGAA-3′, and reverse primer, 5′-GCCTCCACAGAGCGAAAC-3′. All reactions were performed in triplicate and *glyceraldehyde-3-phosphate dehydrogenase* (***GAPDH***; GenBank accession no. NM_204305.1) was used as an internal control. The primer sequences for chicken *GAPDH* were as follows: forward primer, 5′-AGAACATCATCCCAGCGT-3′, and reverse primer, 5′-AGCCTTCACTACCCTCTTG-3′. Dissociation curves were analyzed using the Dissociation Curve 1.0 software (Applied Biosystems) for each PCR reaction to detect and eliminate possible primer-dimer artifacts. The relative amount of each *PPARγ* to *GAPDH* was described using the Eq. $[2]^{-\Delta C_T}$, where $\Delta C_T = C_{T\ PPAR\gamma} - C_{T\ GAPDH}$ (Livak and Schmittgen, 2001).

Statistical Analysis

Deoxyribonucleic acid methylation data from bisulfite sequencing were analyzed and visualized using BiQ Analyzer (http://biq-analyzer.bioinf.mpi-inf.mpg.de/). The percentage of methylation for individual chicken was calculated by dividing the total number of methylated CpG sites by the total number of CpG sites, using BiQ Analyzer software (Bock et al., 2005). The Shapiro-Wilk test was used to test whether the data were normally distributed. The 3 × 2 factorial analysis was performed using the GLM procedure of JMP 8.0.2 (SAS Inst., Inc., Cary, NC), with the following models:

$$Y = \mu + L + T + L \times T + e \qquad [1]$$

and

$$Z = \mu + L + T + L \times T + e. \qquad [2]$$

The model [1] was used for methylation level analysis, in which Y is the *PPARγ* methylation level. The model [2] was used for expression level analysis, in which Z is the *PPARγ* expression level. In both model [1] and model [2], μ is the population mean, T is the fixed effect of the age, L is the line (broiler lines selected by high and low abdominal fat content) as fixed effect, and e is the random error, $L \times T$ as interaction of L by T. In the NEAUHLF population, only male birds were slaughtered; therefore, the sex effect was not included in the models. Comparison between 2 groups was performed by *t*-test. Comparison among more than 2 groups was performed by Tukey's honestly significant difference test.

Pearson's r was used to assess the degree of correlation between the methylation and mRNA expression levels. Difference was considered significant at $P < 0.05$ unless otherwise specified.

RESULTS

Deoxyribonucleic Acid Methylation of Chicken PPARγ Promoter in Adipose Tissue

Genomic DNA was bisulfite treated and analyzed by BSP. We investigated methylation status within the 875 bp region (from –1,175 to –301 bp upstream of the translation start codon ATG) of *PPARγ* gene. The chicken *PPARγ* promoter sequence is listed in supplemental 1 (available in the online version of this paper). Our previous study showed chicken *PPARγ* promoter harbors no CpG island but only CpG dinucleotides (Ding et al., 2011). The analyzed promoter region contains 6 CpG dinucleotides, which are located at positions –1,014, –796, –625, –548, –435, and –383 bp, respectively (Fig. 1A). As shown in Fig. 1B, between- and within-clone mosaic methylation was detected in the 2 chicken lines. To quantitatively compare DNA methylation status of *PPARγ* promoter in the fat and lean chicken lines, we calculated the percentage of methylation for individual chicken (Table 1). The 3 × 2 factorial analysis indicated that the DNA methylation level of *PPARγ* gene was significantly associated with the broiler lines ($P < 0.0001$) and was significantly higher in lean broiler line than in fat broiler line ($P < 0.0001$; Fig. 1C). Additionally, the comparison between the 2 chicken lines at each age showed that the DNA methylation levels of *PPARγ* gene were significantly higher in lean than fat chickens at 2 wk of age (79.9 to 64.5%; $P < 0.0001$), at 3 wk of age (66.7 to 58.3%; $P < 0.0001$), and at 7 wk of age (50.0 to 42.7%; $P = 0.0004$; Fig. 1D). In addition, the DNA methylation levels were also significantly associated with the age of broilers ($P < 0.0001$) and significantly different among all the 3 selected ages and decreased with age ($P < 0.0001$; Fig. 1D). The DNA methylation was also associated with the interaction of line by age ($P = 0.0074$). Analysis of CV revealed that in general the magnitude of CV was higher in fat line than in lean line (Table 1).

