

Transcriptomics Analysis of Daheng Broilers Reveals that PLIN2 Regulates Chicken Preadipocyte Proliferation, Differentiation and Apoptosis

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Abstract

Intramuscular fat content, an important meat quality trait, strongly affects flavor, juiciness, and tenderness. Sex hormones regulate lipid metabolism, and female hormones stimulate fat deposition, thereby making the female chickens always fatter than males. In this study, the effect of sex on IMF deposition was screened following transcriptomics in chickens. Results confirmed significantly higher IMF content of 150-day female chickens as compared to the male chickens. The female chickens manifested higher serum TG, LDL-C, and VLDL, and significantly lower HDL-C contents than male chickens. Moreover, differential expression of genes involved in lipid metabolism were obtained in the muscle and liver between female and male chicken, which could partly interpret the possible reasons for the sex-mediated differences of IMF content. Cellular results revealed that inhibition of *PLIN2* significantly inhibited chicken preadipocyte proliferation and induces apoptosis of preadipocytes, as well as promoted adipocyte differentiation.

Introduction

During the past few decades, a notable increase has been experienced in the demand for poultry meat (Petracci et al. 2015). As far as nutrition is concerned, poultry meat, particularly breast meat, quench the demand of the modern consumer for a high degree of unsaturation fatty acids and low fat, sodium, and cholesterol levels (Petracci and Cavani 2012). Nowadays, the importance of meat quality in the poultry industry could be validated by the enhanced sale of chicken meat as parts or further-processed products (Li et al. 2019). Intramuscular fat (IMF) refers to the amount of lipids deposited within the muscle, including myometrium, myofibrils, and endometrium. It mostly comprises triglycerides (TGs), phospholipids, and cholesterol (Liu et al. 2019). Among the meat quality traits, the major role is played by the content of IMF owing to its strong effect on flavor, juiciness, and tenderness (Zerehdaran et al. 2004; Hocquette et al. 2010; Warner et al. 2010). Previous observations substantiated the impact of sex hormones in regulating lipid metabolism, and female hormones stimulated fat deposition are responsible for the fact that female chickens are always fatter than males (Gawrieh 2015). Importantly, higher IMF content is always associated with higher plasma lipid and fatty acid metabolism related gene expression (Saez et al. 2009; Chen et al. 2017).

Transcriptome encompasses the pool of all gene transcripts transcribed from a tissue or cell at a certain developmental stage or functional state (Yin et al. 2019). With expanded knowledge on the extent and complexity of transcriptomes, RNA sequencing (RNA-Seq) has been established as one of the powerful next-generation sequencing techniques (Wang et al. 2011). Thus far, RNA-Seq has elucidated the mechanism of lipid deposition in bull (Jeong et al. 2013), sheep (Miao et al. 2015), Squabs (Ye et al. 2016), and pig (Ramayo-Caldas et al. 2012). Many candidate genes attributed to chicken lipid metabolism have been identified, such as *fatty acid binding protein (FABP)* (Shi et al. 2010), *peroxisome proliferator-activated receptor- γ (PPAR γ)* (Royan and Navidshad 2016), *follicle-stimulating hormone receptor (FSHR)* (Cui et al. 2012a). Although, previous studies have analyzed the transcriptome of chicken breast muscle (Cui et al. 2012b), and identified some potential candidate genes. However, the molecular

mechanisms underlying chicken IMF deposition have not been fully elucidated and further validation need to be performed.

The present study was conducted to: (1) demonstrate the differences of IMF content and serum lipid level between male chickens and female chickens; (2) identify candidate genes involved in the lipid metabolism through transcriptome analysis of muscle and liver tissues between female chickens and male chickens; (3) explore the function of the differentially expressed gene between female chickens and male chickens in chicken primary intramuscular preadipocytes.

Materials And Methods

Animals

A total of 200 1-day-old female and male Da-Heng broilers were reared in the Sichuan Da-Heng Poultry Breeding Company (Chengdu, China) with one cage for each bird. All birds were simultaneously enrolled for the experiment and were kept under the same management system with free access to feed and water at all times. The experiments were conducted following national institutes of health guide for the care and use of laboratory animals. At the rearing period of 150 days of age, 3 female chickens and 3 male chickens were randomly selected for sample collection. Before slaughtering, birds were deprived of feed overnight (12 h) but provided with water. All the experiments were performed in accordance with the principles and procedures outlined by Sichuan Agricultural University's Animal Care and Use Committee.

Sample collection

A total of 2 mL of blood was drained from the brachial vein. After breathing anesthesia until unconsciousness, the birds were sacrificed by cutting the carotid arteries. After slaughter, roughly 1–2 g of pectoral muscle samples (on the left side) and liver were rapidly sampled and immediately snap-frozen using liquid nitrogen and stored at -80°C until they were analyzed using RNA-seq technology. To determine the intramuscular fat content, the left breast muscles were quickly frozen at -20°C . Meanwhile, 13 tissues of three female chicken were also collected and immediately snap-frozen in liquid nitrogen. All tissues were stored at -80°C until RNA isolation.

Serum Lipid Measurement and IMF content determination

The blood was incubated at room temperature for 1 h. Thereafter, serum was separated by centrifugation at 1500 g/min for 15 min. The serum was stored at -80°C in glass tubes to prevent oxidative degradation. The levels of serum triglycerides (TG), total cholesterol (TC), high density lipoprotein (HDL), low-density lipoprotein (LDL), and very low-density lipoprotein (VLDL) were estimated by colorimetric enzymatic methods with the help of commercial kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

For the determination of IMF content, breast muscles were homogenized individually in a meat grinder after being thawed. About 20 g of muscle were weighed and dried at 105°C for 12 h, followed by cooling

in a desiccator for 12 h. The IMF contents in breast muscles were measured by placing dried samples into a Soxhlet extraction device (VELP Scientifica, SER 148, Usmate, Italy) and extracted for 24 h. The results were expressed as percentages.

RNA extraction and transcriptome analysis

The RNA-Seq analysis involved three animals from female chickens and male chickens. Total RNA was isolated from the tissues and cells with the help of TRIzol total RNA extraction kit (Invitrogen, Carlsbad, USA) following the manufacturer's instructions. Agilent 2100 Bioanalyzer (Agilent Technologies) and agarose gel electrophoresis were conducted to validate the integrity of RNA. The purity of RNA was determined by the NanoPhotometer spectrophotometer (Thermo Scientific). Only qualified RNA samples were considered acceptable for sequencing.

FastQC v0.10.1 was adopted to estimate the quality of the raw data. Removal of adapter sequences and low-quality sequences would clean the raw data, which were then mapped to the reference genome of Gallus 5.0 using HISAT v2.2.4. Differentially expressed genes (DEGs) were identified by comparing the fragments per kilobase per million reads (FPKM) of two groups using DESeq2. The genes with a false discovery rate (FDR) < 0.05 and fold changes ≥ 2 or ≤ 0.5 ($|\log_2 FC| \geq 1$) were considered differentially expressed genes (DEGs). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment of the DEGs was explored using the DAVID database (<http://david.abcc.ncifcrf.gov/>) (Dennis et al. 2003). The calculated p-value was gone through FDR Correction, taking FDR ≤ 0.05 as a threshold.

cDNA synthesis and real-time qRT-PCR analysis

The PrimeScript™ RT Reagent Kit was employed for the reverse transcription of total RNA. Primers used for quantitative real-time (qRT-PCR) were listed in Table 1. The qRT-PCR was conducted in a CFX-96 (Bio-Rad, Inc., Richmond, CA, USA) qRT-PCR system using a 10 μ L reaction volume with 1 μ L of cDNA, 0.5 μ L of the forward and reverse primers (10 μ M) for each gene, 5 μ L of TB Green Premix Ex Taq (Tli RNase H Plus) (TaKaRa), and 3 μ L of double-distilled H₂O. All mRNA expression levels were normalized to GAPDH mRNA level, thereafter computing the mean expression level in each group.

