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Expression and functional analysis of Krüppel-like factor 2 in chicken adipose tissue[1]

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ABSTRACT: Studies in mammalian species showed that Krüppel-like factor 2 (KLF2) regulates adipogenesis. However, its role in birds is unclear. The objective of the current study was to explore the expression and function of *KLF2* in chicken adipogenesis. Results showed that chicken *KLF2* (*Gallus gallus KLF2* [*gKLF2*]) was greatly expressed in abdominal adipose tissue, and its transcripts fluctuated during adipose tissue development. In addition, *gKLF2* transcripts in abdominal adipose tissue of lean broilers were greater at 1 wk of age but lower at 3, 5, and 8 wk of age than those in fat broilers ($P < 0.05$). The *gKLF2* was more greatly expressed in preadipocytes than in mature adipocytes ($P < 0.05$), and its expression level decreased during the preadipocyte differentiation in vitro ($P < 0.05$). The functional analysis showed that gKLF2 overexpression inhibited chicken preadipocyte differentiation ($P < 0.05$), accompanied by the reduced expression of CCAAT/enhancer binding protein α (*C/EBPα*) and peroxisome proliferator-activated receptor γ (*PPARγ*) and the elevated expression of GATA binding protein 2 (*GATA2*). Additionally, the luciferase reporter assays showed that gKLF2 overexpression suppressed the promoter activities of chicken *C/EBPα* and *PPARγ* ($P < 0.05$). In conclusion, our results indicated that gKLF2 inhibits chicken adipogenesis, at least in part, through inhibition of *PPARγ* and *C/EBPα* expression.

Key words: adipogenesis, chicken, expression, Krüppel-like factor 2

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INTRODUCTION

Krüppel-like factor 2 (**KLF2**), initially described as lung-specific Krüppel-like factor (**LKLF**; Anderson et al., 1995), is a member of the Krüppel-like factor (**KLF**) family (Kaczynski et al., 2003). Human KLF2 contains an inhibitory and transactivating domains at the N terminus, and its inhibitory domain binds specifically to WW domain-containing E3 ubiquitin protein ligase 1 (**WWP1**), which attenuates its transactivation and promotes its degradation (Conkright et al., 2001; Zhang et al., 2004). Both mouse and human *KLF2* are greatly expressed in lung tissue, but with limited expression in heart, muscle, spleen, lymphoid, pancreas, adipose tissue, and other tissues (Anderson et al., 1995; Wani et al., 1999a; Su et al., 2004).

Deletion of *KLF2* in mice results in embryonic lethality between E11.5 and E13.5 (Wani et al., 1998). Chimeric mice derived from *KLF2*$^{-/-}$ embryonic stem cells demonstrated that KLF2 is essential for lung development (Wani et al., 1999b). In addition, KLF2 regulates T cell differentiation and the circulation of peripheral T cells (Kuo et al., 1997; Carlson et al., 2006). Overexpression of KLF2 inhibits 3T3-L1 preadipocyte differentiation, coupled with the downregulation of peroxisome proliferator-activated receptor γ (***PPARγ***), CCAAT/enhancer binding protein α (***C/EBPα***), and adipocyte determination- and differentiation-dependent factor 1/sterol regulatory element-binding protein-1c (***ADD1/SREBP-1c***; Banerjee et al., 2003). Studies in mouse embryonic fibroblasts derived from *KLF2*$^{-/-}$ mouse embryos showed that KLF2 does not affect the commitment of multipotent stem cells into the preadipocytic lineage but suppresses the transition from preadipocytes into adipocytes (Wu et al., 2005).

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Although *KLF2* has been extensively studied in mammals, little is known about its role in birds. We hypothesize that KLF2 may also play a role in chicken adipogenesis. In the current study, we investigated the expression characteristics and function of chicken *KLF2* (*Gallus gallus KLF2* [***gKLF2***]) in adipose tissue.

MATERIALS AND METHODS

Experimental Birds and Management

Animal work was conducted according to the guidelines for the care and use of experimental animals 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 (Harbin, P.R. China). In total, 108 male birds (fat line, n = 57, and lean line, n = 51) from the 14th generation population of Northeast Agricultural University broiler lines divergently selected for abdominal fat content (**NEAUHLF**) were used. Information regarding NEAUHLF has been published previously (Guo et al., 2011). Briefly, after 14 generations of divergent selection for abdominal fatness, the abdominal fat percentage of the fat broiler line at 7 wk of age was 4.45 times as that of the lean line. All birds were kept in similar environmental conditions and had free access to feed and water. 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 12 wk of age, all birds were fed a grower diet (3,000 kcal of ME/kg and 190 g/kg of CP).

Tissues

In total, 108 male birds (3–6 birds for each broiler line and for each age from 1 to 12 wk of age) were slaughtered after fasting for 6 h. At each week of age, the abdominal fat was collected after slaughtering. For the birds slaughtered at 7 wk of age, 14 other tissue samples, including liver, duodenum, jejunum, ileum, pectoralis, leg muscle, gizzard, heart, spleen, kidney, pancreas, proventriculus, brain, and testes, were also collected. After washing with 0.75% NaCl solution, all the tissues collected were snap-frozen in liquid nitrogen and stored at –80°C until RNA extraction.

