ANIMAL GENETICS Immunogenetics, Molecular Genetics and Functional Genomics

doi: 10.1111/age.12326

CpG site DNA methylation of the *CCAAT/enhancer-binding protein, alpha* promoter in chicken lines divergently selected for fatness

Yuan Gao*, Yingning Sun*†, Kui Duan*, Hongyan Shi*, Shouzhi Wang*, Hui Li* and Ning Wang*

*Key Laboratory of Chicken Genetics and Breeding, Ministry of Agriculture, Key Laboratory of Animal Genetics, Breeding and Reproduction, Education Department of Heilongjiang Province. College of Animal Science and Technology, Northeast Agricultural University, Harbin, Heilongjiang 150030, China. †College of Life Science and Agriculture Forestry, Qiqihar University, Qiqihar, Heilongjiang 161006, China.

Summary

CCAAT/enhancer-binding protein (C/EBP), alpha (CEBPA) is a master regulator of adipogenesis and, together with peroxisome proliferator-activated receptor gamma (PPARG), plays a critical role in adipocyte differentiation. Previous studies have demonstrated that *CEBPA* is regulated by DNA methylation and involved in the osteogenesis and adipogenesis of mouse C3H10T1/2 and bone marrow stromal cells. However, it is unclear whether *CEBPA* is regulated by DNA methylation in adipose tissues. Thus, the objectives of the present study were to investigate CpG site methylation in a 357-bp *CEBPA* promoter region and to assess the correlation between promoter CpG site methylation and *CEBPA* gene expression in the abdominal adipose tissues of Northeast Agricultural University broiler lines divergently selected for abdominal fat content. The results showed that the methylation percentage of the analyzed *CEBPA* promoter region was significantly higher in lean broilers than in fat broilers at 2 weeks (80.3% vs. 43.4%, $P < 0.0001$), 3 weeks (95.4% vs. 74.0%, $P < 0.0001$) and 7 weeks of age (82.6% vs. 57.2%, $P < 0.0001$). Real-time quantitative RT-PCR analysis showed that *CEBPA* expression was significantly higher in the fat vs. the lean line at 2 weeks of age ($P = 0.0013$) but not at 3 or 7 weeks of age. The correlation analysis showed that only at 2 weeks of age was the methylation percentage negatively correlated with *CEBPA* expression (Pearson's $r = -0.8312$, $P = 0.0029$). Of all seven tested CpGs, only two, the CpGs at −1494 and −1478 bp, displayed a significantly negative correlation with *CEBPA* mRNA expression. These results suggest that the *CEBPA* is methylated in adipose tissue and may regulate chicken early adipose development.

Keywords adipogenesis, adipose, Northeast Agricultural University broiler lines divergently selected for abdominal fat content (NEAUHLF)

Introduction

Adipose tissue plays an essential role in energy homeostasis. White adipose tissue stores excess energy as triglyceride and releases free fatty acids during periods of energy starvation, whereas brown adipose tissue produces body heat. Adipose tissue is also a major secretory and endocrine organ that secretes numerous adipocytokines, which are involved in diverse physiological and pathological processes including metabolism, reproduction and immunity. Adipogenesis, the formation of specified mature adipocytes, is elaborately regulated by a cascade of transcription factors. Among these, CCAAT/enhancer-binding protein (C/EBP), alpha (CEBPA) and peroxisome proliferator-activated receptor gamma (PPARG) are two master regulators of adipogenesis that positively regulate each other and cooperate to activate adipogenesis (Wu *et al.* 1999; Liu *et al.* 2010).

DNA methylation is an epigenetic modification that plays an important role in embryonic development, tumorigenesis, aging and other diseases (Gopalakrishnan *et al.* 2008;

Address for correspondence

Ning Wang, College of Animal Science and Technology, Northeast Agricultural University, No. 59 Mucai Road, Harbin, 150030 Heilongjiang, China.
E-mail: wangning@neau.edu.cn

Yuan Gao and Yingning Sun are co-first authors and contributed equally to this work.

Accepted for publication 27 May 2015

 © 2015 Stichting International Foundation for Animal Genetics,

Hannum *et al.* 2013). In general, DNA methylation suppresses gene expression by inhibiting the association of DNA binding factors through steric hindrance (Bird 2007) or by recruiting transcriptional corepressors at methylated CpG sites (Klose & Bird 2006). *CEBPA* is a well-studied gene of major interest in methylation studies (Rishi *et al.* 2010; Annamaneni *et al.* 2014; Musialik *et al.* 2014). Its methylation is regulated by a functional RNA arising from the *CEBPA* locus that binds DNA methyltransferase 1 and prevents methylation of the *CEBPA* locus (Di Ruscio *et al.* 2013).

In vitro studies have demonstrated that, in mammals, *CEBPA* is regulated by DNA methylation and controls the balance between osteogenesis and adipogenesis. In C3H10T1/2 cells, *CEBPA* promoter methylation controls the balance between osteogenic and adipogenic differentiation (Fan *et al.* 2009). It has been shown that dexamethasone inhibits *CEBPA* promoter hypermethylation and shifts the differentiation of mouse bone marrow stromal cells (BMSCs) from osteoblasts to adipocytes during osteoblastogenesis (Li *et al.* 2013). During 3T3-L1 pre-adipocyte differentiation, *CEBPA* promoter is hypomethylated in 3T3-L1 pre-adipocytes but hypermethylated in 3T3-L1 adipocytes (Li *et al.* 2010). These data may hint that that *CEBPA* may be regulated by DNA methylation and involved in *in vivo* adipogenesis and adipose development.