Chicken primary intramuscular preadipocyte isolation, culture, and induction of differentiation

Chicken primary intramuscular preadipocytes were isolated from two-week-old Daheng female chickens using the method described by Zhang et al., with some modifications (Zhang et al. 2018). In brief, breast muscle tissues were collected under sterile conditions, followed by washing with phosphate-buffered saline (PBS) supplemented 1% penicillin-streptomycin (Solarbio, Beijing, China). The washed tissue was minced into 1-mm³ pieces using surgical scissors and digested for 1.5 h using a mixture of collagenase type I and collagenase type II (Solarbio, Beijing, China). After filtering the digested cell suspension with 200- and 500-mesh screens, centrifugation was done at 1000 rpm for 10 min. After adherence for 2 h, intramuscular adipocytes were plated onto a 60-mm culture plate at a density of 1×10^5 cells/mL. Cells were expanded in Dulbecco's modified Eagle medium (DMEM; Gibco, Australia),

supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), and 1% penicillin-streptomycin. On reaching 80–90% confluency, cells were exposed to an induced differentiation medium consisting of growth medium supplemented with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 μ M dexamethasone (DEX), 10 μ g/mL insulin (INS), and 300 μ M oleate (dissolved in DMSO) (Sigma).

Cell transfection

Cultivated in 6-well plates, cells were transfected with *PLIN2*-siRNAs (sense: GCUGGUGAUGUCUACGAAATT; antisense: UUUCGUAGACAUCACCAGCTT) (Sangon Biotech, Shanghai, China) when the fusion degree of myoblasts reached 70%. The Lipofectamine 3000 Reagent (Invitrogen, USA) was used as a transfection reagent, and cells were harvested after 48 h.

Cell proliferation and apoptosis assays

Cell counting kit-8 (CCK-8) and 5-Ethynyl-20-Deoxyuridine (EdU) assays were used to evaluate the cell proliferation activity. For the CCK-8 assay, the cells were seeded in 96-well plates, and after 0, 12, 24, 36, and 48 h of transfection, 10 μ L of cck-8 (Bestbio, Shanghai, China) was added and incubated for 2 h at 37 °C, and the absorbance was measured using a Model 680 Microplate Reader (Bio-Rad, Hercules, CA, USA) by optical density at a wavelength of 450 nm. For the EdU assay, cells were seeded in 96-well plates and transfected when they reached to a density of approximately 50%. After transfection for 48 h, the cells were exposed to 50 μ M EdU (Ribobio, Guangzhou, China) for 2 h at 37 °C. After that, the cells were fixed and stained with Apollo and Hoechst 33,342 according to the manufacturer's instructions. Finally, we used a fluorescence microscope (OLYMPUS, Tokyo, Japan) to capture three fields which randomly selected and the number of stained cells was counted by the Image-Pro Plus 6.0 software. Additionally, to evaluate the effect of *PLIN2* on apoptosis, Apoptosis and Necrosis Assay Kit (Beyotime; catalog number: C1056) was used following the manufacturer's instructions.

Oil red O staining and quantification

The muscle tissues were sectioned with a freezing microtome, fixed with 4% paraformaldehyde for 15 min, stained with Oil Red O (Servicebio, Wuhan, China) for 10 min at room temperature and then visualized under a microscope. Cells were washed with PBS three times and fixed with 10% formaldehyde for 30 min. Each slice within each group was randomly selected 3 images in the field of vision. For cellular Oil Red O staining, cells were washed with PBS twice and fixed with 10% formaldehyde for 20 min. After washing with isopropanol for 5 min, the fixed cells were incubated with Oil Red O solution for 30 min. The images were collected at three points in the field by a digital microscope (BA400Digital, China). Subsequently, Oil Red O was eluted with 100% isopropanol and quantified by a microplate reader (Thermo Fisher Scientific) at 510 nm.

Western blot

Cells were collected and proteins were extracted and the concentration was evaluated by a bicinchoninic acid (BCA) protein assay kit (BestBio, Shanghai, China). Then, proteins were subjected to SDS

polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The PVDF membrane was incubated at room temperature with 5% defatted milk powder for 1 h and incubated overnight at 4 °C, with primary antibodies specific for anti-CDK2 (Abcam), anti-Bcl-2 (Affinity), anti-C/EBP β (Immunoway), anti- β -actin (Affinity). Subsequently, the PVDF membrane was washed three times with washing buffer and incubated with HRP-labeled secondary antibody (Santa Cruz Biotechnology Inc., Heidelberg, Germany) for 1 h at room temperature. Finally, results were observed using the chemiluminescence (ECL) system (Beyotime, Shanghai, China) and was analyzed by ImageJ software (National Health Institute, Bethesda, MD, USA).

Statistical analysis

All statistical analyses were performed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Data were presented as least square mean \pm standard error of the mean (SEM) based on at least three replicates for each treatment. * $P < 0.05$; ** $P < 0.01$.

Results

IMF content and serum lipid parameters

The amount of IMF was estimated by Oil Red O staining the neutral lipids in breast muscle with the aim to visualize the differences in IMF deposition of male and female chickens. Our results revealed that the density and size of lipid droplets in the female chicken group were higher as compared to those of the male chicken group. The IMF content reflected a sex-dependent pattern with significantly higher levels in 150-day female chickens ($P < 0.05$) (Fig. 1A). Besides, the estimated serum lipid parameters (TG, TC, HDL-C, LDL-C, and VLDL-C) highlighted significantly higher serum TG, LDL-C, and VLDL-C ($P < 0.05$), whereas significantly lower HDL-C ($P < 0.01$) in the female chickens than the male chickens (Fig. 1B, D, E, F, G). However, there was no statistical difference in TC between 150-day males and females (Fig. 1C).

Differentially expressed genes analysis

RNA-Seq experiment derived overall 83.14 Gb clean data and 597.82 million reads. Aligned with the reference genome, the mapping frequencies were found to vary from 82.38% to 93.53% for each sample. Among the mapped reads, an average of 82.66% of the total mapped reads was mapped to exons, 7.38% mapped to introns, and 9.96% mapped to the intergenic regions.