Cloning of KLF2 *and Plasmid Construction*

Total RNA from chicken abdominal fat tissue was reverse transcribed using oligo(dT)-anchor primer and ImProm-II reverse transcriptase (Promega, Madison, WI). The full-length coding region of KLF2 was amplified by PCR using a set of primers, *gKLF2*-F1 (5′-ATGAATTCCCATGGCGCTGAGCGATAC-3′) and *gKLF2*-R1 5′-TTCTCGAGCTACATGTGCCGCTTCATGTG-3′), which were designed according to the predicted chicken *KLF2* sequences (GenBank accession XM_418264), and *Eco*RI and *Xho*I (underlined) were introduced, respectively. ExTaq (Takara, Dalian, China) was used to perform the PCR reaction. The amplified products were separated on a 0.8% agarose gel and the desired band was purified. The purified chicken *KLF2* cDNA was cloned into pMD-18T vector (Takara) and verified by direct sequencing. The DNAMAN software (version 6.0; Lynnon Biosoft, Quebec, Canada) was used for sequence analysis. The full-length coding region of *gKLF2* was released from the pMD-18T-*gKLF2* plasmid by treating with *Eco*RI and *Xho*I (Takara) and subcloned into pCMV-myc vector (Clontech, Palo Alto, CA) to gain the *KLF2*-overexpression vector, pCMV-myc-*gKLF2*.

Table 1. Primers used for quantitative reverse transcription--PCR

Gene[1]	Reference	Primers[2] (5′–3′)
GATA2	NM_001003797	F: aactgtggagcaaccgctac
		R: agtccgcaggcattacaaac
KLF2	JQ687128	F: ataccatcctgccctccttc
		R: ctgcccatggaaaggataaa
C/EBPα	X66844	F: agctcgacccgctgtac
		R: tgtctttttggatttgc
PPARγ	NM_001001460	F: caactcacttatggcta
		R: cttatttctgcttttct
GAPDH	NM_204305	F: ctgtcaaggctgagaacc
		R: gataacacgcttagcacca

[1]*GATA2* = GATA binding protein 2; *KLF2* = Krüppel-like factor 2; *C/EBPα* = CCAAT/enhancer binding protein alpha; *PPARγ* = Peroxisome proliferator-activated receptor gamma; *GAPDH* = glyceraldehyde 3-phosphate dehydrogenase.

[2]F = forward; R = reverse.

The plasmid of pGL3-basic-*PPARγ* (–1,978/–82) is a luciferase reporter plasmid of pGL3-basic (Promega) containing the chicken *PPARγ* promoter (nucleotides –1,978 to –82 bp, relative to the start site of AB045597.1), and the plasmid of pGL3-basic-*C/EBPα* (–1,863/+332) is a luciferase reporter plasmid of pGL3-basic (Promega) containing the chicken *C/EBPα* promoter (nucleotides –1,863 to +332 bp, relative to the start site of X66844.1).

Preparation of Stromal–Vascular Cell and Fat Cell Fractions and Chicken Preadipocyte Culture

Chicken stromal–vascular and fat cells were isolated according to the following procedure. First, abdominal fat tissue (3–5 g) was isolated from 12-d-old chickens, minced, and incubated with 2 mg/mL of collagenase I (Sigma-Aldrich, St. Louis, MO) for 1 h in a shaking water bath (180 rpm at 37°C). The suspension was then passed through a 100- and 600-μm nylon cell strainer (BD Falcon, New York,

NY) to remove undigested tissue. The filtrate was centrifuged (200 × *g* for 10 min at room temperature). The top layer (fat cell fraction) and the pellet (stromal–vascular cell fraction) were collected as chicken fat and stromal–vascular cells, respectively. The separated chicken stromal–vascular cells (chicken preadipocytes) were seeded at a density of 1×10^5 cells/cm^2 in Dulbecco's modified Eagle's medium (**DMEM**)/F12 medium (Gibco, New York, NY) supplemented with 10% fetal calf serum (Gibco) and maintained at 37°C in a humidified, 5% CO_2 atmosphere.

Until about 70 to 90% confluency (Day 3–4), cells were passaged and seeded into 6-well plates at a density of 1×10^5 cells/cm^2. After 12 h, when cells reached 60 to 80% confluency, they were transfected with pCMV-myc-*gKLF2* or empty vector (pCMV-myc) using the FuGENE HD transfection reagent (Roche, Mannheim, Germany) according to the manufacturer's recommendations. At 24 h after transfection, 160 μ*M* oleate, prepared by dissolving the sodium oleate (Sigma-Aldrich) in double distilled water, was added into the medium to induce preadipocyte differentiation for 48 h.

Oil Red O Staining

Oil red O staining of intracellular lipid droplets was performed in 6-well plates. Chicken preadipocytes induced to differentiate for 48 h were washed with PBS and fixed in 10% formaldehyde for 10 min. After rinsing with distilled water, they were stained with 0.5% oil red O working solution, prepared by vigorously mixing 3 parts of a stock solution (0.5% oil red O in isopropanol; Sigma-Aldrich) with 2 parts of water for 5 min and filtering through a 0.4-μm filter. Excess staining was removed by rinsing twice with PBS. The dye was extracted by isopropanol incubation for 15 min at room temperature. Quantitative assessment was obtained by spectrophotometric analysis of absorbance of a 3-fold dilution of the extracted dye at 500 nm.

Ribonucleic Acid Isolation and Quantitative Reverse Transcription-PCR

Total RNA of tissues (each 100 mg) and cells was extracted using Trizol (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Total RNA was treated with DNase I (Takara), and RNA quality was assessed by visualization of the 18S and 28S ribosomal RNA bands on denaturing formaldehyde agarose gel. Only the RNA with a 28S:18S ratio between 1.8 and 2.1 was used for reverse transcription. Reverse transcription was performed using 1 μg of total RNA, an oligo(dT) anchor primer, and ImProm-II reverse transcriptase (Promega). Reverse transcription conditions for each cDNA amplification were 25°C for 5 min, 42°C for 60 min, and 70°C for 15 min.

