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Cloning, characterization and expression of Peking duck fatty acid synthase during adipocyte differentiation

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ABSTRACT

Background: Fatty acid synthase (FAS) is a key enzyme of *de novo* lipogenesis (DNL), which has been cloned from several species: *Gallus gallus*, *Mus musculus*, *Homo sapiens*, but not from *Anas platyrhynchos*. The current study was conducted to obtain the full-length coding sequence of Peking duck FAS and investigate its expression during adipocyte differentiation.
Results: We have isolated a 7654 bp fragment from Peking duck adipocytes that corresponds to the FAS gene. The cloned fragment contains an open reading frame of 7545 bp, encodes a 2515 amino acid protein, and displays high nucleotide and amino acid homology to avian FAS orthologs. Twelve hour treatment of oleic acid significantly up-regulated the expression of FAS in duck preadipocytes ($P < 0.05$). However, 1000 μM treatment of oleic acid exhibited lipotoxic effect on cell viability ($P < 0.05$). In addition, during the first 24 h of duck adipocyte differentiation FAS was induced; however, after 24 h its expression level declined ($P < 0.05$).
Conclusion: We have successfully cloned and characterized Peking duck FAS. FAS was induced during adipocyte differentiation and by oleic acid treatment. These findings suggest that Peking duck FAS plays a similar role to mammalian FAS during adipocyte differentiation.

1. Introduction

Fatty acid synthase (FAS, EC 2.3.1.85) is a key multifunctional enzyme that catalyzes the synthesis of saturated long-chain fatty acids, predominately palmitate, by using malonyl-CoA as a two-carbon donor [1]. It has been determined that FAS consists of two identical subunits, each of which contains seven unique functional domains: β-ketoacyl synthase (KS), malonyl/acetyl transferase (MAT), dehydratase (DH), enoyl reductase (ER), β-ketoacyl reductase (KR), acyl carrier protein (ACP), and the thioesterase (TE) [2]. Among them the TE domain of FAS plays an essential role in determining the final chain length of the product [3].

As FAS is critical to the synthesis of fatty acids, it is highly expressed in lipogenic tissues such as the liver, adipose tissue and lactating mammary glands [4]. In both rodents and mammals, there is a positive correlation between FAS mRNA expression level and body fat content [5,6,7]. Moreover, inhibition of FAS reduces food intake and facilitates weight lose [8]. Therefore, FAS would be a reasonable therapeutic target for the treatment of obesity [5,9]. In chicken, FAS mRNA levels are strongly correlated with hepatic fat content [10]. Due to the low capacity of adipose tissue DNL in avian species, there is little information regarding the roles of FAS in adipocyte differentiation. However, down-regulation of FAS in 3T3L1 cells, either with inhibitors or by RNA interference, leads to decreased lipid droplet accumulation, demonstrating FAS plays a key role in adipocyte differentiation [11,12]. To date, it is unclear whether FAS can play a similar role in duck adipocyte differentiation.

In avian species, the addition of fatty acids (mainly oleic acid) is needed to supplement the hormonal cocktail containing dexamethasone (DEX), insulin, and 3-isobutyl-1-methylxanthine (IBMX) to induce adipocyte differentiation in cell culture [13]. Compelling evidence, from studies conducted in chicken and mouse, has shown that oleic acid can significantly stimulate the expression of several marker genes related to adipocyte differentiation, such as FAS, adipocyte fatty acid-binding protein-4 (FABP4), and peroxisome proliferator-activated receptor-γ (PPARγ) [14,15]. Moreover, it has been identified that oleic acid by itself can also promote adipogenesis [15,16,17]. However, it is unknown whether oleic acid can play a similar role in duck adipocyte differentiation by stimulating adipocyte-related gene expression.

Until now, the FAS gene has been successfully cloned from several species, such as rat, chicken, pig, sheep, and human, but not duck [18,19,20]. In the current study, we cloned the full-length coding

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sequence of duck FAS gene and detected the expression level of FAS in duck preadipocytes exposed to oleic acid. Furthermore, the expression level of FAS during duck adipocyte differentiation was investigated. This work will have the potential to increase our understanding of the functional roles of FAS during duck adipocyte differentiation.