The DESeq2 program detected a total of 1281 transcripts as DEGs in the breast muscle tissue between male chickens and female chickens, of which 219 transcripts were upregulated, and 1062 transcripts were down-regulated in female chickens (Fig. 2A). In the liver tissue, a total of 598 DEGs were identified, of which 360 transcripts were upregulated and the remaining 238 transcripts were down-regulated in female chickens (Fig. 2B). The lipid metabolism-related DEGs in the breast muscle and liver were summarized in Table S1 and Table S2, respectively. Among the male and female chickens, 58 DEGs were common in the breast muscle and liver. Among these common DEGs, 10 genes, including *ETNK2*, *PLIN2*,

OSBPL10, *MFSD2A*, *SLC51B*, *GCNT2*, *ALDH7A1*, *CPT1A*, *LSS*, and *MSMO1*, were relevant for lipid transport and metabolism. Among the DEGs, lipid biosynthetic process and lipid storage-related genes in pectoralis muscle tissue include *ACSL1*, *MSMO1*, *HACD4*, *SLC27A1*, *LSS*, *LPIN1*, *DGAT2*, and *APOB* that were upregulated in female chickens, indicating a significant role in lipid deposition of female chickens. As compared to the male chicken group, the expression of *PLCZ1*, *LPIN2*, and *DDHD2*, associated with lipid or fatty acid catabolic process, was significantly lower in the female chickens. Moreover, significant down-regulation of *NR4A3* and *PPARA*, which participate in positive regulation of fatty acid oxidation, was witnessed in female chickens. Furthermore, some genes involved in long-chain fatty acid transport (*THBS1*, *PLIN2*), phospholipid transport (*PLTP*, *MFSD2A*, *OSBPL5*), cholesterol transport (*LIPG*, *ABCA1*), were significantly down-regulated in female chickens. These findings suggested the probable association of these genes with the higher IMF content in the female chickens compared with that in the male chickens. In terms of DEGs in the liver, up-regulation was evident in the genes belonging to the PPAR signaling pathway, such as *ACOX1*, *ACOX3*, *ACAA1*, *CPT1A*, *FABP1*, and *FABP3*, in the female chickens. Additionally, significantly higher expression of the fatty acid biosynthetic process-related genes, including *HADHA*, *ELOVL2*, *ABHD2*, *ABHD3*, *ABDH6*, and *ABCD3*, was observed than that in the female chicken group. However, the female chicken group manifested down-regulation of the genes involved in the steroid biosynthetic process (*DHCR24*, *SQLE*, *NSDHL*, *CYP51A1*, *LSS*, *MVD*, *CYP17A1*, *HSD17B7*, *DHCR7*, *MSMO1*). **KEGG pathway analysis of the DEGs**

The significantly enriched pathways obtained from the KEGG pathway analysis, conducted based on the known DEGs, are illustrated in Fig. 3. We identified 238 DEGs annotated into 174 pathways in FBM vs. MBM (Fig. 3A) and 197 DEGs annotated into 160 pathways in FL vs. ML (Fig. 3B). Since the homeostasis between synthesis, transport, and degradation of lipids regulates the IMF content (Zhang et al. 2017), we focused mainly on the pathways directly involved in lipid metabolism. The breast muscle and liver of female and male chickens witnessed five common significantly enriched pathways (metabolic pathways, fatty acid degradation, fatty acid biosynthesis, glycolysis/gluconeogenesis, and PPAR signaling pathway) ($P < 0.05$). Moreover, the DEGs in the liver tissue were also found to be significantly enriched in steroid biosynthesis, steroid hormone biosynthesis, fatty acid metabolism, citrate cycle, and five amino acid-related metabolism pathways ($P < 0.05$).

3.4 Gene expression validation of DEGs by qRT-PCR

The results of the RNA-Seq analysis were further confirmed by validating the expression data of randomly selected ten DEGs. Results revealed expression of five DEGs (*APOB*, *SLC27A1*, *DGAT2*, *PLTP*, *LIPG*) in the muscle, whereas five DEGs (*CPT1A*, *ACAA1*, *FABP3*, *DHCR24*, *MSMO1*) were expressed in the liver. Consistency of the qRT-PCR result with the RNA-Seq data with a correlation coefficient of 0.986 authenticated the accuracy of RNA-Seq (Fig. 4).

3.5 *PLIN2* promoted intramuscular preadipocyte proliferation, differentiation, and inhibited apoptosis

PLIN2, a common DSG in the breast muscle and liver, was studied to further explore the biological significance of candidate genes. The tissue expression patterns of *PLIN2* were determined by analyzing

13 tissues of female chickens using qRT-PCR. High expression of *PLIN2* in the liver, subcutaneous fat, and abdominal fat indicated its potential role in the lipid metabolism of chicken (Fig. 5). Transfection with siRNA knocked down the *PLIN2* expression, which helped to elucidate the regulatory role of *PLIN2* in chicken intramuscular preadipocyte proliferation, apoptosis and differentiation. Compared with the negative control (NC) group, transfection with siRNA significantly downregulated the mRNA expression level of *PLIN2* (Fig. 6A).

To explore the role of *PLIN2* on chicken intramuscular preadipocyte proliferation, we detected the mRNA expression of cell-proliferation-related genes, including Cyclin D1 (*CCND1*), Cyclin D2 (*CCND2*), cyclin dependent kinase 2 (*CDK2*), proliferating cell nuclear antigen (*PCNA*) and a marker of proliferation Ki-67 (*Ki67*). Results showed that the mRNA expression levels of *CCND1*, *CCND2*, *CDK2*, *PCNA* and *Ki67* were all significantly decreased after transfected with si-*PLIN2*, compared with the negative control (NC) group (Fig. 6B). The protein expression level of *CDK2* was also detected by western blotting and the result was consistent with the qRT-PCR results (Fig. 6C). Furthermore, CCK-8 results showed that the cell vitality of the si-*PLIN2* transfected group was significantly lower than that of the NC group after 12, 24, 36, and 48h of transfection (Fig. 6D). EdU assay results showed that the percentage of EdU positive cells were significantly reduced in si-*PLIN2* transfected group (Fig. 6E and F).

To investigate the role of *PLIN2* in chicken intramuscular preadipocyte differentiation, we examined the mRNA expression levels of the adipogenic transcripts *PPAR γ* , *FABP4*, *C/EBP α* and *C/EBP β* . Results showed that mRNA expression levels of these genes were all downregulated after transfected with si-*PLIN2* (Fig. 7A). The protein expression level of *C/EBP β* was consistent with the qRT-PCR results (Fig. 7B). Besides, oil red O staining corroborated a decrease in the number of intracellular lipid droplets in the siRNA-*PLIN2* group (Fig. 7C), and the OD₅₁₀ values of the siRNA-*PLIN2* group were also significantly lower as compared to that of the NC group (Fig. 7D). Cumulatively, these results indicated *PLIN2* promoted intramuscular preadipocyte proliferation, differentiation, and inhibited apoptosis.

To further investigate whether *PLIN2* could also modulate preadipocyte apoptosis, the mRNA expression levels of crucial mediators of apoptosis, including *caspase-3*, *caspase-8* and *Bcl-2* were examined. We found the mRNA expression levels of *caspase-3*, *caspase-8*, *caspase-9* were upregulated and *Bcl-2* mRNA and protein expression levels were downregulated after transfected with si-*PLIN2* (Fig. 8A and B). The Apoptosis and Necrosis Assay results suggested that the interference with *PLIN2* significantly increased preadipocyte apoptosis when compared with the NC group (Fig. 8C and D).