Quantitative reverse transcription (**RT-**) PCR was used to analyze gene expression, and the expression of glyceraldehyde 3-phosphate dehydrogenase (***GAPDH***) was used as the internal reference of target gene expression. Real-time PCR was performed using the SYBR Premix Ex Taq kit (Takara) on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). Part (1 μL) of each RT reaction product was amplified in a 20-μL PCR reaction system. Reaction mixtures were incubated in an ABI Prism 7500 sequence detection system (Applied Biosystems) programmed to conduct 1 cycle at 95°C for 30 s and 40 cycles at 95°C for 5 s and at 60°C for 34 s. 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 expression level of target gene to GAPDH was determined using $2^{-\Delta\text{cycle threshold value}}$ (**CT**) method, in which $\Delta C_T = C_T$ (target gene) – C_T (*GAPDH*). In addition, semiquantitative RT-PCR was performed using Ex Taq (Takara). The primers used for quantitative RT-PCR were designed to cross introns. The primer information is presented in Table 1. The conditions used for semiquantitative RT-PCR are shown in Table 2.

Luciferase Reporter Assays

The DF-1 cells were grown in DMEM/F12 medium (Gibco) supplemented with 10% fetal calf serum (Gibco) and plated in 12-well dishes. A fixed amount of total DNA (1 μg) was used to transfect cells in each well using the FuGENE HD transfection reagent (Roche). The components of transfection mixture and their quantity per well are listed in Table 3. After transfection and incubation for 48 h, the cells were lysed in 250 μL of 1x passive lysis buffer (Promega), and portions of the lysate were subjected to assays for Firefly and Renilla luciferase using the Dual-Luciferase Reporter Assay System (Promega). Promoter activities were expressed as the ratio of Firefly:Renilla luciferase activity.

Western Blot Assays

Chicken preadipocytes transfected with pCMV-myc-*gKLF2* or pCMV-myc plasmid for 2 d were lysed in radioimmunoprecipitation assay buffer (PBS, pH 7.4, containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor cocktail). The cell lysates were added into 5x denaturing loading buffer and boiled for 5 min. Cell lysates were separated on 5 to 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. After incubation with the primary antibody for myc-tag (1:200; Clontech) or chicken GAPDH (1:1,000; Beyotime, Beijing, China), a secondary horseradish peroxide-conjugated antibody was added. The BeyoECL Plus kit (Beyotime) was used for detection.

Table 2. Conditions for semiquantitative reverse transcription-PCR in DF1 cells

Gene[1]	Initial denaturation	Denaturation	Annealing	Extension	Cycle number	Final extension
GATA2	94°C for 7 min	94°C for 30 s	55°C for 30 s	72°C for 30 s	32	72°C for 7 min
C/EBPα	94°C for 7 min	94°C for 30 s	62°C for 30 s	72°C for 30 s	34	72°C for 7 min
KLF2	94°C for 7 min	94°C for 30 s	61°C for 30 s	72°C for 30 s	34	72°C for 7 min
PPARγ	94°C for 7 min	94°C for 30 s	60°C for 30 s	72°C for 30 s	36	72°C for 7 min
GAPDH	94°C for 7 min	94°C for 30 s	58°C for 30 s	72°C for 30 s	27	72°C for 7 min

[1]*GATA2* = GATA binding protein 2; *C/EBPα* = CCAAT/enhancer binding protein alpha; *KLF2* = Krüppel-like factor 2; *PPARγ* = Peroxisome proliferator-activated receptor gamma; *GAPDH* = glyceraldehyde 3-phosphate dehydrogenase.

Statistical Analyses

All data are presented as means plus SD. The Shapiro-Wilk test was used to test the normality of data. Comparison between 2 groups was performed by Student's *t* test. Statistical analyses among more than 2 groups were performed by PROC GLM procedure followed by the Duncan's multiple test, with the following models:

$$Y = \mu + A + L + (A \times L) + e \quad [1] \text{ and}$$

$$Y = \mu + F + e \quad [2].$$

Model [1] was used for tissue samples, in which Y is the dependent variable (*gKLF2* mRNA expression level), μ is the population mean, A is the broiler age as fixed effect, L is the broiler line as fixed effect, $A \times L$ as interaction of $A \times L$, and e is the random error. Model [2] was used for cell samples, in which Y is the dependent variable (the *gKLF2* expression level or promoter activity), μ is the population mean, F is various factors (time point of differentiation or concentration of the plasmid of pCMV-myc-*gKLF2*) as the fixed effect, and e is the random error. Differences were considered significant at $P < 0.05$ unless otherwise indicated. All analyses were performed using the SAS software system (version 9.2; SAS Inst. Inc., Cary, NC)

RESULTS

Cloning and Sequence Analysis of the Chicken KLF2

Cloning and sequencing showed that the full-length coding sequence of *gKLF2* was 1,143 bp long, encoding 380 AA, and the deduced protein sequence of *gKLF2* was identical to the chicken predicted KLF2 protein (GenBank accession: XP_418264.1; AA identity = 100%). The DNA sequence was submitted to GenBank database under accession number JQ687128. In addition, sequence analysis showed that gKLF2 protein sequence has a low similarity to its human (GenBank accession: NP_057354) and murine (GenBank accession: NP_032478) orthologs (<60% AA identity).

Tissue Expression of gKLF2

Real-time RT-PCR analysis of the tissue expression pattern of *gKLF2* in 7-wk-old broilers from NEAUHLF showed that *gKLF2* was expressed in all 15 chicken tissues tested. The *gKLF2* had a great expression level in abdominal fat tissues; a medium expression level in pancreas, gizzard, and spleen; and a low expression level in duodenum, brain, pectoralis, proventriculus, heart, ileum, kidney, jejunum, liver, leg muscle, and testes (Fig. 1). Additionally, the *gKLF2* transcripts in pectoralis, jejunum, leg muscle, and pancreas of fat males were significantly greater than those of lean males at 7 wk of age ($P < 0.05$; Fig. 1).