Lipogenesis and adipogenesis have a number of important differences between mammals and birds. First, in most mammals, lipogenesis occurs in both adipose tissue and liver, whereas in birds, the liver is the major site for lipogenesis, and avian adipose tissue growth depends on the availability of plasma triglycerides, which are transported as components of lipoprotein (Hermier 1997). Second, the *in vitro* and *in vivo* gene expression patterns of pro-adipogenic and anti-adipogenic transcription factors differ between mammals and birds (Matsubara *et al.* 2005). Third, unlike mammalian adipogenesis, chicken adipogenesis is insulin independent and requires the presence of exogenous fatty acids (Matsubara *et al.* 2008). These differences suggest mammals and birds do not use completely identical molecular mechanisms for adipogenesis and lipogenesis.

CEBPA and PPARG are master regulators of adipogenesis (Wu *et al.* 1999). Our previous study showed that *PPARG* promoter methylation levels were significantly higher in lean broilers compared with fat broilers of Northeast Agricultural University broiler lines and that the *PPARG* promoter methylation was negatively correlated with *PPARG* expression in adipose tissues (Sun *et al.* 2014), suggesting that DNA methylation may play a critical role in chicken adipogenesis and adipose development. A recent study found that folate supplementation increased the methylation level of chicken *CEBPA* promoter and reduced *CEBPA* expression in cultured adipocytes (Yu *et al.* 2014). However, to date, it remains unclear whether *CEBPA* is regulated by DNA methylation in adipogenesis and adipose tissue development. Therefore, the objectives of this study were to investigate CpG site methylation in a 357-bp *CEBPA* promoter region (−1568 to −1212 bp upstream of the *CEBPA* translation start site) and to assess the correlation between promoter CpG site methylation and *CEBPA* gene expression in the abdominal adipose tissues of Northeast Agricultural University broiler lines divergently selected for abdominal fat content (NEAUHLF).

Materials and methods

Animals and tissues

Chickens from generation 14 of NEAUHLF were used. NEAUHLF have been divergently selected since 1996 using the abdominal fat percentage (AFP = abdominal fat weight/ body weight) and plasma very low-density lipoprotein (VLDL) concentration as selection criteria (Liu *et al.* 2007). The G0 generation of the NEAUHLF derived from the commercial Arbour Acres grandsire line. Briefly, plasma VLDL concentrations of 90 male and 208 female chickens were measured at 29 weeks of age, and 50 birds (10 males and 40 females) with the lowest and highest plasma VLDL concentration were selected to form the G0 generation of the putative lean and fat lines (Li & Yang 1997). In both lean and fat lines, females were artificially inseminated with semen of the selected males from the same line to generate the G1 generation of lean and fat lines. From G1 to G14, each line was raised in two hatches. Plasma VLDL concentrations were measured for all males, and the AFP of the males in the first hatch was measured after slaughter at 7 weeks of age. Sib birds from the families with lower (lean line) or higher (fat line) AFP than the average value for the population were selected as candidates for breeding, considering the plasma VLDL concentration and the body weights of males in the second hatch and the egg production of females in both hatches (Zhang *et al.* 2012). After 14 generations of selection, the AFP of the fat line at 7 weeks of age was 4.45 times higher than that of the lean line.

All animal work was conducted according to the guidelines for experimental animal studies, which were established by the Ministry of Science and Technology of the People's Republic of China (Approval number 2006–398) and were approved by the Laboratory Animal Management Committee of Northeast Agricultural University. These birds were kept under the same environmental conditions and had free access to feed and water. Commercial corn- and soya bean-based diets that meet all National Research Council requirements (NRC, 1994) were provided. From hatch to 3 weeks of age, all birds received a starter feed (3000 kcal of ME/kg and 210 g/kg of CP), and from 4 to 7 weeks of age, all birds were fed a grower diet (3100 kcal of ME/kg and 190 g/kg of CP). In total, 30 male birds (five birds per line per time point) were slaughtered at 2, 3 and

7 weeks of age. The abdominal fat tissues were collected, snap-frozen and stored in liquid nitrogen until the extraction of genomic DNA and total RNA.

DNA extraction and bisulfite modification

Frozen abdominal fat tissues were thawed, and genomic DNA was isolated from the abdominal fat tissues of NEAUHLF at 2, 3 and 7 weeks of age using TRIzol (Invitrogen), according to the manufacturer's protocol. Genomic DNA quantification was performed on a NanoVue Spectrophotometer (GE Healthcare). Sodium bisulfite modification was performed using an EZ DNA Methylation-Gold kit (Zymo Research), according to the manufacturer's recommendations. In this process, unmethylated cytosine residues were converted to thymine, whereas the methylated cytosines remained unchanged (Frommer *et al.* 1992).