Discussion

IMF content is predominant among the several factors affecting meat quality. IMF deposition is influenced by an important factor- gender, and it is generally witnessed that the content was higher in female chicken than that of male chicken (Guo-Bin et al. 2010; Yang et al. 2015). In accordance with previous studies, the results of the present study also demonstrated a sex-dependent significantly higher IMF content in 150-day female Daheng broilers than male chickens. The equilibrium between lipid

synthesis, transport, uptake, and catabolism determines the lipid deposition (Yan et al. 2015). In mammals, adipocytes are the major site for de novo synthesis of fatty acids, whereas, in the case of chicken, lipid biosynthesis mainly takes place in the liver (Leveille et al. 1975). After being synthesized in the liver, fatty acids are exported to muscle and adipose tissue by the peripheral vascular system (Zhuo et al. 2015). Thus, chicken IMF accumulation depends on the transport and uptake of blood lipids as well as subsequent lipogenesis in the muscle (Wang et al. 2012). Serum lipid parameters (TG, TC, HDL-C, LDL-C, and VLDL-C) were estimated between 150-day female chickens and male chickens. According to Dominique et al., a two-fold higher plasma concentration of VLDL was evident in the fat chicken line than in the lean chicken line (Dominique et al.). It is documented that serum VLDL can be considered as an indirect indicator for fat deposition (Hermier 1997). Moreover, the fat deposition rate was also significantly correlated with the serum TG concentration, and greater TG concentration was always associated with higher IMF in the breast muscle of chickens (Chen et al. 2017). The present study revealed significantly higher contents of VLDL and TG concentration in the female chicken as compared to that of the male chicken, which signified an augmented circulating lipid flux. Homeostasis between de novo synthesis, dietary intake, and metabolism is reflected by the total body cholesterol (TC) pool (Griffin and Lichtenstein 2013). LDL and HDL are responsible for cholesterol transport in the blood. Blood cholesterol is delivered throughout the body by LDL-C, and HDL-C could recycle cholesterol from the body tissues back to the liver, which turns it into bile and excretes it through the gastrointestinal tract (P.O. et al. 2016). Therefore, it is rational to infer that higher LDL-C concentration and lower HDL-C concentration in the female chickens could affect increased lipid deposition in pectoralis muscle tissue.

The key role played by IMF content in multiple quality traits establishes it as an important index for superior-quality chicken breeding (Ye et al. 2014). To elucidate the molecular regulation of IMF deposition, the expression profile of breast muscle and liver between female and male chickens was investigated. In poultry, lipid synthesis predominates in the liver, and the IMF content primarily relies on the net balance among lipid synthesis, transport, uptake, and catabolism (Yan et al. 2015). Numerous key genes and metabolic pathways are involved in these processes. The differential deposition mechanism of IMF in different breeds and ages has been addressed in previous studies (Liu et al. 2017; Zhang et al. 2018). The present study screened the DEGs associated with lipid metabolism in breast muscle and liver between female chicken and male chickens, with the goal to unearth the molecular mechanisms affecting IMF in different genders.

Previous studies have documented the liver as the site for more than 70% of the de novo synthesis of fatty acids for poultry and the critical role it plays in lipid synthesis, degradation, and transport (Leveille et al. 1968). These findings motivated the scientists to comprehend the genetic difference in hepatic lipid metabolism (Li et al. 2016; Li et al. 2015). Fatty acid-binding proteins (FABPs) have attracted interest from many researchers owing to their diverse biological functions, including fatty acid oxidation (Gan et al. 2015), transport (Storch and Thumser 2000), and lipid storage (He et al. 2013). *FABP1*, *FABP3*, and *FABP4* were widely reported as vital candidate genes for IMF deposition in chicken (Gao et al. 2015; Li et al. 2015). According to Li et al., the liver of laying hens manifested over-expression of *FABP1* and *FABP3* levels compared to the juvenile hens to meet the requirement of laying eggs (Li et al. 2015). This was in

agreement with the findings of the present study that also revealed significant up-regulation of both *FABP1* and *FABP3* in the liver of female chickens compared with male chickens, thereby signifying their role in promoting lipid metabolism in the PPAR signaling pathway in female chickens. Meanwhile, over-expression was also noted for *APOB*, another lipid transport-related gene, in female chickens than in male chickens (Lee et al. 1996). The female chicken group also witnessed up-regulation of some fatty acid biosynthesis-related genes, including *ELOVL2* (Morais et al. 2009), *HSD3B1* (Lavoie and King 2009). Furthermore, among all the significant DEGs in breast muscle tissue, several functional genes have been recognized as candidate genes for IMF, such as *ACSL1* (Widmann et al. 2011), *DGAT2* (Cui et al. 2011), and *CPT1A* (Qiu et al. 2017).

The present study confirmed the involvement of a number of potential genes affecting IMF. However, their molecular mechanisms are still ambiguous. Here, we selected a common DEG in breast muscle and liver to explore the molecular mechanism in IMF deposition. At the cellular level, variability in IMF content is fundamentally correlated to the number and size of intramuscular adipocytes, differentiated by intramuscular preadipocytes (Hocquette et al. 2010). The present study tried to ascertain the role of *PLIN2* in the process of intramuscular preadipocyte proliferation, differentiation, and apoptosis. Perilipins 2 (*PLIN2*), a member of the perilipin family, is a major group of lipid droplets-associated proteins with both structural and regulatory functions (Brasaemle 2007). Research has documented the role of *PLIN2* in lipid droplet accumulation in other species (Bildirici et al. 2018; Tsai et al. 2017; Dahlhoff et al. 2013), while the effect of the *PLIN2* gene on lipid metabolism in chicken preadipocytes is rarely reported. In the present study, knockdown of *PLIN2* significantly inhibited the expression of proliferation-related genes (*CCND1*, *CCND2*, *CDK2*, *PCNA* and *Ki67*) and inhibited the cell vitality and reduced the proliferating cells ratio. These results suggested that *PLIN2* could promote chicken intramuscular preadipocyte proliferation by regulating factors involved in the control of cell proliferation.

During the process of precursor adipocytes differentiated into mature fat cells, lipid droplets gradually formed and get larger (Li and Raza 2020). Adipogenic differentiation related marker genes include *PPAR γ* , *FABP4*, *C/EBP α* and *C/EBP β* (Li and Raza 2020). After interfere of *PLIN2*, the expression of these key genes all decreased. Studies have shown a key role for *C/EBP β* in regulating adipocytes differentiated (Raciti et al. 2018). Here, *C/EBP β* protein expression level also decreased in si-*PLIN2* group. Besides, a decreased lipid accumulation in the si-*PLIN2* group was also highlighted from the Oil Red O staining results. These results suggest that *PLIN2* could promote chicken intramuscular preadipocyte differentiation.

Caspase-3 and *caspase-8* are crucial mediators of apoptosis (Porter and Jänicke 1999; Kruidering and Evan 2000; Würstle et al. 2012). Expectantly, *caspase-3* and *caspase-8* expressions increased in intramuscular preadipocytes transfected with the si-*PLIN2*, strongly suggesting that *PLIN2* inhibits apoptosis in intramuscular preadipocytes. *Bcl-2*, a central player in the genetic program of eukaryotic cells that favors survival and inhibits apoptosis, can block p53-dependent apoptosis (Chiou et al. 1994). Using qRT-PCR and western blot, we found that *PLIN2* promoted *Bcl-2* mRNA and protein expression in chicken intramuscular preadipocytes. Besides, the interfere with *PLIN2* significantly increased

preadipocyte apoptosis when compared with the NC. These results suggest that *PLIN2* could inhibit chicken intramuscular preadipocyte apoptosis.

