

Molecular Cloning and the Developmental Patterns of the *PLIN* Gene in Goose

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Abstract: PLIN plays a central role in the regulation of adipocyte metabolism which drives triacylglycerol storage in adipocytes and requires to hormonally stimulated lipolysis by cellular lipases. However, there is no report about the molecular structure and the expression profile of PLIN in goose. In this study, we cloned the goose *PLIN* gene and predicted bioinformatics. Quantitative Real Time PCR (qRT-PCR) assays were developed for accurate measurement of PLIN mRNA levels in Zhejiang White goose (ZW) and Landes goose different tissues from different ages (0-9 weeks). The results showed that goose PLIN cDNA sequence encoded an open reading frame of 527 Amino Acids (AA). The molecular phylogenetic tree among species were constructed that the mammal grouped together, *Bombyx mori* became another branch while the goose and chicken became another branch. It was found that PLIN mRNA were highly expressed in adipose tissues and to a lesser extent in cardiac and skeletal muscle and the difference is extreme significant between the fat and other tissues ($p < 0.01$) yet, no expression in liver. In addition, there was a significantly age-related and breed-related change in fat tissues ($p < 0.05$) and PLIN mRNA expression in ZW fat higher than that of Landes goose. All these results showed that the expression of PLIN mRNA in adipose tissues exhibited specific developmental changes, age-related and breeding patterns. The patterns of *PLIN* gene suggested that it played an important role in geese fat development. What's more, further study is needed to reconfirm its function in a large population and in other breeds with different genetics background.

Key words: Geese, *PLIN* gene, cloning, sequence analysis, expression, genetic background

INTRODUCTION

It was generally accepted that geese have been domesticated about 3,000 years ago. However, geese have never been exploited commercially as common as chickens or even ducks. The Chinese goose is relatively small in body size. The ZW breed is one of the popular breeds in Zhejiang Province of the Southern China which has orange shanks, long neck, pure the white feathers. Besides, it is popular with several superior traits such as delightful appearance, delicious favorable meat quality and high nutritional value. Landes goose is famous for its well fatty liver production in the world (Felix *et al.*, 1983; Hermier *et al.*, 1999; Mourot *et al.*, 2000).

In mammalian adipose tissue, droplets store energy in the form of neutral lipids, predominantly triglycerides and sterol esters. Perilipin, encoded by the PLIN gene is a member of PAT family proteins (adipophilin/ADRP, TIP47, S3-12 and MLDP/OXPAT/LSDP5) (Dalen *et al.*,

2007; Wolins *et al.*, 2006; Yamaguchi *et al.*, 2006) which binds on the surface of droplets and shares close similarity between the N terminal regions whereas the C terminal domain shows a lower degree of homology (Lu *et al.*, 2001; Miura *et al.*, 2002). Perilipin was firstly identified in the mouse epididymal adipocytes (Greenberg *et al.*, 1991). Human *PLIN* gene located on chromosome 15q26, gene length 15 kb, including nine exons, rat *PLIN* gene located on chromosome 7D2, gene length 11.5 kb including nine exons, exists in four isoforms (A, B C and D) (Lu *et al.*, 2001) which is the products of differentially spliced transcripts from a single gene and share a common N terminus but has distinct C termini. Perilipin A was found in surrounding neutral lipid storage droplets in adipocytes and steroidogenic cells, Perilipin B expressed lowly in above two cells, perilipins C and D expressed in steroidogenic cells and the cholesterol ester-rich lipid storage droplets (Lafontan and Langin, 2009). Perilipin A has double effect on triglyceride

metabolism which drives triacylglycerol storage by regulating the rate of basal lipolysis and is also required to maximize hormonally stimulated lipolysis. Under basal conditions, perilipin A forms a barrier on the surfaces of lipid droplets and restricts the access of cytosolic lipases including hormone sensitive lipase to lipid droplets thus promoting triacylglycerol storage. Perilipin A knockout mice showed that increased substantially in basal lipolysis and reduced in adipose mass and can resistant to diet-induced obesity (Martinez-Botas *et al.*, 2000; Tansey *et al.*, 2001).