Bisulfite sequencing PCR (BSP)

According to the published DNA sequence in the promoter region of the chicken *CEBPA* gene (Ding *et al.* 2011), the 357-bp region (−1568 to −1212 bp upstream of the *CEBPA* translation start site) was amplified from bisulfite-modified chicken genomic DNA by BSP. The primer (forward, 5′-TTTTTATTGATATTGAAAGGTGA-3′; reverse, 5′-CAATAAAAACCCCAATATAACTATAA-3′) was designed using METHYL PRIMER EXPRESS SOFTWARE – v1.0 (Applied Biosystems Inc.). Amplification conditions were as follows: 94 °C for 10 min, 35 cycles at 94 °C for 1 min, 60.3 °C for 50 s and 72 °C for 50 s, followed by a final extension at 72 °C for 2 min. The amplified PCR products were purified, ligated into pEASY-T1-vector (Trans) and transformed into Trans-T1 competent *Escherichia coli* cells (Trans). Ten ampicillin-resistant colonies for each sample were randomly picked to subculture for plasmid extraction and sequencing (Invitrogen) (Sun *et al.* 2012).

RNA isolation and quantitative real-time RT-PCR

Total RNA was extracted from the same samples used for bisulfite sequencing PCR, using TRIzol (Invitrogen), according to the manufacturer's protocol, and treated using recombinant shrimp DNase (Promega Corp.) to eliminate DNA contamination. RNA quantification was performed using a NanoVue Spectrophotometer (GE Healthcare), and RNA quality was assessed by visualization of the 18S and 28S ribosomal RNA bands on denaturing formaldehyde agarose gel. The quality of RNA is considered to be good if the intensity ratio of 28s/18s is around two. Total RNA (1 μg) was reverse-transcribed using ImProm-II reverse transcriptase (Promega Corp.) for 5 min at 25 °C, 60 min at 42 °C and 15 min at 70 °C. Chicken *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*; GenBank accession no. NM_204305.1) was used as an endogenous control. PCR amplification of chicken *CEBPA* was performed using the primers (forward, 5′-GGAGCAAGCCAACTTCTACGC-3′; reverse, 5′-CTCGTTCTCGCAGATGTCGC-3′), resulting in an amplicon length of 169 bp. PCR amplification of chicken *GAPDH* was performed using the primers (forward, 5′-AGAACATCATCCCAGCGT-3′; reverse, 5′-AGCCTTCAC TACCCTCTTG-3′, resulting in an amplicon length of 181 bp. The SYBR Green quantitative PCR was performed using the ABI Prism 7500 sequence detection system (Applied Biosystems) in a total volume of 10 μl, containing 1 μl of cDNA sample, 0.1 μM of each primer and 1X SYBR Green PCR Master Mix (Applied Biosystems). SYBR Green PCR conditions were 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Dissociation curves were analyzed using DISSOCIATION CURVE 1.0 software (Applied Biosystems) for each PCR to detect and eliminate possible primer–dimer artifacts. The relative amount of *CEBPA* to *GAPDH* was calculated using the formula $2^{-\Delta CT}$, where $\Delta C_T = C_{T\ CEBPA} - C_{T\ GAPDH}$ (Livak & Schmittgen 2001). Each assay was conducted in duplicate and repeated a minimum of three times.

Statistical analysis

DNA methylation data from bisulfite sequencing were analyzed and visualized using BIQ ANALYZER (biq-analyzer.bioinf.mpi-inf.mpg.de). The percentage of methylated CpGs was calculated by the number of methylated CpGs divided by the total number of CpGs analyzed, using BIQ ANALYZER software (Bock *et al.* 2005). Normality of data distribution was assessed using the Shapiro–Wilk test, and the non-normal distribution data were converted into normal distribution data for further statistical analysis, using MINITAB 16 Box–Cox. The 3 × 2 factorial analyses were performed using the GLM procedure of JMP 8.0.2 (SAS Institute, Inc.), with the following models:

$$Y = \mu + L + T + L \times T + e \quad (1)$$

$$Z = \mu + L + T + L \times T + e. \quad (2)$$

Model [1] was used for methylation level analysis, where Y is the *CEBPA* methylation level. Model [2] was used for expression level analysis, where Z is the *CEBPA* expression level. In both models, 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. Comparison between two groups was performed by t-test. Comparison among more than two groups was performed by Tukey's honestly significant difference (HSD) test.

Pearson's r was used to assess the degree of correlation between the methylation and mRNA expression levels. Significance was determined as $P < 0.05$, unless otherwise specified.

© 2015 Stichting International Foundation for Animal Genetics, 46, 410–417

Results

DNA methylation of chicken *CEBPA* in adipose tissues

Our previous study showed that a large CpG island was located in the −1512 to −61 bp promoter region of *CEBPA* (Ding *et al.* 2011). In the present study, the methylation status of the 357-bp region (from −1568 to −1212 bp upstream of the translation start site) of the *CEBPA* gene promoter was investigated using the bisulfite sequencing method. This analyzed region contains a total of seven CpG dinucleotides (CpGs) located at −1494, −1478, −1419, −1370, −1255, −1252 and −1243 bp (Fig. 1a).