Conclusion

Findings from this study concluded significantly higher IMF content of 150-day female chickens in comparison to that of male chickens. The female chickens also demonstrated higher serum TG, LDL-C, and VLDL, and significantly lower HDL-C than male chickens. Furthermore, DEGs involved in lipid metabolism in muscle and liver were identified between female chicken and male chickens, which could partially interpret possible reasons for the differences in IMF content. Cellular results indicates that *PLIN2* could promote chicken IMF deposition by promoting intramuscular preadipocyte proliferation, differentiation, and inhibiting apoptosis. Therefore, *PLIN2* may be considered as a molecular marker for poultry meat quality and applying this gene in early breed selection.

Declarations

Data availability

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation. All the data are available in the SRA database with accession number PRJNA732829.

Author Contributions

Conceptualization, J.L. and C.Y.; methodology, J.L. and P.R.; software, J.L. and Z.L.; investigation, J.L., C.Y., D.Z. and L.W.; resources, C.Y. and X.J.; writing—original draft preparation, J.L.; writing—review and editing, C.Y., X.J., P.R. and Y.L.; supervision, X.J. and Y.L.; project administration, Y.L.; funding acquisition, Y.L. and C.Y. All authors have read and agreed to the published version of the manuscript.

Ethics Statement

The animal study was reviewed and approved by the Ethics Committee for Animal Experiments of Sichuan agricultural university.

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Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation. All the data are available in the SRA database with accession number PRJNA732829.

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Supplemental Tables

Tables S1 and S2 are not available with this version.

Figures

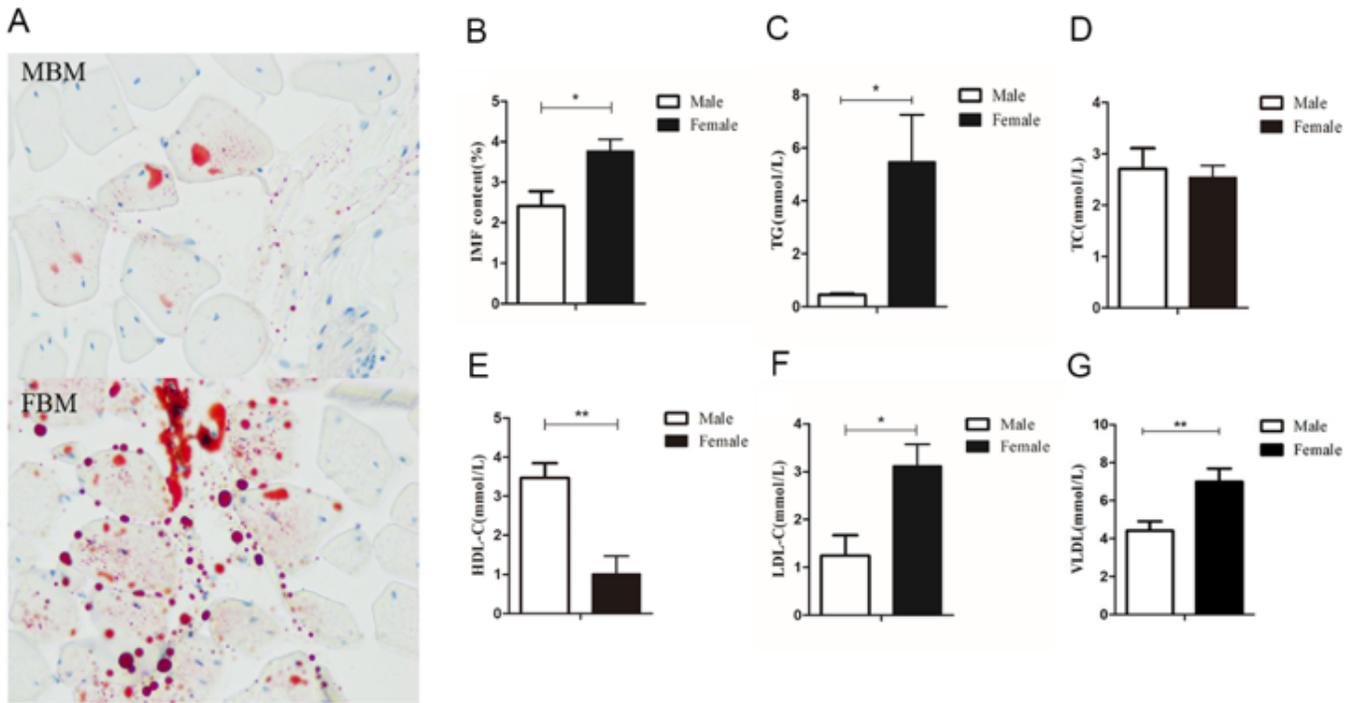


Figure 1

IMF content and serum lipid measurement. (A) Oil Red O staining for neutral lipids in breast muscle of male chicken and female chicken (400× magnification). (B) IMF content of male chicken and female chicken. (C, D, E, F, G) The levels of serum triglycerides (TG), total cholesterol (TC), high density lipoprotein (HDL), low density lipoprotein (LDL) and very low density lipoprotein (VLDL) between male chicken and female chicken, respectively. *p < 0.05; **p < 0.01