Further analysis showed that these 6 CpGs were not randomly methylated in the lean and fat chicken lines (Fig. 1B and 1E). The CpG at position –383 bp was uniquely unmethylated in fat line but predominantly methylated in lean line at 2 wk of age, and the CpG at position –435 bp was more frequently methylated in lean line than in fat line at 3 and 7 wk of age (Fig. 1B and 1E). The CpG at position –548 bp was mainly methylated in the 2 chicken lines at 2 and 3 wk of age but was unmethylated in the 2 chicken lines at 7 wk of age (Fig. 1B and 1E). The methylation patterns for the 3 CpGs at positions –1,014, –796, and –625 bp were similar in both the lean and fat chicken lines (Fig. 1E). The CpGs at positions –1,014 and –625 bp were consistently methylated

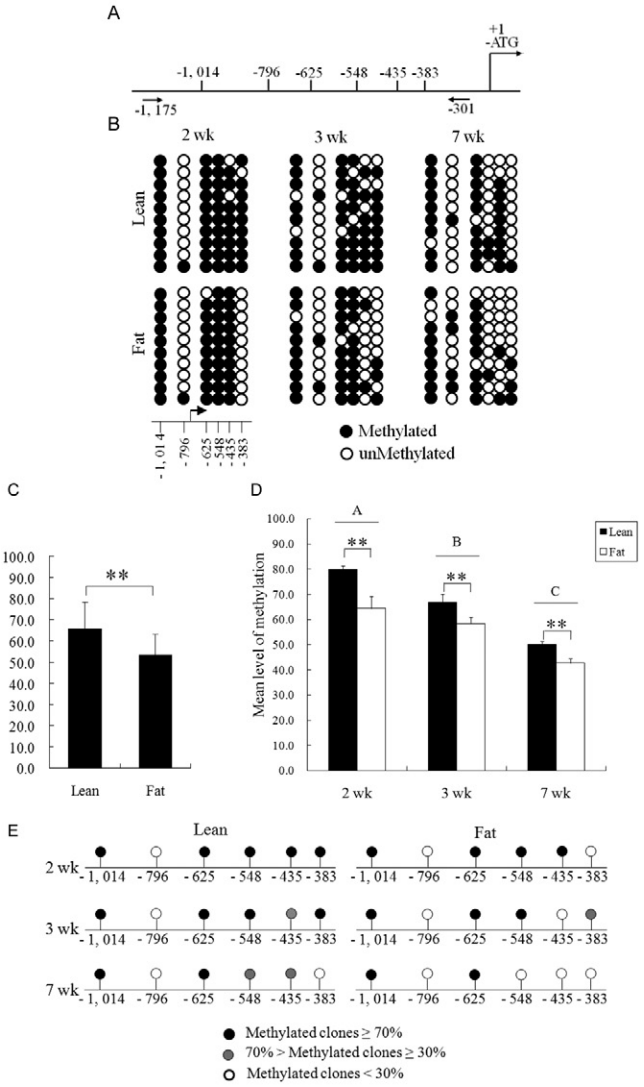

Figure 1. Comparison of *PPARγ* promoter methylation in fat and lean chicken lines. (A) Schematic diagram of *PPARγ* gene promoter. The arrow pairs denote the positions of primers used for BSP. The translational start codon of chicken *PPARγ* gene was represented by ATG. Short vertical lines indicate the positions of the CpG sites in the *PPARγ* gene promoter. All numbered positions are relative to the adenine of the translational ATG start codon of chicken *PPARγ*. (B) Bisulfite genomic sequencing results of *PPARγ* gene promoter in the 2 chicken lines at 2, 3 and 7 wk of age (1 bird per chicken line per time point shown as a representative). Ten clones per sample were sequenced. Each row represents 1 clone with 1 circle symbolizing 1 CpG site. The methylation status of each CpG site is aligned corresponding to their genomic order (represented at the bottom of the results for lean line at 2 wk of age). (C) The mean methylation levels of *PPARγ* promoter in adipose tissues of lean and fat broilers (mean ± SD). Double asterisks (**) indicate significant difference in DNA methylation between the 2 chicken lines (GLM followed by *t*-test, $P < 0.01$). (D) The mean methylation levels of *PPARγ* promoter in adipose tissues of lean and fat broilers at 2, 3 and 7 wk of age ($n = 5$, mean ± SD). Double asterisks (**) indicate significant difference in DNA methylation between the 2 chicken lines (GLM followed by *t*-test, $P < 0.01$). The different uppercase letters above error bars indicate significant difference in *PPARγ* promoter methylation level among ages (GLM followed by Tukey's honestly significant difference test). (E) The methylation status of each CpG site in the sequenced *PPARγ* promoter region. Short vertical lines indicate the positions of the CpG sites. The unfilled (white), filled (gray), and filled (black) circles represent CpG sites with methylation levels < 30%, 70% > methylation levels ≥ 30%, and methylation levels ≥ 70%, respectively.