To quantitatively compare the DNA methylation status of this region in fat and lean chicken lines of NEAUHLF, we calculated the methylation percentage of these seven CpGs for individual chickens. The Shapiro–Wilk test suggested that the samples followed a normal distribution ($P = 0.0537$). The 3×2 factorial analysis indicated that the *CEBPA* methylation level was significantly associated with the broiler lines ($P < 0.0001$) and was significantly higher in the lean compared with the fat broiler line

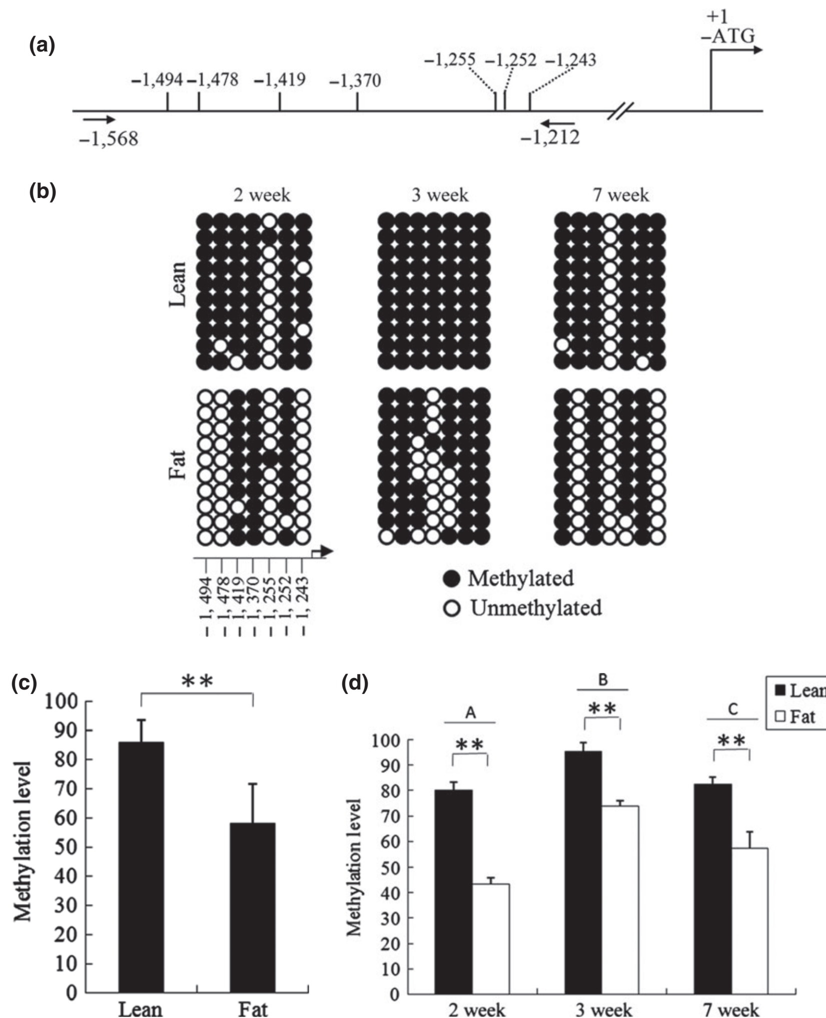

Figure 1 Comparison of CpG site methylation of chicken CEBPA promoter in the lean and fat chicken lines of NEAUHLF. (a) Schematic diagram of CEBPA gene promoter. The arrow pairs denote the positions of primers used for bisulfite sequencing PCR. Short vertical lines indicate the positions of the CpG sites in the CEBPA gene promoter. All numbered positions are relative to the adenine of the translation start site of chicken CEBPA. (b) Bisulfite genomic sequencing results of the selected promoter region of CEBPA gene in the lean and fat chicken lines at 2, 3 and 7 weeks of age (one bird per chicken line per time point shown as a representative). Ten clones per sample were randomly picked and sequenced. Each row represents one clone with one circle symbolizing one CpG site. The methylation status of each CpG site is aligned corresponding to its genomic order (represented at the bottom of the results for the lean line at 2 weeks of age). (c) The mean methylation levels of the selected CEBPA promoter region in adipose tissues of lean and fat broiler lines (mean ± SD). (d) The mean methylation levels of the selected CEBPA promoter region in adipose tissues of lean and fat broilers at 2, 3 and 7 weeks of age ($n = 5$, mean ± SD). The different uppercase letters above error bars indicate significant difference in CEBPA DNA methylation level among ages (GLM followed by Tukey's HSD test). Double asterisks (**) indicate significant difference in DNA methylation between the two chicken lines (GLM followed by *t*-test, $P < 0.01$).

© 2015 Stichting International Foundation for Animal Genetics,

($P < 0.0001$) (Fig. 1b, c). Additionally, the comparison between the two chicken lines at all three tested ages showed that DNA methylation levels of *CEBPA* were significantly higher in lean than fat chickens at 2 weeks (80.3% vs. 43.4%, $P < 0.0001$), 3 weeks (95.4% vs. 74.0%, $P < 0.0001$) and 7 weeks of age (82.6% vs. 57.2%, $P < 0.0001$) (Fig. 1d). In both chicken lines, DNA methylation levels varied with age in the abdominal adipose tissues and peaked at 3 weeks of age ($P < 0.0001$) (Fig. 1d). DNA methylation was also significantly associated with the interaction of line by age ($P = 0.0004$).