Expression Pattern of the gKLF2 *during Adipose Tissue Development*

Real-time RT-PCR was used to analyze the *gKLF2* expression in abdominal fat tissues of 1- to 12-wk-old broilers from NEAUHLF, and the results showed that *gKLF2* was expressed in all the chicken abdominal fat tissues tested. In addition, statistical analysis indicated that the relative *gKLF2* mRNA level (*gKLF2*:chicken *GAPDH* [*Gallus gallus GAPDH, gGAPDH*] ratio) in chicken abdominal fat tissue was significantly associated with the age of broilers ($P < 0.0001$). The *gKLF2* expression decreased at early stage of development (1 to 3 wk of age), reached its nadir at 3 wk of age, and increased slowly afterward (from 4 to 12 wk of age; $P < 0.01$; Fig. 2).

In addition, no significant association between the broiler line (selected by high and low abdominal fat content, respectively) and the relative *gKLF2* mRNA level (*gKLF2*:*gGAPDH* ratio) in chicken abdominal fat tissue was detected ($P = 0.5756$). However, the relative *gKLF2* mRNA level (*gKLF2*:*gGAPDH* ratio) in chicken abdominal fat tissue was significantly associated with the interaction of line × age ($P = 0.0002$). Comparison of *gKLF2* expression in the abdominal fat tissue between fat and lean broilers at each age showed that, at 1 wk of age, the *gKLF2* expression was significantly greater in lean males than in fat males, and at 3, 5, and 8 wk of age, the *gKLF2* expression was significantly lower in lean males than in fat males ($P < 0.05$; Fig. 2).

Table 3. The components of transfection mixtures for luciferase assay (per well)

Group[1]	Promoter reporter plasmids	pRL-TK[2]	The gKLF2 overexpression plasmid mixtures[3]
PPARγ	pGL3-basic-*PPARγ* (–1,985/–89), 400 ng	8 ng	600 ng
C/EBPα	pGL3-basic-*C/EBPα* (–2,214/–19), 200 ng	10 ng	800 ng

[1]*C/EBPα* = CCAAT/enhancer binding protein alpha; *PPARγ* = Peroxisome proliferator-activated receptor gamma.

[2]Promega, Madison, WI.

[3]The *gKLF2* (*Gallus gallus KLF2*) overexpression plasmid mixtures: Five different plasmid mixtures of pCMV-myc-*gKLF2* and pCMV-myc were used, and the mass ratios of pCMV-myc-*gKLF2*:pCMV-myc of these 5 plasmid mixtures were 3:0, 2:1, 1:1, 1:2, 0:3, respectively.

Characterization of gKLF2 *Expression during Preadipocyte Differentiation*

The chicken preadipocytes (stromal–vascular cell fraction) and mature adipocytes (fat cell fraction) were isolated from abdominal adipose tissue of broilers, and the *gKLF2* expression was analyzed using real-time RT-PCR. The result showed that *gKLF2* was expressed in both chicken preadipocytes and mature adipocytes, and its expression was significantly greater in preadipocytes than in mature adipocytes ($P < 0.01$; Fig. 3A). In addition, *gKLF2* expression decreased during the chicken preadipocyte differentiation induced by oleate in vitro ($P < 0.05$; Fig. 3B).

Overexpression of gKLF2 in Chicken Preadipocytes

The *gKLF2* mRNA expression was greater in preadipocytes than in mature adipocytes, suggesting that gKLF2 might play a potentially inhibitory role in chicken adipogenesis. To test this hypothesis, a gKLF2 overexpression plasmid, pCMV-myc-*gKLF2*, which could express chicken KLF2 in chicken preadipocytes (Fig. 4A and 4D), was constructed. Transfection assay showed that, compared with empty vector (pCMV-myc)-transfected chicken preadipocytes, gKLF2-overexpressing chicken preadipocytes exhibited a decrease in the intracellular lipid accumulation, as evidenced by oil red O staining (Fig. 4B) and a quantitative assessment ($P < 0.05$; Fig. 4C). Consistently, the expression levels of pro-adipogenic differentiation marker genes, *PPARγ* and *C/EBPα*, were decreased ($P < 0.05$) and the expression of anti-adipogenic differentiation marker gene GATA binding protein 2 (***GATA2***) was increased ($P < 0.05$; Fig. 4D). Our results provide evidence that KLF2 inhibits chicken adipogenesis.

Effect of gKLF2 on the Transcription of Chicken C/EBPα, PPARγ, *and* GATA2

To understand the mechanism that gKLF2 inhibits chicken adipocyte differentiation, we investigated the effect of gKLF2 on the transcription of chicken *C/EBPα*, *PPARγ*, and *GATA2* using luciferase reporter assay and semiquantitative RT-PCR in DF1 cells. Luciferase reporter assay showed that gKLF2 overexpression significantly suppressed chicken *PPARγ* (–1,978/–82) and *C/EBPα* promoter activities (–1,863/+332) and its suppressing effect was dose dependent ($P < 0.05$; Fig. 5A). Semiquantitative RT-PCR analysis showed that in the DF1 cells gKLF2 overexpression decreased endogenous *PPARγ* expression and increased endogenous *GATA2* expression (Fig. 5B). Unfortunately, the expression of *C/EBPα* was not detected in DF1 cells (Fig. 5B), so the effect of gKLF2 overexpression on the endogenous *C/EBPα* expression in DF1 cells was not observed.