Table 1. The percentage of DNA methylation of the *PPARγ* promoter in the tested individual chicken

Line	Age, wk	Individual's percentage of DNA methylation[1]					CV
		n1	n2	n3	n4	n5[2]	
Lean line	2	80.0	77.6	81.7	80.0	80.0	0.016
	3	68.3	65.0	70.0	61.7	68.3	0.045
	7	50.0	48.3	50.0	51.7	50.0	0.022
Fat line	2	66.7	68.9	66.7	63.3	56.7	0.067
	3	56.7	60.0	58.3	61.7	55.0	0.041
	7	43.3	41.7	45.0	43.3	40.0	0.040

[1]The percentage of methylation for individual chicken was calculated by dividing the total number of methylated CpG sites by the total number of CpG sites, using BiQ Analyzer software (http://biq-analyzer.bioinf.mpi-inf.mpg.de/).

[2]n1, n2, n3, n4, and n5 refer to the 5 individual chickens for each chicken line at each time point.

in both the chicken lines, but the CpG at position –796 bp was predominately unmethylated (Fig. 1E).

Peroxisome Proliferator-Activated Receptor γ Expression in Adipose Tissue

To evaluate the degree of correlation between *PPARγ* promoter methylation and *PPARγ* mRNA expression, we examined *PPARγ* gene expression. The 3 × 2 factorial analysis showed that the *PPARγ* gene expression level was associated with the line ($P < 0.0001$) and the age ($P = 0.0023$) but not with the interaction of line by age ($P = 0.7596$). Comparing the *PPARγ* mRNA expression between the 2 chicken lines, we observed that *PPARγ* mRNA expression was significantly higher in fat than lean chicken lines ($P < 0.0001$; Fig. 2A). *Peroxisome proliferator-activated receptor γ* mRNA expression in the fat line was approximately threefold greater than that observed in the lean line at 2 and 3 wk of age and twofold greater than that observed in the lean line at 7 wk of age (Fig. 2B). As opposed to *PPARγ* promoter methylation, *PPARγ* mRNA expression in both the chicken lines increased with age, and the *PPARγ* mRNA expression was significantly higher in 7 wk of age than the other 2 ages ($P < 0.0001$; Fig. 2B). The correlation analysis showed that there was a significant negative correlation between *PPARγ* promoter methylation and mRNA expression (Pearson's $r = –0.653$, $P = 0.0057$).

DISUSSION

Deoxyribonucleic acid methylation is a heritable modification that favors genomic integrity and ensures proper regulation of gene expression. In multicellular eukaryotes, DNA methylation seems to be confined to cytosine bases and is associated with inhibition of gene expression (Bird and Wolffe, 1999). The role of DNA