Until now, no information about geese PLIN sequence structure and function has been reported. The purpose of the study is to clone geese PLIN cDNA identify the structure of perilipin, discriminate from other species, analysis the PLIN mRNA expression patterns of Zhejiang white goose and Landes goose different tissues from different ages (0-9 weeks).

MATERIALS AND METHODS

Experiment birds and sample collecting: ZW and Landes geese were selected from the Pinghu Breeding Company in Zhejiang province and reared under similar management conditions including a standard diet. Firstly, birds were housed on the deep-litter bedding and transferred to the growing pens located. Geese were killed at the following points: posthatch day (P) 1, 14, 21, 28, 35, 42, 49, 56 and 63. At each point, sampled 4 individuals from every breed. Pectoralis muscle, liver, leg muscle, heart, whole brain, subcutaneous fat and abdominal fat were quickly sampled and frozen in liquid nitrogen.

RT-PCR primers: Based on the sequences (Accession No: EU620701), the following two primer pairs (PLIN-1F: ACGATGACGGCGAAGAAGAAT, PLIN-1R: TCTTCTG GCAACAGGTACTCCA; PLIN-2F: GAGAAGCTGATG GAGTACCTGT, PLIN-2R: GGTCAGTCCTTCTGTAGGC) were designed using Oligo 6.0 software to amplify the goose PLIN cDNA, the length of the amplified fragment were 569 and 1019 bp, respectively.

Cloning: Total RNA was extracted from adipose tissues according to the protocol specified by the TRIZOL Reagent kit (Invitrogen). Following DNase treatment (Fermentas Co, Canada), 1 ng total RNA was used for first-strand cDNA synthesis using SYBR PrimeScript™ RT-PCR Kit (TaKaRa Biotechnology (Dalian) Co.). The reaction was performed in a volume of 10 µL containing 5×PrimerScript Buffer, 10 mM of each dNTPs, 40 U µL⁻¹

RNase Inhibitor, 2.5 µM oligo-dT Primer. The reverse transcription was maintained at 37°C for 15 min and ended with an incubation at 85°C for 5 sec.

Cloning of goose PLIN cDNA was amplified in a volume of 50 µL consisting of 25 µL Master mix (Tiangen, Peking, PRC), 4 µL reverse transcription product (fsc DNA from abdominal fat), 1.2 µL 10 mol each of gene-specific primers and the rest of nuclease free water. Amplification conditions consisted of an initial 1 min denaturation at 94°C; 35 cycles of at 94°C 30 sec, 50 sec annealing at 58°C and 1 min extension at 72°C and a final 2 min extension at 72°C. The band was excised and purified using Gel Extraction Kit (OMEGA) and cloned using the plasmid PMD-19T vector (TaKaRa Biotechnology (Dalian) Co.) and transformed into *E. coli* DH5a (Tiangen, Peking, PRC). Plasmid DNA was isolated from positive recombinants and fully sequenced.

Sequence and phylogenetic analysis: Reading frames in obtained goose cDNA was finished via NCBI ORF tool (<http://www.ncbi.nlm.nih.gov/gorf/>). The amino acid similarity among different species was analyzed using the BLASTp program with BLOSUM62 scoring matrix. The putative hydrophobicity, molecular mass and isoelectric point of the deduced amino acid sequence were predicted using Bioedit software and phosphorylation locus was predicted by NetPhos 2.0 (<http://www.cbs.dtu.dk/services/NetPhos/>). The conserve domain prediction was finished by NCBI CCD tool (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.