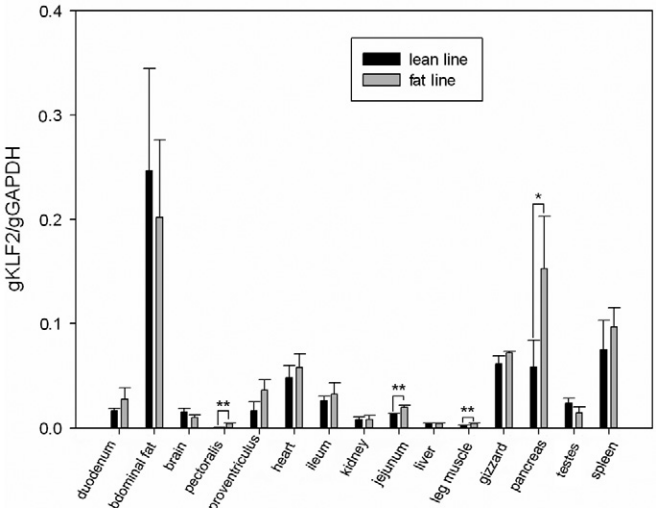

Figure 1. Tissue expression characterization of chicken Krüppel-like factor 2 (*Gallus gallus KLF2* [*gKLF2*]) in 7-wk-old broilers. The *gKLF2* mRNA expression in various tissues of 7-wk-old Northeast Agricultural University broiler lines divergently selected for abdominal fat content (NEAUHLF) male broilers (each line $n = 3$) was detected by real-time reverse transcription-PCR. Chicken glyceraldehyde 3-phosphate dehydrogenase (*Gallus gallus GAPDH* [*gGAPDH*]) was used as the reference gene. The diagram shows the relative quantification of *gKLF2* expression in the designated tissues. Error bars represent the SD of 3 replicates. Asterisks indicate significant difference between fat and lean broilers (Student's *t* test): $*P < 0.05$ or $**P < 0.01$.

DISUSSION

Krüppel-like factors in mammalian species are encoded by a large gene family (Kaczynski et al., 2003). There are also many *KLF*-like genes in the chicken genome demonstrated by gene prediction; however, to date, few of them have been studied. In the current study, for the first time, the existence of the chicken *KLF2* was validated by cloning and sequencing. In addition, sequence analysis showed that chicken KLF2 protein sequence has a low similarity to its human and murine orthologs.

The tissue expression analysis showed that, similar to the expression pattern of human and mouse *KLF2* (Su et al., 2004), *gKLF2* was expressed in many different

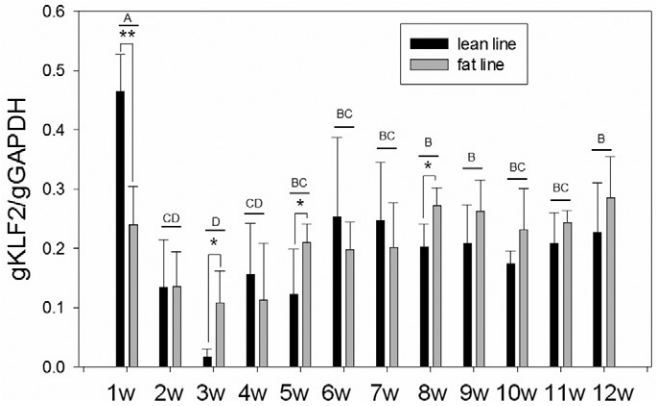

Figure 2. Expression pattern of chicken Krüppel-like factor 2 (*Gallus gallus KLF2* [*gKLF2*]) during chicken abdominal fat tissue development. The *gKLF2* expression in abdominal fat tissues of male broilers were analyzed using real-time reverse transcription-PCR. Chicken glyceraldehyde 3-phosphate dehydrogenase (*Gallus gallus GAPDH* [*gGAPDH*]) was used as the internal control. The diagram shows the relative quantification of *gKLF2* mRNA expression during adipose development. Error bars represent the SD of several biological replicates (each age, each line $n = 3$ to 6). Asterisks indicate significant difference between fat and lean broilers (Student's t test): $^{*}P < 0.05$ and $^{**}P < 0.01$. $^{A–C}$The different uppercase letters above columns indicate significant difference among various ages (Duncan's multiple test, $P < 0.01$). 1w to 12w = 1 to 12 wk of age.

chicken tissues. However, unlike human and mouse *KLF2*, *gKLF2* had a great expression level in the adipose tissues. Real-time RT-PCR analysis showed that *gKLF2* was expressed consecutively in chicken abdominal adipose tissue from 1 to 12 wk of age and its expression levels significantly associated with the age of broilers, suggested that KLF2 regulate the development and growth of chicken abdominal adipose tissue. The *gKLF2* transcripts decreased at early stage of abdominal adipose tissue development (1 to 3 wk of age) and *gKLF2* mRNA expression level was greater in lean broilers than in fat broilers at 1 wk of age, suggesting that gKLF2 play a negative role at the early stage of chicken adipose tissue development. The *gKLF2* transcripts increased slowly afterward at the late stage of chicken abdominal adipose tissue development (from 4 to 12 wk of age), and the *gKLF2* expression level was greater in fat broilers than in lean broilers at 3, 5, and 8 wk of age, suggesting that gKLF2 might also play roles in the late developmental stages of chicken abdominal adipose tissue. *In vivo* gene expression is dynamic and complex and affected by many factors such as age. It is hard to explain why the *gKLF2* mRNA expression level of abdominal fat tissues was significantly greater in lean broilers than in fat broilers at 1 wk of age but significantly lower in lean broilers than in fat broilers at 3, 5, and 8 wk of age. These data suggest that gKLF2 may play a role in chicken adipose development and contribute to difference in fat traits between the lean and fat chicken lines.