Further analysis showed that six of the seven CpGs analyzed were differentially methylated in the lean and fat chicken lines (Fig. 1b, Table 1). The CpGs at −1494, −1478 and −1243 bp were unmethylated in the fat line but predominantly methylated in the lean line at 2 weeks of age. The CpGs at −1419, −1370 and −1255 bp were more frequently methylated in the lean line than in the fat line at 3 weeks of age (Fig. 1b, Table 1). The CpGs at −1478 and −1243 bp were methylated in the lean line but mainly unmethylated in the fat line at 7 weeks of age (Fig. 1b, Table 1). Additionally, the CpG at −1370 bp was mainly methylated in the lean line at 2 and 3 weeks of age and in the fat line at 2 weeks of age but was unmethylated in the fat line at 3 and 7 weeks of age and in the lean line at 7 weeks of age (Fig. 1b, Table 1). The CpG at −1252 bp was predominantly methylated in both the lean and fat lines at all three tested ages (Fig. 1b, Table 1).

CEBPA expression in adipose tissues

To evaluate the degree of correlation between the CpG site methylation and mRNA expression of *CEBPA*, we also performed quantitative real-time RT-PCR expression analysis of *CEBPA* in the lean and fat broiler lines of NEAUHLF. The Shapiro–Wilk test suggested that the expression data did not follow a normal distribution ($P = 0.0037$), so we converted them into normally distributed data ($P = 0.2229$) using the MINITAB 16 Box–Cox. The 3×2 factorial analysis showed that the *CEBPA* expression level was not associated with line ($P = 0.1048$) or age ($P = 0.3143$) but was significantly associated with the interaction of line by age ($P = 0.0048$). *CEBPA* mRNA expression was higher in the fat line than in the lean line, but this difference did not reach statistical significance ($P = 0.0505$, Fig. 2a), probably due to the small sample size ($n = 5$ for each line per time point) and high variations in *CEBPA* expression among the tested samples. In both the lean and fat lines, no significant difference in *CEBPA* mRNA expression among the three tested ages was observed (Fig. 2b). However, comparison between the lean and fat lines showed that, at 2 weeks of age, *CEBPA* expression was significantly higher in the fat line than in the lean line ($P = 0.0013$, Fig. 2b), but no significant difference was observed at 3 and 7 weeks of age ($P = 0.9159$ and $P = 0.3709$ respectively Fig. 2b).

Based on the calculated methylation percentage and the quantitative real-time RT-PCR results for individual chickens, we performed a correlation analysis. The results revealed a significant negative correlation between promoter CpG site methylation and *CEBPA* mRNA expression at 2 weeks of age (Pearson's $r = -0.8312$, $P = 0.0029$) but not at 3 (Pearson's $r = 0.0695$, $P = 0.8960$) and 7 weeks of age (Pearson's $r = 0.4535$, $P = 0.3664$). Furthermore, we tested the correlation between each DNA methylation site and *CEBPA* gene expression. The results showed that, of all seven tested CpGs, two CpGs, the CpG at −1494 bp (Pearson's $r = -0.5713$, $P = 0.0050$) and the CpG at −1478 bp (Pearson's $r = -0.4890$, $P = 0.0290$), displayed a significantly negative correlation with *CEBPA* mRNA expression (Table 2).

Discussion

In the present study, we demonstrated that CpG site methylation in the chicken *CEBPA* promoter was significantly higher in lean lines than in fat lines at all three tested ages and that there was a significantly negative correlation between the CpG site methylation and *CEBPA* mRNA

Table 1 CpG site methylation status of the selected *CEBPA* promoter region in lean and fat lines of NEAUHLF.

		Mean methylation levels of each CpG site (%)[1]						
Line	Age (week)	−1494 bp	−1478 bp	−1419 bp	−1370 bp	−1255 bp	−1252 bp	−1243 bp
Lean	2	94 ± 1.10**	92 ± 0.89**	82 ± 1.67	96 ± 1.10	18 ± 1.67	98 ± 0.89	82 ± 1.67**
	3	98 ± 0.89	98 ± 0.89	96 ± 1.10**	88 ± 1.67**	94 ± 1.10**	98 ± 0.89	98 ± 0.89
	7	98 ± 0.89	96 ± 1.10	98 ± 0.89	12 ± 0.89	88 ± 1.67	98 ± 0.89	98 ± 0.89
Fat	2	14 ± 1.79**	6 ± 1.79**	84 ± 2.68	90 ± 2.00	12 ± 2.61	94 ± 1.79	4 ± 1.10**
	3	96 ± 1.10	96 ± 1.10	58 ± 1.67**	14 ± 2.28**	56 ± 1.79**	98 ± 0.89	98 ± 0.89
	7	94 ± 1.79	4 ± 1.10**	94 ± 1.79	8 ± 0.89	80 ± 1.41	96 ± 1.10	2 ± 0.89**

[1]The mean methylation levels for each CpG site at each time point was calculated by dividing the total number of methylated CpG sites by the total number of CpG sites (mean ± SE, $n = 5$). The seven tested CpG sites were located at −1494, −1478, −1419, −1370, −1255, −1252 and −1243 bp respectively upstream of the CEBPA translation start site.
**Significant difference in DNA methylation between the two chicken lines (Student's *t*-test, $P < 0.01$).