2. Materials and methods

2.1. Duck preadipocytes isolation and culture

Duck preadipocytes were isolated from Peking duck 1 week after hatching as previously described [13]. Briefly, Peking ducks were rapidly decapitated and subcutaneous adipose tissue was sterilely dissected from the leg. Then the adipose tissue was minced into fine sections using scissors and incubated in digestion buffer (PBS (-), 4% BSA (Gibco, USA), 0.1% collagenase type I (Gibco, USA)) at 37°C in a shaking water bath for 40–60 min. Growth medium (Dulbecco's modified Eagle's Medium/nutrient mixture F12 Ham's (V/V, 1:1; Gibco, USA), 10% fetal calf serum (Gibco, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, USA) were used to end the digestion. The resulting mixture was filtered through 20 μm nylon screens to remove undigested tissue and large cell aggregates. These filtered cells were then centrifuged at 300 × g for 10 min at room temperature to separate floating adipocytes from the stromal-vascular cells. The preadipocytes were seeded at a density of 1 × 104 cells/cm^2 and cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. After the cells reached full confluence, oleic acid was added in the medium to induce preadipocyte differentiation. During this time, the FAS expression and the cell viability were measured.

2.2. Cloning the full-length coding sequence of FAS

Based on the FAS sequences of *Gallus gallus* (NM_205155.2), *Meleagris gallopavo* (XM_003211461.1), and *Anser cygnoides* (EU770327.1) deposited in the GenBank database, we designed and selected 11 pairs of gene-specific primers (namely FAS-P1 to FAS-P11) using DNAMAN 7.0, Primer Premier 5.0, and Oligo 7.0 software. PCR protocols are available upon request. The amplified products were verified by 1.5% agarose gel electrophoresis and purified with a gel extraction kit (Omega, USA). The purified products were then ligated into the pMD-19T vector (Biomed, China) and sequenced by Invitrogen Corporation (Applied Invitrogen, China). The primer sequences and PCR product lengths are listed in Table 1.

Table 1
Primer sequences used in the current study.

Primer ID	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size (bp)
FAS-P1	CCGCCTACGCAGTAACAG	CTCACATTGGCAGAAGAC	980
FAS-P2	CAGCGGCAGTTGGTCAGT	ATCGCCCTCGCCAATAAG	1018
FAS-P3	AGATGAGGCTTTGAAGAACA	TGAACGAGGTTTAGGGTGT	559
FAS-P4	TACCAGCCTGCCACAACT	TTCCCATTCCCTGACACT	940
FAS-P5	TACCTTGTGCTGGCTTGG	CCTGTGACTGGTCATGTT	1062
FAS-P6	CTCCCACCCTGGAAAAAT	AGACAGTTCACCATGCCC	1053
FAS-P7	ATCCCCTGCCAAAACACC	AGTTTGCGGTGTCTTGCTC	1068
FAS-P8	AAGCAGCCATTGCCATTG	CAAGCCCAAATCCTCCTA	651
FAS-P9	ATGGTGTGGTAAAGCCCC	TTGATTGTAAGAAGTCGG	1093
FAS-P10	TTCGGGCACCCTTCATCT	GCTGGGAGCACATTTCAA	958
FAS-P11	GAGTCTGGCATCCTATTA	GAAGAGTTCCTTGGGGTC	765
RT-FAS	TGGGAGTAACACTGATGGC	TCCAGGCTTGATACCACA	109
β-Actin	CAACGAGCGGTTCAGGTGT	TGGAGTTGAAGGTGGTCTCG	92

Note: FAS-P1 to FAS-P11: Primers for gene cloning. RT-FAS: Primer for qRT-PCR. β-actin: Reference gene for data normalization.

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from the cultured cells at different differentiation stages using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions and quantified by spectrophotometric absorbance at 260 nm. First stand cDNA was synthesized from 10 μg of total RNA using a cDNA synthesis kit (TaKaRa, Japan). The newly synthesized cDNA product was immediately stored at -20°C for further study.

2.4. Cell viability assay

Duck preadipocytes were seeded on 96-well plates and treated with different concentrations of oleic acid for 24 h. Cell viability was determined using a commercial Cell Counting Kit-8 (CCK-8, Beijing Zomanbio biotechnology, China) as previously described [21]. 10 μL of CCK-8 reagent was added to each well and incubated at 37°C for 2-4 h until the media became yellow. Absorbance was measured at wavelength of 450 nm using a microplate reader.