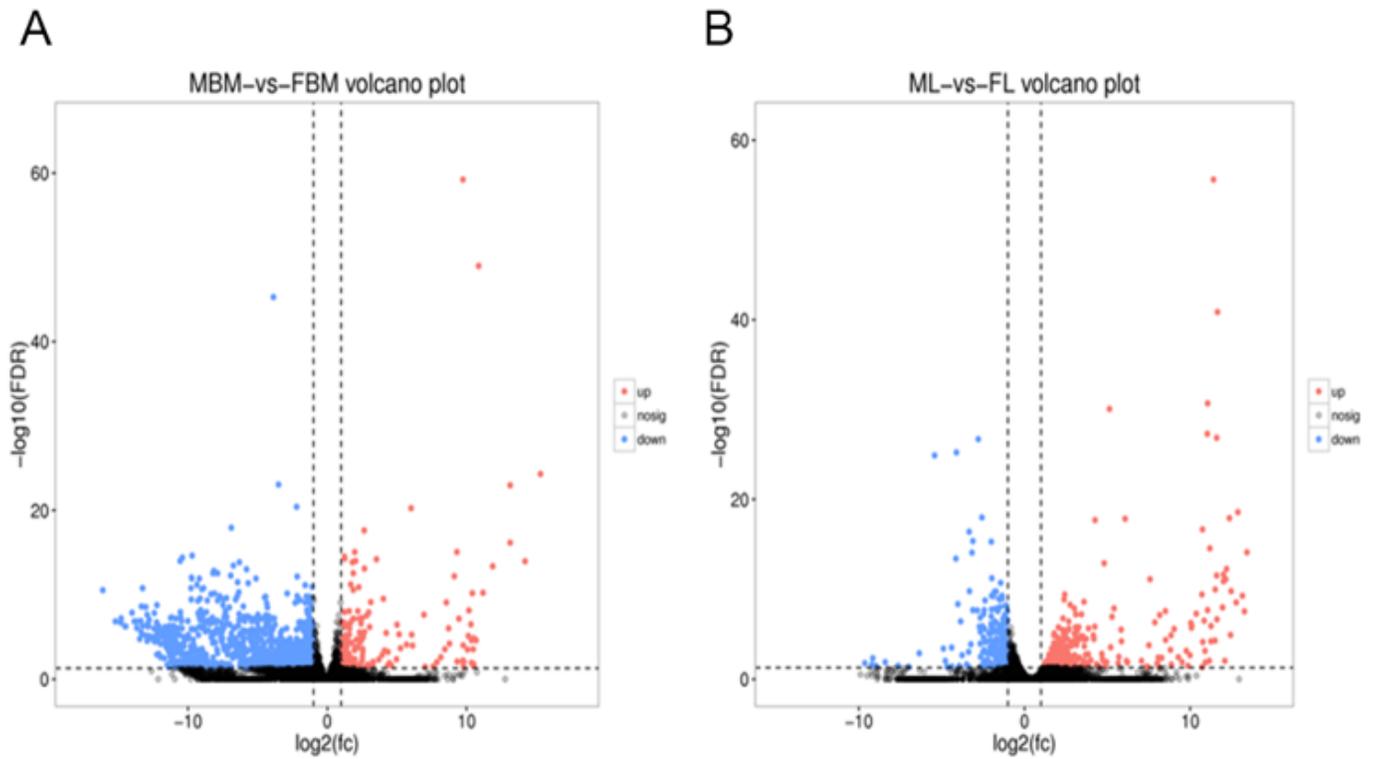


Figure 2

(A) Differentially expressed volcano diagram and up-down regulation in breast muscle of male chicken and female chicken. (B) Differentially expressed volcano diagram and up-down regulation in liver tissue of male chicken and female chicken. MBM: breast muscle of male chicken; FBM: breast muscle of female chicken; ML: liver of male chicken; FL: liver of female chicken.

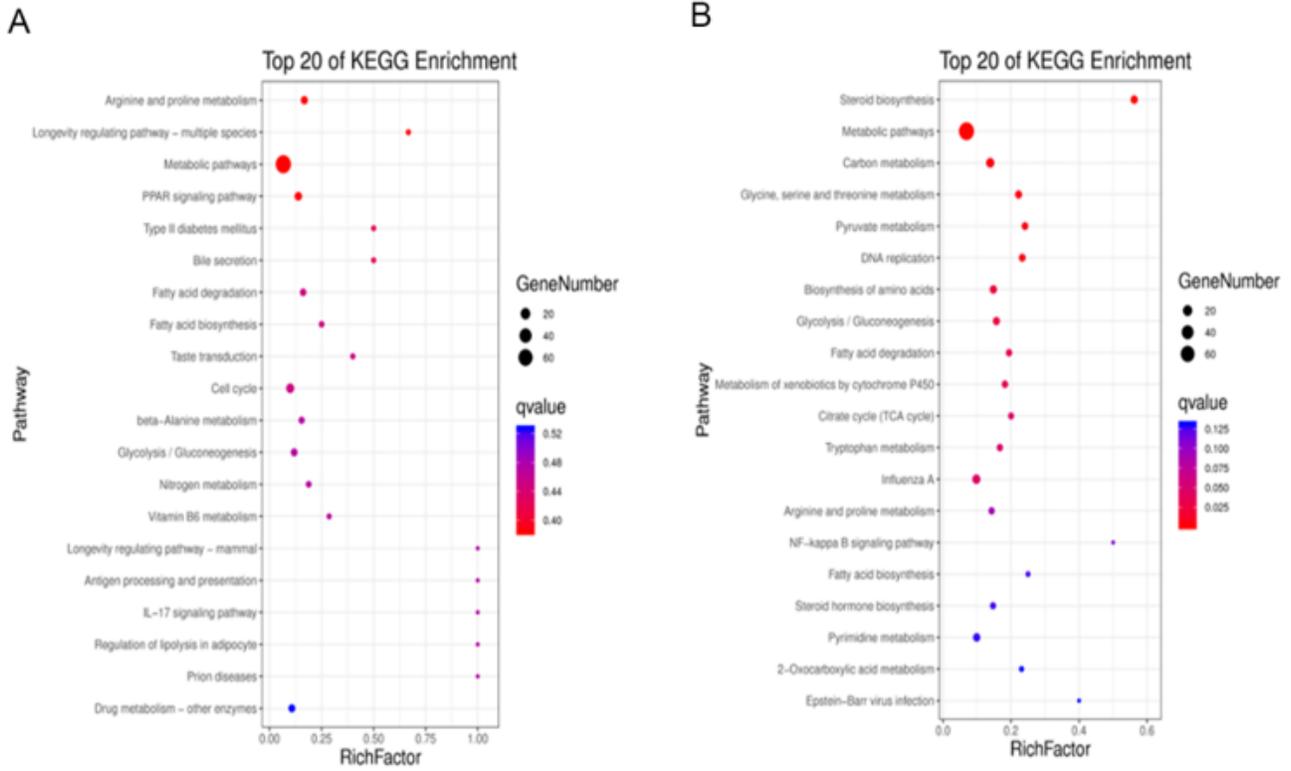


Figure 3

Top 20 significantly enriched pathways of FBM vs MBM (A) and FL vs ML (B) based on differentially expressed genes (DEGs) by KEGG pathway enrichment analysis. MBM: breast muscle of male chicken; FBM: breast muscle of female chicken; ML: liver of male chicken; FL: liver of female chicken.

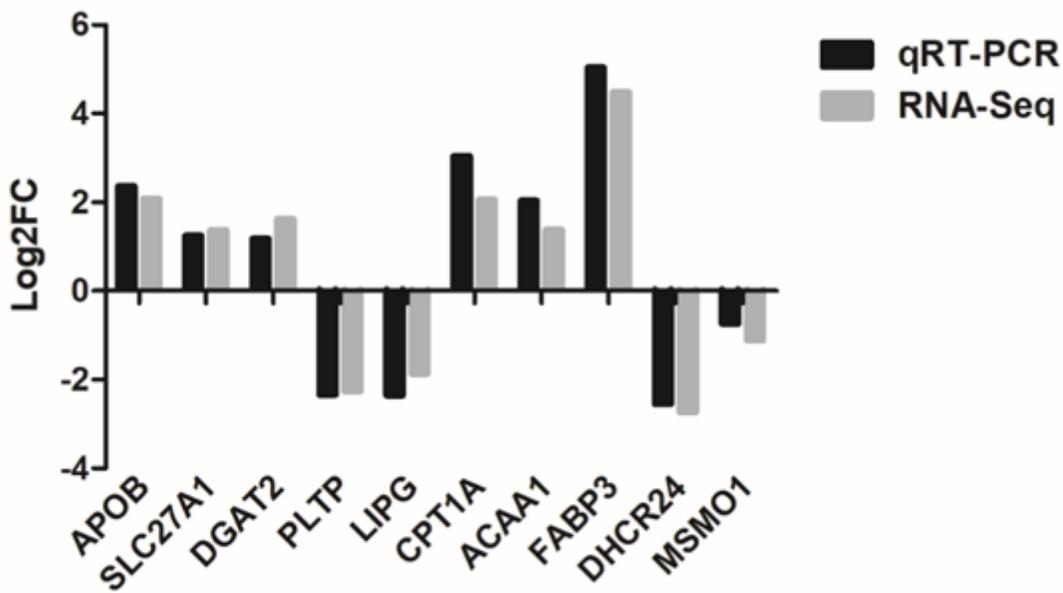


Figure 4

Results of qPCR validation.