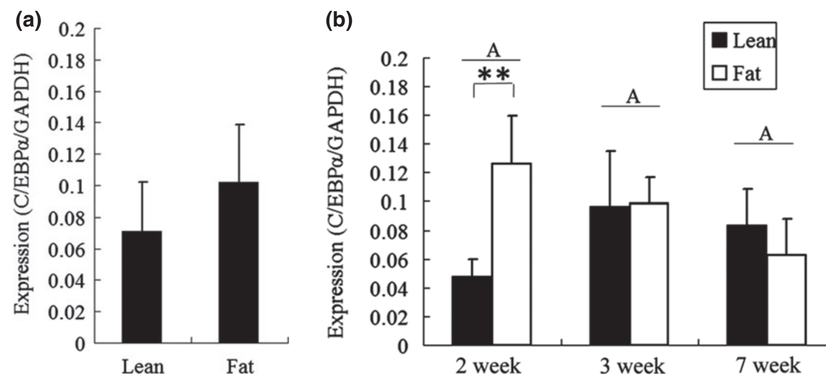

Figure 2 *CEBPA* expression in abdominal adipose tissues of the lean and fat chicken lines of NEAUHLF. (a) The mean expression levels of *CEBPA* in adipose tissues of lean and fat broilers ($n = 15$, mean ± SD). Columns with vertical bars represent the mean ± SD from 15 individual chickens. *CEBPA* expression seemed to be higher in the fat compared with the lean chicken line; however, this difference did not reach statistical significance (GLM followed by *t*-test, $P = 0.0505$). (b) The mean expression levels of *CEBPA* in adipose tissues of lean and fat broilers at 2, 3 and 7 weeks of age ($n = 5$, mean ± SD). Double asterisks (**) indicate significant difference in CEBPA mRNA expression between the two chicken lines (GLM followed by *t*-test, $P < 0.01$). The different uppercase letters above error bars indicate significant difference in CEBPA mRNA expression levels among the selected ages (GLM followed Tukey's HSD test, $P < 0.01$).

Table 2 Correlations between each single DNA methylation site and *CEBPA* gene expression.

	CpG sites						
	−1494 bp	−1478 bp	−1419 bp	−1370 bp	−1255 bp	−1252 bp	−1243 bp
Pearson's *r*	−0.5713	−0.4890	0.0788	0.2259	−0.1741	0.0856	−0.2940
P-value	0.0050	0.0290	0.7270	0.3120	0.4390	0.7050	0.1840

expression at 2 weeks of age. Previous *in vitro* studies in mice have shown that *CEBPA* is methylated and controls the balance between osteogenic and adipogenic differentiation of C3H10T1/2 cells and mouse BMSCs (Fan *et al.* 2009; Li *et al.* 2013; Zhao *et al.* 2013). *CEBPA* is differentially methylated in 3T3-L1 pre-adipocytes and adipocytes (Li *et al.* 2010), and a recent investigation showed that, compared with normal rat offspring, on postnatal day (PND) 21, the offspring of obese rat dam displayed decreased DNA methylation of CpG island shore in the key adipogenic transcription factor genes including *CEBPA* and *PPARG*, in concert with their increased expression. The offspring of obese rat dams gained more body weight and fat mass than normal rat offspring on PND56 but not on PND21 (Borengasser *et al.* 2013). A recent *in vitro* study in chicken showed that folate supplementation reduced CpG site methylation in *CEBPA* promoter and increased *CEBPA* gene expression in cultured adipocytes (Yu *et al.* 2014). Taken together with our results, these data suggest that, in spite of significant differences in adipogenesis between mammals and birds, DNA methylation may regulate *CEBPA* expression in both mammalian and avian adipogenesis and adipose development.

CEBPA and *PPARG* are master regulators of adipogenesis. Our previous study showed that *PPARG* promoter was differentially methylated and negatively correlated with its expression in the abdominal adipose tissues of the lean and fat chicken lines at 2, 3 and 7 weeks of age (Sun *et al.* 2014). In contrast with the findings on the correlation between chicken *PPARG* methylation and its gene expression, in the present study, we found only a significant negative correlation between the methylation percentage of CpG site methylation and *CEBPA* mRNA expression at 2, but not at 3 and 7, weeks of age. This correlation difference may be due to little difference in regulation between *CEBPA* and *PPARG*. Gene regulation is very complex and dynamic and involves epigenetic and genetic factors. Epigenetic factors, such as DNA methylation and histone modifications, and genetic factors simultaneously or sequentially regulate gene expression during development (Ponomarev *et al.* 2011; De Obaldia *et al.* 2013; Rao *et al.* 2013). A previous *in vitro* study showed that 3T3-L1 pre-adipocytes were highly sensitive to methylation inhibitors during the contact-inhibition stage but were less sensitive during late stages of adipogenesis (Guo *et al.* 2009), indicating that DNA methylation effect is stage specific in 3T3-L1 adipogenesis. Therefore, we hypothesize that *CEBPA* may be stage-specifically regulated by DNA methylation in chicken early adipose development.