In addition, unlike mouse *KLF2*, which is only expressed in preadipocytes (Banerjee et al., 2003), *gKLF2* was expressed in both chicken preadipocytes and mature adipocytes. These results shed light that the function of gKLF2 in adipose tissues might be more complex than that of its human and mouse orthologs.

Both *gKLF2* and mouse *KLF2* have the decreased expression pattern during preadipocyte differentiation in vitro, and similar to mouse KLF2, gKLF2 overexpression inhibited chicken preadipocyte differentiation. These results indicated that, consistent with the previous reports in mammalian species (Banerjee et al., 2003; Wu

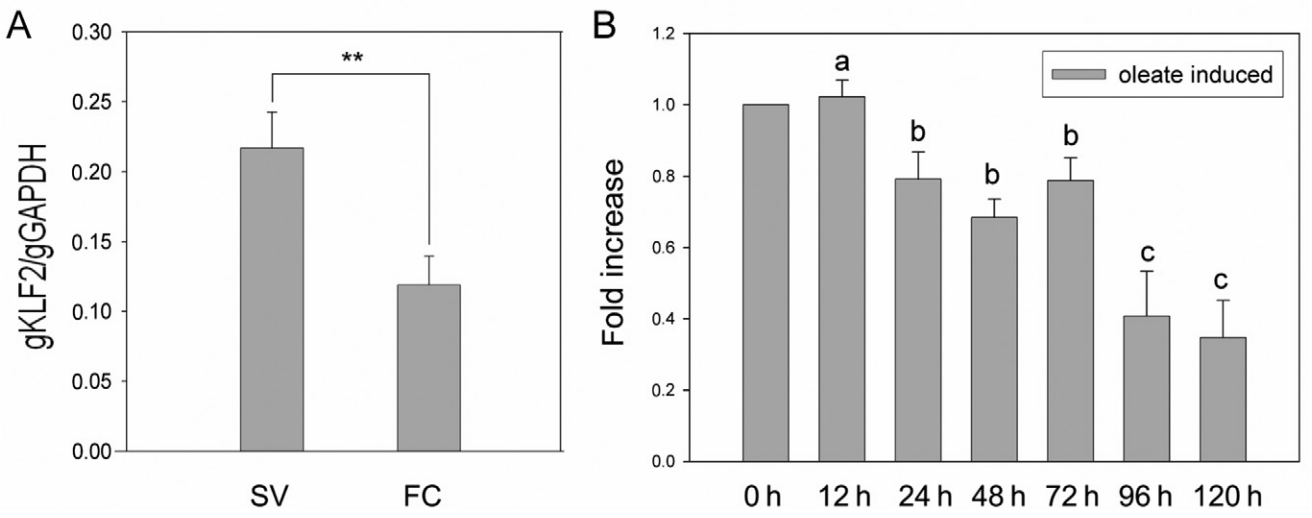

Figure 3. The chicken Krüppel-like factor 2 (*Gallus gallus KLF2* [*gKLF2*]) expression during chicken adipocyte differentiation. The *gKLF2* expression was measured by real-time reverse transcription-PCR. Chicken glyceraldehyde 3-phosphate dehydrogenase (*Gallus gallus GAPDH* [*gGAPDH*]) was used as the internal control. The diagram shows the relative quantification of *gKLF2* expression, Error bars represent SD of 3 replicates. (A) The *gKLF2* expression in chicken preadipocytes and mature adipocytes. Chicken preadipocytes (stromal–vascular cell fraction [SV]) and mature adipocytes (fat cell fraction [FC]) were isolated from abdominal adipose tissue of broilers ($n = 8$) at 12 d of age. Asterisks indicate significant difference (Student's t test, $^{**}P < 0.01$). (B) The *gKLF2* expression pattern during preadipocyte differentiation. Chicken preadipocytes were induced into differentiation by oleate in vitro. Preadipocytes were harvested at the designated time points after treatment with oleate. Untreated cell control, which was harvested at 0 h, was designated as control group. $^{a–c}$The different lowercase letters above columns indicate significant differences among various time points (Duncan's multiple test, $P < 0.05$).