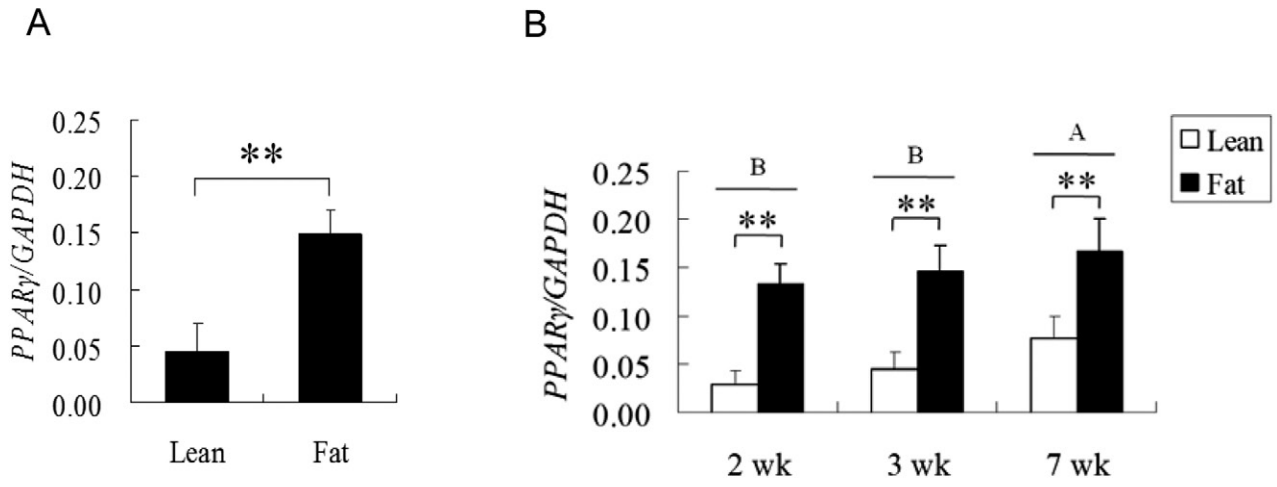

Figure 2. *PPARγ* expression in abdominal adipose tissues of the lean and fat chicken lines. The mRNA expression levels were determined by real-time quantitative Real time reverse-transcription polymerase chain reaction (RT-PCR) and normalized to *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) mRNA measured in parallel experiments, and the results are expressed as mean ± SD. (A) The mean expression levels of *PPARγ* promoter in adipose tissues of lean and fat broilers (mean ± SD). Double asterisks (**) indicate significant difference in *PPARγ* expression between the 2 chicken lines (GLM followed by *t*-test, $P < 0.01$). (B) The mean expression levels of *PPARγ* promoter in adipose tissues of lean and fat broilers at 2, 3 and 7 wk of age ($n = 5$, mean ± SD). Double asterisks (**) indicate significant difference in *PPARγ* expression between the 2 chicken lines (GLM followed by *t*-test, $P < 0.01$). The different uppercase letters above error bars indicate significant difference in *PPARγ* promoter methylation levels among selected ages (GLM followed Tukey's honestly significant difference test, $P < 0.01$).

methylation in adipose development has been a topic of considerable interests in the last few years. Several mammalian studies have shown that DNA methylation, including hypermethylation and hypomethylation, plays an important role in regulating the expression of transcription factors, transcriptional cofactor, and other genes involved in mammalian adipose development and adipogenesis (Bowers et al., 2006; Noer et al., 2007; Shore et al., 2010).

Recent evidence has shown that DNA methylation regulates the expression of *PPARγ* in mammalian adipogenesis. *PPARγ* promoter was hypermethylated in 3T3-L1 preadipocytes and was progressively demethylated on the induction of differentiation, accompanied by an increase in *PPARγ* mRNA expression (Fujiki et al., 2009). Further luciferase reporter assays revealed that *PPARγ* promoter methylation could repress reporter gene expression (Fujiki et al., 2009). The methylation of the CpG at position –437 bp upstream of the transcription start site of *PPARγ* gene in epididymal adipose tissues was higher in diet-induced obesity mice than in wild-type mice, and *PPARγ* mRNA expression was negatively correlated with this CpG methylation (Fujiki et al., 2009). In the present study, we observed that at all 3 selected ages, chicken *PPARγ* promoter DNA methylation was significantly higher in lean than fat chicken lines and *PPARγ* mRNA expression was significantly higher in fat than lean chicken lines, consistent with our previous study showing PPARγ in abdominal adipose tissue was expressed much higher in fat than lean lines at 5 and 7 wk of age by western blotting (Wang et al., 2012). Chicken *PPARγ* gene expression was negatively correlated with its promoter DNA methylation, indicating that chicken *PPARγ* is regulated by DNA methylation during adipose tissue development. Taken together, these data suggest that epigenetical regulation of *PPARγ* gene is conserved in chicken and mammals despite their significant differences in lipogenesis and adipogenesis (Wang et al., 2008; Fujiki et al., 2009; Liu et al., 2010); our finding provides further evidence that epigenetical regulation plays important role in adipose development.

In the present study, among the 6 CpGs, 3 CpGs at positions –548, –435, and –383 bp showed differential methylation between the lean and fat chicken lines, but the other 3 CpG sites at positions –1,014, –796, and –625 bp did not. On the basis of understanding of epigenetic regulation, we speculate that the 3 CpGs may be located within or around the binding sites for transcription factors, and these methylated CpGs may affect protein–DNA interaction, altering *PPARγ* gene regulation and adipose development. These 3 methylated CpGs may be used as epigenetic biomarkers for future chicken breeding improvement. However, this speculation needs to be experimentally confirmed in the future.

Previous studies revealed that *PPARγ* promoter was hypomethylated in uncultured adipose stem cells and maintained hypomethylated despite transcriptional induction (Noer et al., 2006, 2007), which is different from our results and other reports (Harris and Phipps, 2001; Noer et al., 2006, 2007; Fujiki et al., 2009). These data collectively suggest that *PPARγ* promoter methylation may be cell type or context specific.

After 14 generations of selection, NEAUHLF have a striking difference in AFP and abdominal fat content. Many researchers attribute the striking difference to genetic variations, for example, SNP (Liu et al., 2007; Leng et al., 2009; Chen et al., 2012). In the present study, our results showed that *PPARγ* promoter was differentially methylated in the lean and fat chicken lines and that *PPARγ* promoter methylation and *PPARγ* mRNA expression were negative correlated, suggesting DNA methylation variation may also attribute to the chicken fat trait difference in the divergently selected chicken lines. Future studies are needed to understand epigenetic mechanisms controlling chicken adipose tissue development and underlying the different DNA methylation variation in the divergently selected chicken lines.

In conclusion, we demonstrated that chicken *PPARγ* promoter methylation was negatively correlated with *PPARγ* expression and that *PPARγ* promoter methylation levels were decreased with age and significantly higher in lean broilers than fat broilers.

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