We found that six of the seven CpGs investigated showed differential methylation between the lean and fat chicken lines of NEAUHLF. These six CpGs were predicted to be located within or around a number of transcription factor binding sites using TFSEARCH (http://www.cbrc.jp/

research/db/TFSEARCH.html). The CpG at −1478 was predicted to be within a binding site of p300, which has been shown to coactivate *CEBPA* and play indispensable roles in adipocyte differentiation (Erickson *et al.* 2001; Zhao *et al.* 2014). Further correlation analysis showed a significantly negative correlation between the methylation of CpG at −1478 and *CEBPA* mRNA expression. Taken together, it is reasonable to assume that methylation of the CpG at −1478 may regulate *CEBPA* expression by influencing the binding of p300 to *CEBPA* promoter. However, further studies should be carried out to test this hypothesis. A significantly negative correlation was also observed between the methylation of CpG at −1494 and *CEBPA* mRNA expression, but this CpG site was not predicted to be located within any known transcription factor binding sites using TFSEARCH and Mulan (http://mulan.dcode.org/). We speculate that the methylation of CpG at −1494 may recruit methyl-CpG-binding domain proteins (MBDs), which in turn recruit additional proteins, for example histone deacetylases (HDACs) and histone methyltransferases, and alter chromatin structure at the *CEBPA* promoter, thus leading to gene transcription repression.

In the present study, we analyzed only the methylation of a 357-bp region of *CEBPA* promoter, and our results may not reflect the overall methylation status of the *CEBPA* promoter. Therefore, to fully understand the epigenetic regulation of chicken *CEBPA* by DNA methylation, it is necessary to investigate the full-length *CEBPA* promoter methylation in future work.

In summary, we demonstrated that promoter CpG site methylation of *CEBPA* is significantly higher in lean lines than in fat lines and that the promoter CpG site methylation is negatively correlated with *CEBPA* expression at 2 weeks of age. Our results suggest that DNA methylation may regulate *CEBPA* expression at chicken early adipose development.

Acknowledgements

This work was supported by the National Basic Research Program of China (No. 2009CB941604), National 863 project of China (No. 2011AA100301), and China Agriculture Research System (No.CARS-42). The authors declare that they have no conflict of interest.

References

Annamaneni S., Kagita S., Gorre M., Digumarti R.R., Satti V. & Battini M.R. (2014) Methylation status of *CEBPA* gene promoter in chronic myeloid leukemia. *Hematology* **19**, 42–4.

Bird A. (2007) Perceptions of epigenetics. *Nature* **447**, 396–8.

Bock C., Reither S., Mikeska T., Paulsen M., Walter J. & Lengauer T. (2005) BIQ ANALYZER: visualization and quality control for DNA methylation data from bisulfite sequencing. *Bioinformatics* **21**, 4067–8.

Borengasser S.J., Zhong Y., Kang P., Lindsey F., Ronis M.J., Badger T.M., Gomez-Acevedo H. & Shankar K. (2013) Maternal obesity enhances white adipose tissue differentiation and alters genome-scale DNA methylation in male rat offspring. *Endocrinology* **154**, 4113–25.

De Obaldia M.E., Bell J.J., Wang X., Harly C., Yashiro-Ohtani Y., DeLong J.H., Zlotoff D.A., Sultana D.A., Pear W.S. & Bhandoola A. (2013) T cell development requires constraint of the myeloid regulator C/EBP-alpha by the Notch target and transcriptional repressor Hes1. *Nature Immunology* **14**, 1277–84.

Di Ruscio A., Ebralidze A.K., Benoukraf T. *et al.* (2013) DNMT1-interacting RNAs block gene-specific DNA methylation. *Nature* **503**, 371–6.

Ding N., Gao Y., Wang N. & Li H. (2011) Functional analysis of the chicken *PPARgamma* gene 5′-flanking region and *C/EBPalpha*-mediated gene regulation. *Comparative Biochemistry and Physiology. Part B, Biochemistry and Molecular Biology* **158**, 297–303.

Erickson R.L., Hemati N., Ross S.E. & MacDougald O.A. (2001) p300 coactivates the adipogenic transcription factor CCAAT/enhancer-binding protein alpha. *Journal of Biological Chemistry* **276**, 16348–55.

Fan Q., Tang T., Zhang X. & Dai K. (2009) The role of CCAAT/enhancer binding protein (C/EBP)-alpha in osteogenesis of C3H10T1/2 cells induced by BMP-2. *Journal of Cellular and Molecular Medicine* **13**, 2489–505.

Frommer M., McDonald L.E., Millar D.S., Collis C.M., Watt F., Grigg G.W., Molloy P.L. & Paul C.L. (1992) A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 1827–31.

Gopalakrishnan S., Van Emburgh B.O. & Robertson K.D. (2008) DNA methylation in development and human disease. *Mutation Research* **647**, 30–8.

Guo W., Zhang K.M., Tu K., Li Y.X., Zhu L., Xiao H.S., Yang Y. & Wu J.R. (2009) Adipogenesis licensing and execution are disparately linked to cell proliferation. *Cell Research* **19**, 216–23.

Hannum G., Guinney J., Zhao L. *et al.* (2013) Genome-wide methylation profiles reveal quantitative views of human aging rates. *Molecular Cell* **49**, 359–67.

Hermier D. (1997) Lipoprotein metabolism and fattening in poultry. *Journal of Nutrition* **127**, 805S–8S.