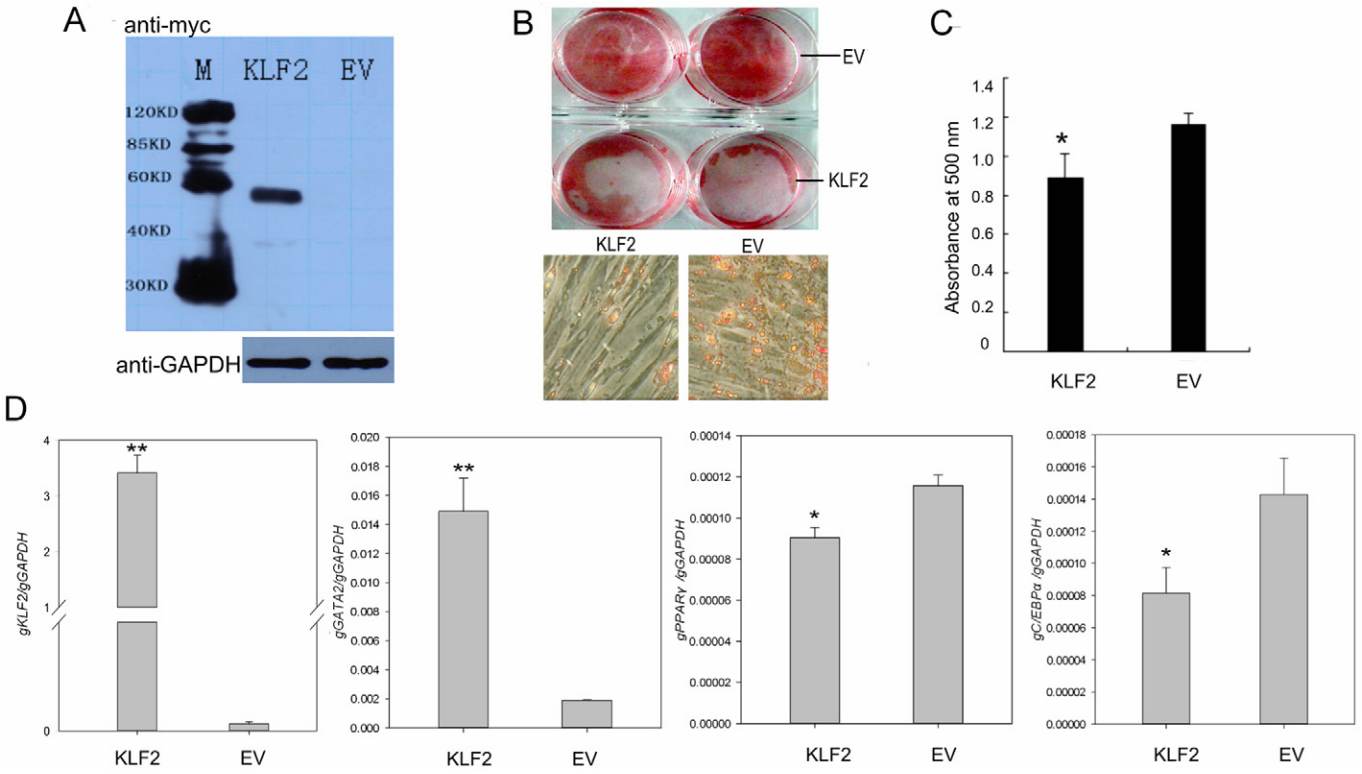

Figure 4. Effect of chicken Krüppel-like factor 2 (*Gallus gallus* KLF2 [gKLF2]) overexpression on chicken preadipocyte differentiation. One day after transfection with pCMV-myc-*gKLF2* (Krüppel-like factor 2 [KLF2]) and pCMV-myc (empty vector [EV]), respectively, chicken preadipocytes were induced into differentiation by oleate for 48 h. (A) Western blot analysis of chicken *KLF2* (*Gallus gallus* Krüppel-like factor 2 [*gKLF2*]) in chicken preadipocytes transfected with pCMV-myc-*gKLF2* and pCMV-myc. (B) Oil red O staining of chicken preadipocytes transfected with pCMV-myc-*gKLF2* and pCMV-myc. (C) The lipid content of chicken preadipocytes measured by absorbance at 500 nm. The diagram shows the absorbance at 500 nm of the extracted oil red O from the preadipocytes transfected with pCMV-myc-*gKLF2* and pCMV-myc, respectively; error bars represent the SD of 3 replicates. (D) Expression analysis of adipogenesis marker genes using real-time reverse transcription-PCR. Chicken glyceraldehyde 3-phosphate dehydrogenase (*Gallus gallus GAPDH* [*gGAPDH*]) was used as the internal control. The diagram shows the quantification of adipogenesis marker gene expression, error bars represent the SD of 3 replicates. Asterisks indicate significant differences (Student's *t* test): $*P < 0.05$ and $**P < 0.01$. The *gGATA2*, *gPPARγ*, and *gC/EBPα* represent chicken GATA binding protein 2, chicken Peroxisome proliferator-activated receptor γ, and chicken CCAAT/enhancer binding protein α, respectively.

et al., 2005), chicken KLF2 is also a negative regulator of preadipocyte differentiation.

Adipogenesis is a complex developmental process involving the coordinated interplay of numerous transcription factors (Lefterova and Lazar, 2009). In addition, PPARγ and C/EBPα are 2 master positive regulators of adipogenesis (Farmer, 2006; Rosen and MacDougald, 2006; Lefterova and Lazar, 2009). In the current study, we found that, similar to mouse KLF2 (Banerjee et al., 2003), gKLF2 overexpression suppressed chicken *PPARγ* promoter activity. Additionally, gKLF2 overexpression suppressed chicken *C/EBPα* promoter activity. These results were consistent with that of gene expression pattern of *C/EBPα* and *PPARγ* in the gKLF2-overexpressing chicken preadipocytes, indicating that gKLF2 inhibits chicken preadipocyte differentiation by repressing the expression of chicken *PPARγ* and *C/EBPα*.

Quantitative RT-PCR analysis showed that gKLF2 overexpression increased *GATA2* mRNA expression in chicken preadipocytes and DF1 cells, which is not observed in other species. Our previous study has showed that GATA2 overexpression suppresses chicken *PPARγ* transcription in DF1 cells (Zhang et al., 2012). Therefore, there are 2 possible ways for gKLF2 to inhibit preadipocyte differentiation. First, like mouse KLF2, gKLF2 directly binds to the promoter of *PPARγ* and represses the expression of *PPARγ* and *C/EBPα*, leading to the inhibition of preadipocyte differentiation. Alternatively, gKLF2 indirectly represses the expression of *PPARγ* by increasing the expression of *GATA2*, a known negative regulator of adipogenesis (Tong et al., 2000), resulting in the inhibition of preadipocyte differentiation.