Expression profiling by qRT-PCR: According to sequence of the cloning goose PLIN gene, we designed one primer (PLIN-3F: AACTGAAGGACACCATCTCCAC, PLIN-3R: GAGTTGCTCCTCGTGTACCTG) for qRT-PCR analysis, another primer (Beta-actin-F: ACCACCGGTATT GTTATGGACT, Beta-actin-R: TTGAAGGTGGTCTCGT GGAT (Su *et al.*, 2009) was used as a reference gene. Total RNA were extracted from all tissues at nine growing points, the cDNA was synthesized using SYBR PrimeScript™ RT-PCR Kit following the manufacturer's instruction.

qRT-PCR analysis was performed on iQ5 Real Time PCR Detection System (BIO-RAD Co.) using SYBR Green detection chemistry. QRT-PCR reaction was performed in a volume of 20 µL containing 2 µL reverse transcription product, 0.8 µL 10 mol of each specific primer, 10 µL SYBR Premix Ex Taq (TaKaRa Biotechnology (Dalian) Co.),

6.4 µL free nuclease water. All reactions were performed in triplicate within a PCR assay and under the same cycling conditions: 95°C for 160 sec; 40 cycles of 95°C for 5 sec; 60°C for 30 sec and a final extension step at 72°C for 15 sec.

Melt curve analysis was performed over a range of 55-95°C in order to verify single production generation at the end of the assay. To exclude that the amplification associated fluorescence was associated with the primer dimers, controls without reverse transcriptase were applied.

Statistical analysis: Relative gene expression data use the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). we grouped all the subjects for data presentation. The results were presented as means±SD. The mean, SD and CV are then determined from the triplicate samples at each time point. Differences of $p < 0.05$ were considered significance.

RESULTS

Molecular cloning of goose *PLIN* gene: Amplified fragments (569 and 1,019 bp) for *PLIN* cDNA were generated and sequenced.

The coding sequence of *PLIN* cDNA with 1,589 nucleotides was obtained by assembling both sequences after being spliced by DNAStar Bioedit software and was deposited in GenBank with accession number GU938447.

Homology analysis of goose *PLIN* gene amino acid:

Using the tool of Open Reading Fragment (ORF) in NCBI, we found an ORF with 1,583 nucleotides which comprised the initiation codon (ATG) and termination codon (TGA), encoded 527 amino acid residues. Analysis with BLASTp revealed the identities of amino acid sequence from red jungle fowl (83%) (NP00120911.1), duck (43%) (XP_001507481.1), human (36%) (NP_002657.3), pig (41%) (NP_001033727.1), ovine (40%) (NP001107245.1), cow (41%) (NP.001077168.1) and *rattus* (39%) (XP_001364257.1), separately.

Character analysis of the deduced perilipin amino acid sequence:

The molecular mass and isoelectric point of the deduced amino acid sequence were 56.93 and 5.55 kDa, respectively, there were 39 phosphorylation sites (26 Serine, 11 Threonine and 2 Tyrosine). Eight obvious hydrophobic regions was found including residues 20-30, 50-70, 130-150, 165-180, 230-250, 265-280, 330-390 and 440-450.

As shown in Fig. 1, the horizontal scale represents the number of amino acid residues and the vertical scale represents the mean hydrophobicity. Points above the zero horizontal line are hydrophobic region and the points below the line are hydrophilic. The conservative domain of goose *PLIN* gene is the typical character of PAT family.

As shown in Fig. 2, the phylogenetic tree based on the amino acid sequence of goose and some other species