Klose R.J. & Bird A.P. (2006) Genomic DNA methylation: the mark and its mediators. *Trends in Biochemical Sciences* **31**, 89–97.

Li H. & Yang S. (1997) The influence of selection for plasma very low density lipoprotein concentration upon production performance in layer type chickens. *Journal of Northeast Agricultural University* **4**, 95–102.

Li H.X., Xiao L., Wang C., Gao J.L. & Zhai Y.G. (2010) Epigenetic regulation of adipocyte differentiation and adipogenesis. *Journal of Zhejiang University Science B* **11**, 784–91.

Li J., Zhang N., Huang X., Xu J., Fernandes J.C., Dai K. & Zhang X. (2013) Dexamethasone shifts bone marrow stromal cells from osteoblasts to adipocytes by *C/EBPalpha* promoter methylation. *Cell Death and Disease* **4**, e832.

Liu S., Wang S.Z., Li Z.H. & Li H. (2007) Association of single nucleotide polymorphism of chicken uncoupling protein gene with muscle and fatness traits. *Journal of Animal Breeding and Genetics* **124**, 230–5.

Liu S., Wang Y., Wang L., Wang N., Li Y. & Li H. (2010) Transdifferentiation of fibroblasts into adipocyte-like cells by chicken adipogenic transcription factors. *Comparative Biochemistry and Physiology. Part A, Molecular and Integrative Physiology* **156**, 502–8.

Livak K.J. & Schmittgen T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(–delta delta C(T)) method. *Methods* **25**, 402–8.

Matsubara Y., Sato K., Ishii H. & Akiba Y. (2005) Changes in mRNA expression of regulatory factors involved in adipocyte differentiation during fatty acid induced adipogenesis in chicken. *Comparative Biochemistry and Physiology. Part A, Molecular and Integrative Physiology* **141**, 108–15.

Matsubara Y., Endo T. & Kano K. (2008) Fatty acids but not dexamethasone are essential inducers for chick adipocyte differentiation *in vitro*. *Comparative Biochemistry and Physiology. Part A, Molecular and Integrative Physiology* **151**, 511–8.

Musialik E., Bujko M., Kober P. *et al.* (2014) Comparison of promoter DNA methylation and expression levels of genes encoding CCAAT/enhancer binding proteins in AML patients. *Leukemia Research* **38**, 850–6.

Ponomarev E.D., Veremeyko T., Barteneva N., Krichevsky A.M. & Weiner H.L. (2011) MicroRNA-124 promotes microglia quiescence and suppresses EAE by deactivating macrophages via the C/EBP-alpha-PU.1 pathway. *Natural Medicines* **17**, 64–70.

Rao K.N., Smuda C., Gregory G.D., Min B. & Brown M.A. (2013) Ikaros limits basophil development by suppressing *C/EBP-alpha* expression. *Blood* **122**, 2572–81.

Rishi V., Bhattacharya P., Chatterjee R., Rozenberg J., Zhao J., Glass K., Fitzgerald P. & Vinson C. (2010) CpG methylation of half-CRE sequences creates C/EBPalpha binding sites that activate some tissue-specific genes. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 20311–6.

Sun X.F., Sun Z.Y., Pan B., Li L. & Shen W. (2012) Alteration in methylation pattern of oncogene *Akt1* promoter region in bladder cancer. *Molecular Biology Reports* **39**, 5631–6.

Sun Y.N., Gao Y., Qiao S.P., Wang S.Z., Duan K., Wang Y.X., Li H. & Wang N. (2014) Epigenetic DNA methylation in the promoters of peroxisome proliferator-activated receptor gamma in chicken lines divergently selected for fatness. *Journal of Animal Science* **92**, 48–53.

Wu Z., Rosen E.D., Brun R., Hauser S., Adelmant G., Troy A.E., McKeon C., Darlington G.J. & Spiegelman B.M. (1999) Cross-regulation of C/EBP alpha and PPAR gamma controls the transcriptional pathway of adipogenesis and insulin sensitivity. *Molecular Cell* **3**, 151–8.

Yu X., Liu R., Zhao G., Zheng M., Chen J. & Wen J. (2014) Folate supplementation modifies CCAAT/enhancer-binding protein alpha methylation to mediate differentiation of preadipocytes in chickens. *Poultry Science* **93**, 1–8.

Zhang H., Hu X., Wang Z. *et al.* (2012) Selection signature analysis implicates the PC1/PCSK1 region for chicken abdominal fat content. *PLoS ONE* **7**, e40736.

Zhao Q.H., Wang S.G., Liu S.X., Li J.P., Zhang Y.X., Sun Z.Y., Fan Q.M. & Tian J.W. (2013) PPARgamma forms a bridge between DNA methylation and histone acetylation at the *C/EBPalpha* gene promoter to regulate the balance between osteogenesis and adipogenesis of bone marrow stromal cells. *FEBS Journal* **280**, 5801–14.

Zhao Y., Zhang Y.D., Zhang Y.Y. *et al.* (2014) p300-dependent acetylation of activating transcription factor 5 enhances C/EBPbeta transactivation of C/EBPalpha during 3T3-L1 differentiation. *Molecular and Cellular Biology* **34**, 315–24.