Notably, the *C/EBPa* promoter is active in DF1 cells and responsive to gKLF2; however, *C/EBPa* expression was not detected in DF1 cells. The discrepancy may be due to the difference in chromatin structure of *C/EBPa* promoter between the *C/EBPa* reporter plasmid and chromosome. The *C/EBPa* promoter reporter plasmid does not have similar chromatin structure with the chromosome; for example, it does not have histones, so it may not completely reflect functionality of *C/EBPa* promoter. Alternatively, it is also likely that the *C/EBPa* promoter may be genetically or epigenetically altered in DF1 cells; for example, the *C/EBPa* promoter may be

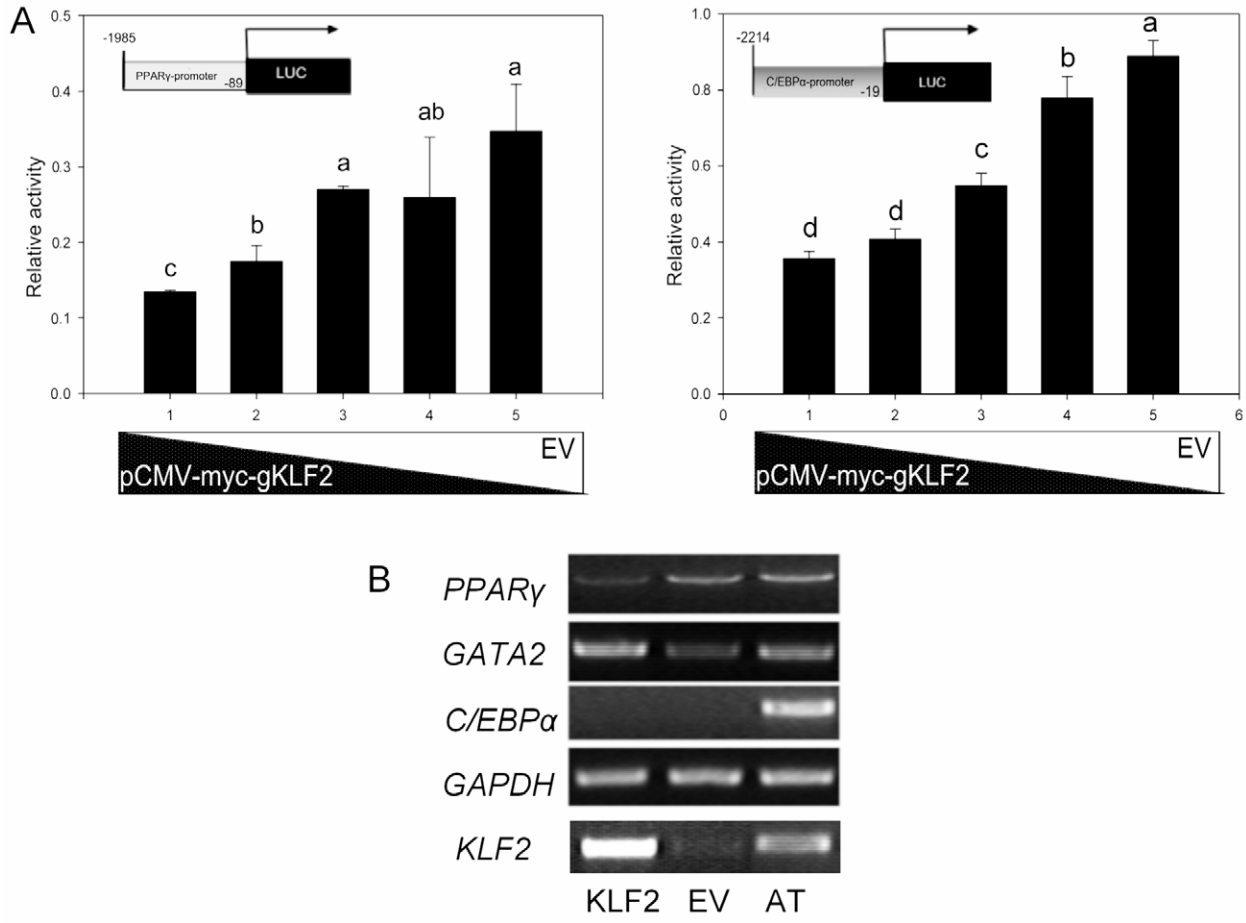

Figure 5. Effects of Krüppel-like factor 2 [KLF2] overexpression on the transcription of chicken peroxisome proliferator-activated receptor γ (*PPARγ*), CCAAT/enhancer binding protein α (*C/EBPα*), and GATA binding protein 2 (*GATA2*). (A) Effects of chicken KLF2 (*Gallus gallus* KLF2 [gKLF2]) overexpression on the promoter activities of *PPARγ* and *C/EBPα*. The diagrams show the quantification of promoter activities (ratios of Firefly:Renilla luciferase activity). Error bars represent the SD of 3 replicates. The mass ratios of pCMV-myc-*gKLF2*:pCMV-myc (empty vector [EV]) in the 5 plasmid mixtures (designated as 1 to 5) were 3:0, 2:1, 1:1, 1:2, 0:3, respectively. LUC=Luciferase . [a–d]The different lowercase letters above the histograms indicate significant differences (Duncan's multiple test, $P < 0.05$). (B) Semiquantitative reverse transcription-PCR analyses of the *PPARγ* (cycle number: 36), *C/EBPα* (cycle number: 34), *GATA2* (cycle number: 32), *KLF2* (cycle number: 34), and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*; cycle number: 27) expression in DF1 cells. KLF2: cells transfected with pCMV-myc-*gKLF2*; EV: cells transfected with pCMV-myc. AT = chicken abdominal adipose tissue.

lost due to chromosome aberration and gene mutation, resulting in the loss of *C/EBPa* expression. However, it is also possible that this discrepancy may be due to other unknown factors; the exact molecular mechanism needs further investigation.

In summary, we demonstrated that gKLF2 inhibits the chicken preadipocyte differentiation, at least in part, through direct or indirect inhibition of the expression of *PPARγ* and *C/EBPα*.

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