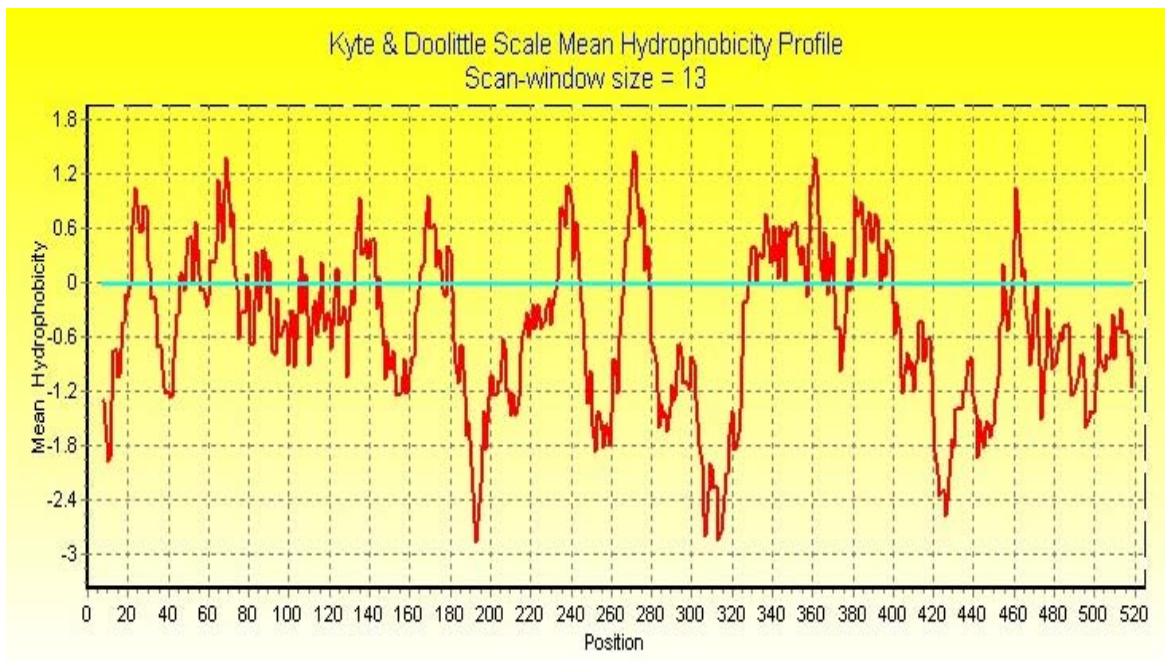


Fig. 1: Goose *PLIN* protein analysis hydrophobic property

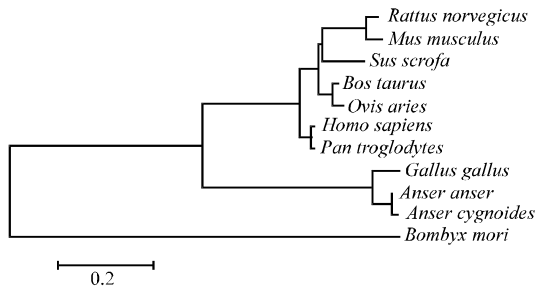


Fig. 2: A schematic trees was predicted by Neighbor-joining using PLIN amino acid sequence of different species which were identified in an NCBI search. Numbers at the branch nodes represent bootstrap values obtained in 1000 replications

was constructed as shown in Fig. 2. The phylogenetic analysis suggested that the mammal were grouped together, Bombyx mori become another branch while the goose and chicken became another branches.

The relative temporal expression of *PLIN* gene during goose development: As shown in Fig. 3, tissue distribution data showed that *PLIN* mRNAs were highly expressed in adipose tissues and to a lesser extent in heart, skeletal muscle and brain and no expression in liver. In brain, a little level expressed in Landes goose only at P1 and P63. The level was the lowest in pectorals muscle, only detected at P56, yet the expressions in leg muscle were detected in other growing points except for P1, P49 and P56. In adipose tissues, the expressions of *PLIN*