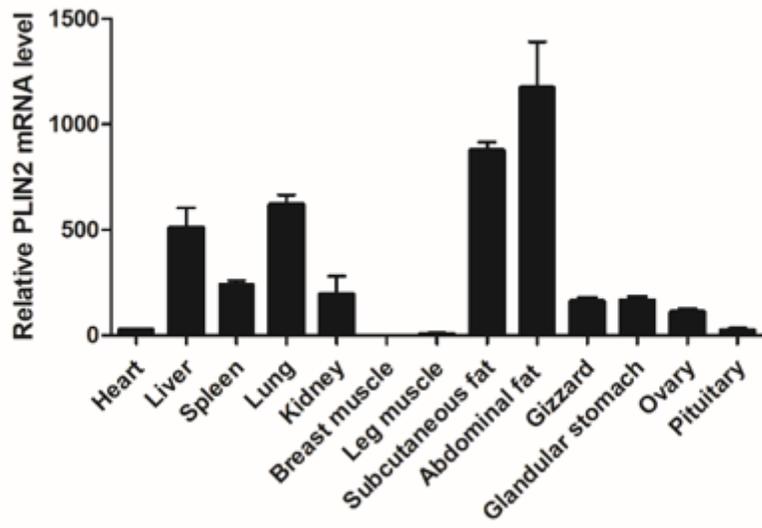


Figure 5

Tissue expression profile of PLIN2 in 150-day Daheng broilers.

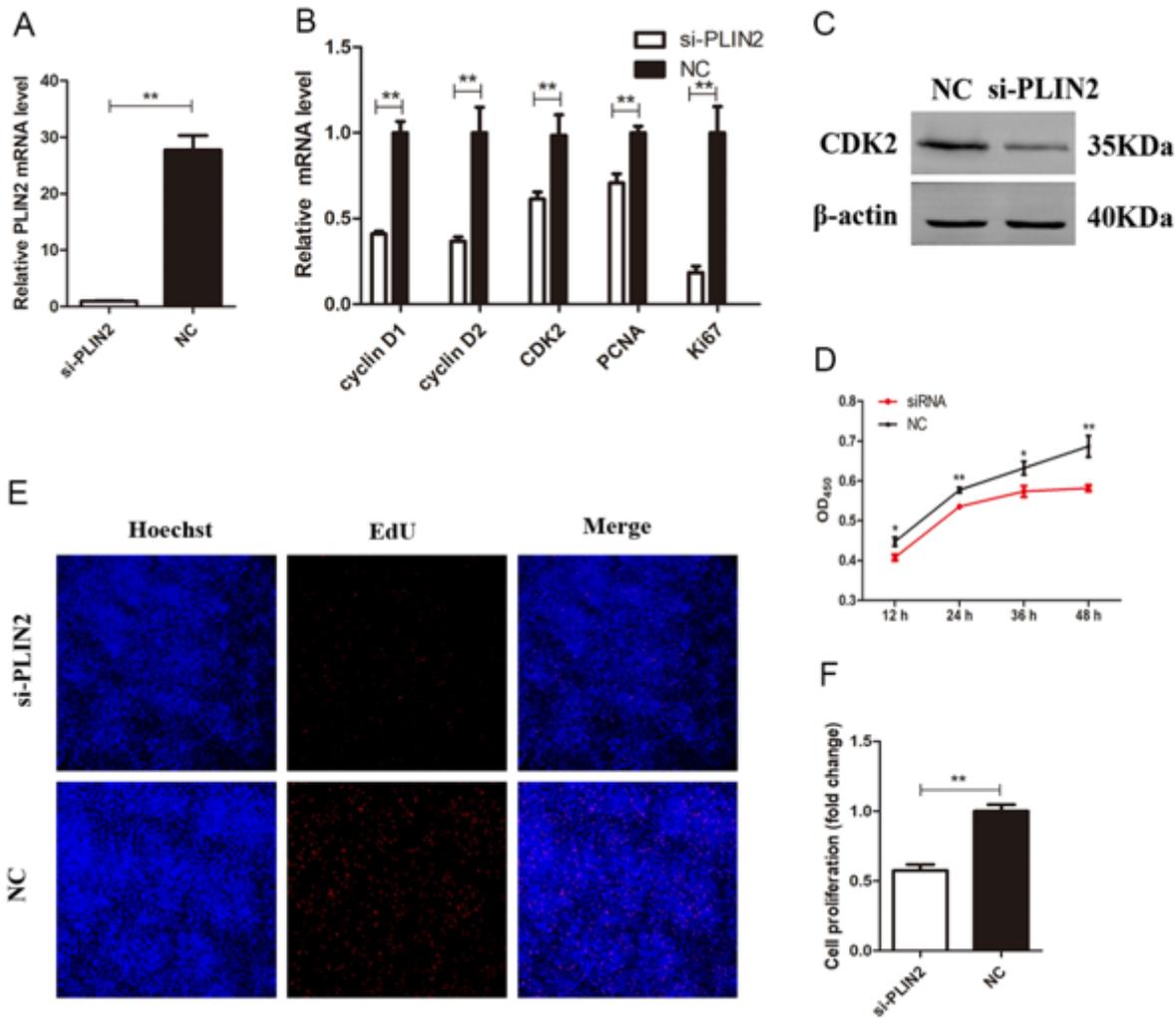


Figure 6

Interference of PLIN2 inhibits the proliferation of chicken intramuscular preadipocyte proliferation. (A) Confirmation of interference efficiency of the PLIN2 gene. (B) The mRNA expression level of cyclinD1, cyclinD2, CDK2, PCNA and Ki67 in si-PLIN2 and NC groups. (C) The protein expression level of CDK2 after transfection with si-PLIN2 and NC. (D) CCK assays for the proliferation activity of chicken intramuscular preadipocyte after transfection with si-PLIN2 and NC at 12 h, 24 h, 36 h and 48 h. (E) si-NC. (E) EdU assays for chicken intramuscular preadipocyte after transfection with si-PLIN2 and NC (100 \times magnification). EdU (red) fluorescence represents proliferation. Hoechst (blue) fluorescence represents nuclei. (F) The percentage of EdU stained cells to total cells. *P < 0.05; **P < 0.01.