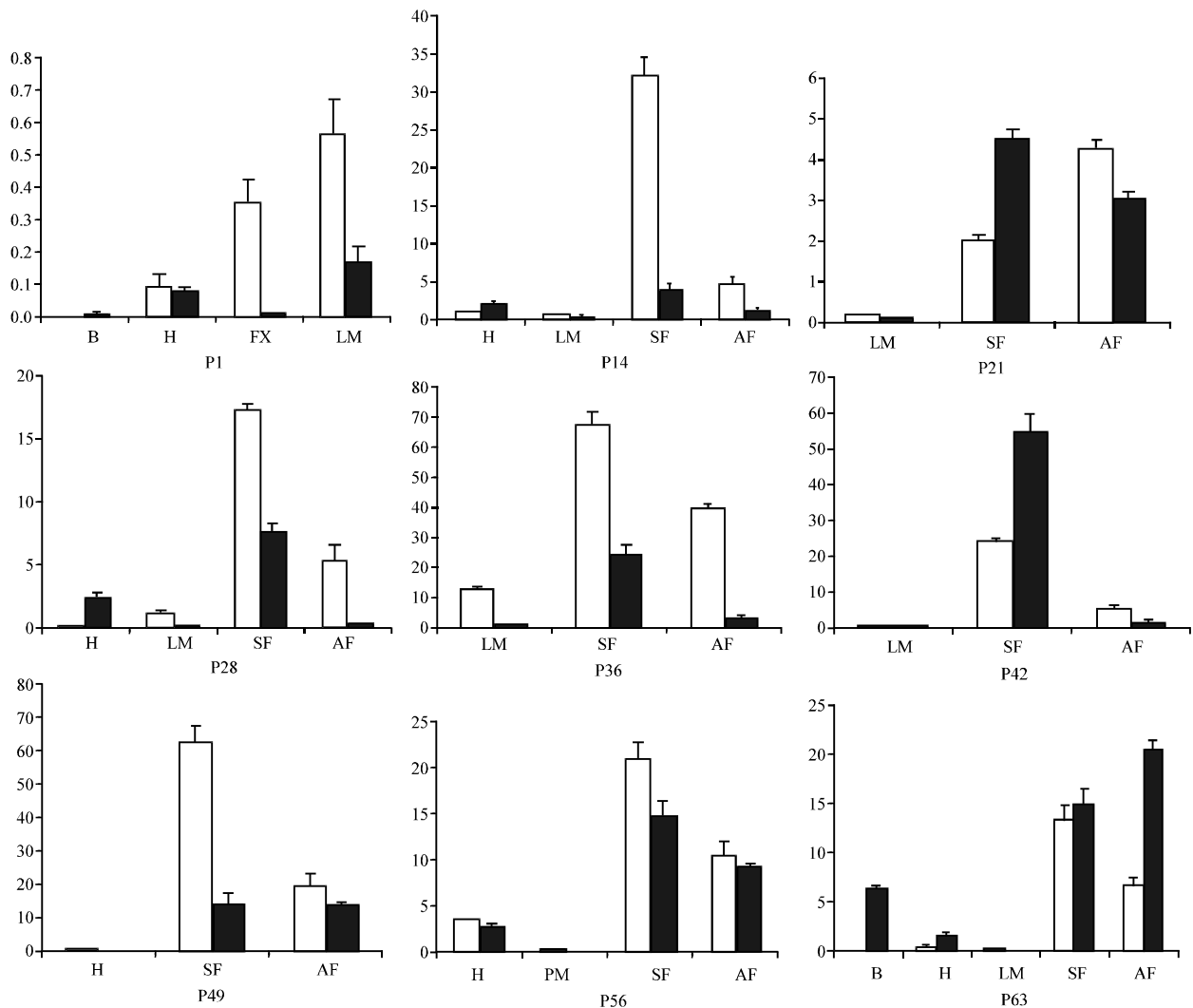


Fig. 3: Goose *PLIN* gene expressed relatively in different tissues of different growing points. All of the values given are the averages of data in triplicate. Pectoralis Muscle (PM), LEG Muscle (LM), Heart (H), whole Brain (B), Subcutaneous Fat (SF) and Abdominal Fat (AF), P posthatch day

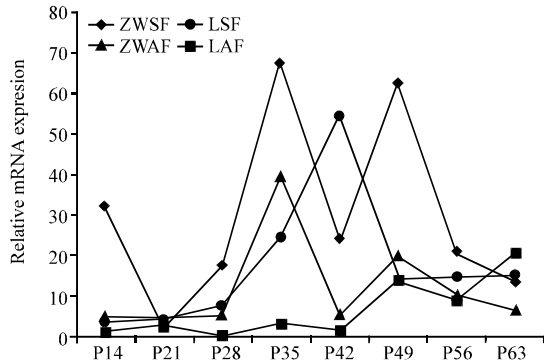


Fig. 4: Goose *PLIN* gene distribution data showed in fat in two breeding. Zhejiang White goose Subcutaneous Fat (ZWSF), Landes Subcutaneous Fat (LSF), Zhejiang White goose Abdominal Fat (ZWAF), Landes Abdominal Fat (LAF)

mRNA were found in every growing points except that we didn't sampled the fat tissues at P1 and significance in two breeds ($p < 0.05$).

As shown in Fig. 4, the ontogenetic expression levels of *PLIN* mRNA in goose adipose tissues is summarized. In subcutaneous fat, the higher values were found in ZW P35 and P49 whereas the expression rose with goose development before the peak at P42 and declined to a plateau in Landes goose. In abdominal fat, the highest value showed in ZW P35 then declined and rose a little at P49 and has a peak at P63 in Landes goose. In the study, it is found that the expressions of the *PLIN* mRNAs in ZW subcutaneous fat were the highest.

DISCUSSION

Several studies have shown Perilipin A functions as both a suppressor of basal lipolysis and a necessary enhancer of PKA-stimulated lipolysis. The six serine residues phosphorylation ensures lipolysis mediating by either HSL or non-HSL lipase (Souza *et al.*, 2002; Sztalryd *et al.*, 2003; Tansey *et al.*, 2003, 2004). To date, the study reported that serine 492 was the function loci in 3T3-L1 cells adipocytes (Marcinkiewicz *et al.*, 2006). In the study, predetermined the structures of goose perilipin including eight obvious hydrophobic regions and 26 serine sites. It remains unclear whether the goose perilipin has the same function which needs further investigation.

Among early study confirmed that perilipin expressed later during adipocyte differentiation (Jiang and Serrero, 1992) and the highest in white and brown adipocytes (Greenberg *et al.*, 1993) with lower levels of expression in steroidogenic cells of the adrenal cortex, testes and ovaries (Servetnick *et al.*, 1995). In this study, we

evaluated the *PLIN* mRNA expression in different goose tissues at different ages between two breeds, the results showed that the expression levels in the abdominal fat and subcutaneous fat tissues were relatively higher than those in other tissues.

The maximum value of the expression was at P35 in ZW adipose tissues on the contrary yet, in Landes goose, the peak was the later development of growing which were P49 and P63. Previous study described that chicks growth of an adipose depot at age of 7 weeks posthatch was a correlation with cell proliferation (Butterwith, 1997). Perilipin was coated the large and mature adipocyte. It seems that the mature period of ZW adipose tissues has earlier than the Landes goose fat.

However, further study is needed to confirm its hypothesis. In addition, the values of *PLIN* mRNA in ZW fat were relatively higher than in Landes goose. All these results showed that the expression of *PLIN* mRNA in adipose tissues exhibits specific developmental changes, age-related and breeding patterns.

In goose, excessive fattening is observed following intensive liver for rapid growth in conjunction with impaired performance. Because of the potential for dysregulation and involvement in multiple functions, lipids are distinctive in virtually every physiological state. But on the study, no expression in liver was observed which was in accordance with the recent report in rat hepatocytes (Servetnick *et al.*, 1995). These findings suggest that perilipin may have limited usefulness in liver metabolic.

CONCLUSION

In study, *PLIN* mRNA expression in ZW fat approaches or exceeds that in Landes goose this suggested that the *PLIN* mRNA has a particular genetic background for increasing adiposity in ZW adipose tissues. Although, the number of samples in each group was not large, further studies are needed to reconfirm its function in a large population and in other breeds with different genetics.

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