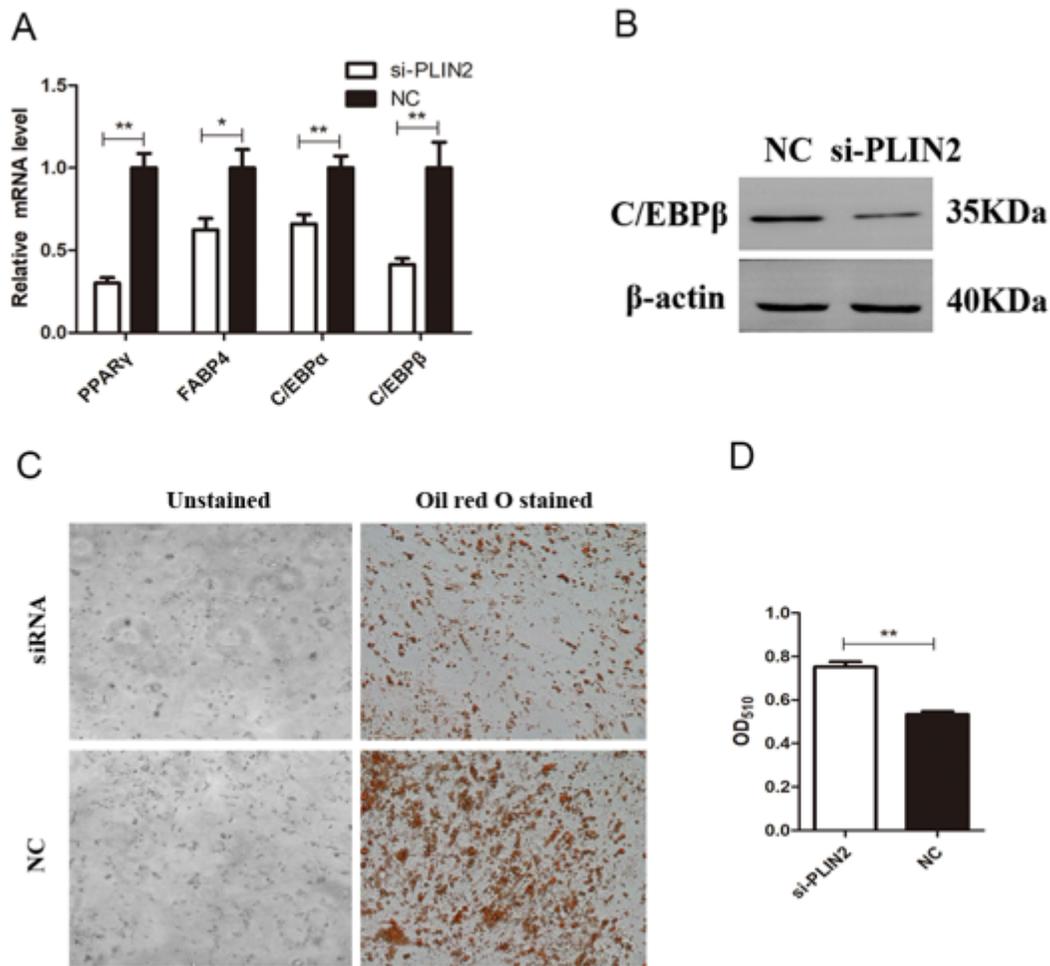


Figure 7

Interference of PLIN2 inhibits the differentiation of chicken intramuscular preadipocyte. (A) The mRNA expression level of PPAR γ , FABP4, C/EBP α and C/EBP β in si-PLIN2 and NC groups. (B) The protein expression level of C/EBP β after transfection with si-PLIN2 and NC. (C) Oil Red O staining of intramuscular adipocytes in the fifth day after IBMX-DEX-INS-Oleate induction (100 \times magnification). (D) Differentiation of adipocytes measured by spectrophotometric analysis. *P < 0.05; **P < 0.01.

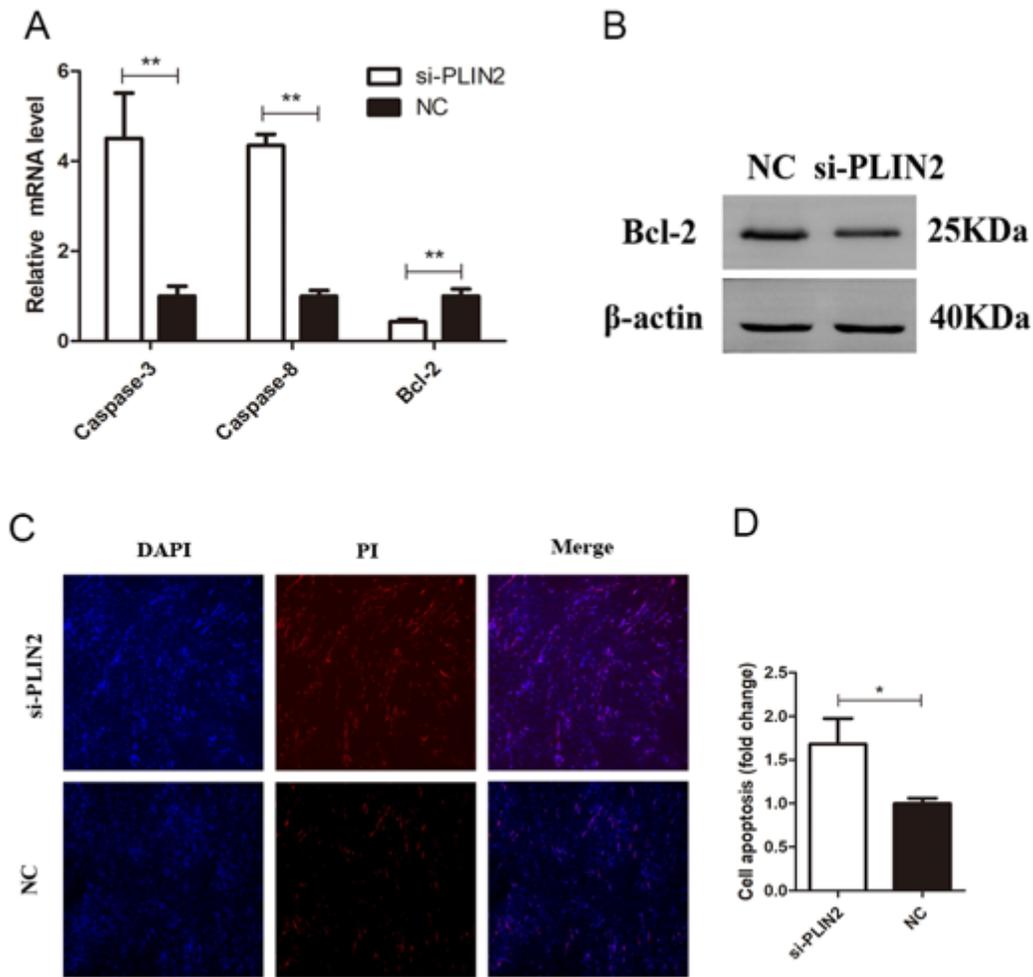


Figure 8

Interference of PLIN2 promotes the apoptosis of chicken intramuscular preadipocyte. (A) The mRNA expression level of caspase-3, caspase-8 and Bcl-2 in si-PLIN2 and NC groups. (B) The protein expression level of Bcl-2 after transfection with si-PLIN2 and NC groups. (C) Effects of the PLIN2 on the apoptosis of chicken intramuscular preadipocyte, determined by propidium iodide (PI) and DAPI double-staining (100 \times magnification). D. The percentage of PI stained cells to total cells. * $P < 0.05$; ** $P < 0.01$.

Supplementary Files

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- [Table1.pdf](#)