2.5. Gene expression measurements

Quantitative real-time PCR (qRT-PCR) analysis was conducted by using SYBR PrimerScript™ RT-PCR kit (TaKaRa, Japan) in the CFX96™ Real-Time System (Bio-Rad, USA). The PCR was carried out in a 25 μL reaction volume, including 2.0 μL cDNA, 12.5 μL of SYBR Premix EX Taq, 8.5 μL of sterile water, and 1.0 μL of each gene-specific primer. The calibrator-normalized relative quantification method using the $2^{-\Delta\Delta CT}$ method was employed [22]. To normalize the target genes in similar cDNA samples, β-actin was selected as the reference gene. All reactions were completed in triplicate, and the data represents the mean of three independent experiments. The specific primers used are listed in Table 1.

2.6. Bioinformatics analysis

BLAST from NCBI was used to determine the similarity between nucleotide and protein sequences. Multiple alignments of the FAS sequences were conducted with the ClustalX multiple alignment software. MEGA 5.0 was used to generate the phylogenetic tree by the Neighbor-Joining (NJ) method based on the sequence of duck FAS and other known FAS sequences. The protein motif sequences and conserved domains were analyzed using NCBI CD-search tool in combination with SMART and PROSITE software.

2.7. Statistical analysis

Results are presented as the mean ± SD. The data were subjected to ANOVA testing, and the means were assessed for significance by Tukey's test using SPSS (version 17). *P* values less than 0.05 were considered significant in all statistical analysis.

3. Results

3.1. Cloning and sequence analysis of FAS from Peking duck

By sequencing and assembling the data, a 7654 bp mRNA of duck FAS was isolated by RT-PCR, which has been submitted to GenBank database with the accession number KF185112. The full-length coding sequence of duck FAS consists of 7545 nucleotides which encodes a 2525 amino acid protein with an estimated molecular mass of 275.0667 kDa and a theoretical isoelectric point of 6.07. The cloned FAS sequence is highly similar to turkey FAS, with 92% and 91% similarity at the nucleotide level and amino acid level, respectively (Table 2).

Table 2
Homology of duck FAS with other species.

Species (Latin name)	Nucleotide (%)	Amino acid (%)
Duck (*Anas platyrnchos*)	99	99
Turkey (*Meleagris gallopavo*)	92	91
Chicken (*Gallus gallus*)	91	90
Pigeon (*Columba livia*)	90	89
Green anole (*Anolis carolinensis*)	76	77
Mouse (*Mus musculus*)	69	65
Goat (*Capra hircus*)	68	65
Human (*Homo sapiens*)	66	65
Pig (*Sus scrofa*)	66	64
Chimpanzee (*Pan troglodytes*)	65	64

3.2. Phylogenetic analysis and alignment

A condensed phylogenetic tree was constructed based on the amino acid sequences of duck FAS and other organisms (Fig. 1). The overall topology of the tree showed that the duck FAS was most similar to those of the other avian species and also had a high similarity to FAS sequences of other organisms, especially *Anolis carolinensis*.

As indicated in Fig. 2a, multiple sequence alignment of duck FAS with other known FAS amino acid sequences revealed that they were highly conserved among different species, especially in the regions of FAS family signatures such as the KS domain, TE domain, and the ACP domain. The deduced FAS amino acid sequences were identified to have seven main functional domains by using SMART and NCBI CD-search software. Meanwhile, in the linear domain map of duck FAS (Fig. 2b), it could be seen that there were seven functional domains corresponding to KS (1–239, 243–360), AT (492–809), DH (865–1060), ER (1543–1859), KR (1890–2070), ACP (2129–2196), and TE (2245–2504).

3.3. Effect of different concentrations of oleic acid on duck FAS mRNA expression and cell viability

To investigate the effect of oleic acid on FAS gene expression and cell viability, duck preadipocytes were treated with different concentrations of oleic acid for 12 h. As shown in Fig. 3, duck FAS mRNA expression level was up-regulated with increasing concentrations of oleic acid compared to the control group ($P < 0.05$). However, FAS expression level decreased by treating the cells with 1000 μM oleic acid ($P < 0.05$). Meanwhile, it is shown in Fig. 4 that incubation of duck preadipocytes with growing concentrations of oleic acid led to a significant increase of cell viability ($P < 0.05$). However, a high concentration of oleic acid (1000 μM) exhibited a lipotoxic effect by significantly reducing the cell viability ($P < 0.05$).

3.4. Expression of FAS mRNA during duck adipocyte differentiation

Duck preadipocytes were induced to differentiate by the addition of 300 μM oleic acid and expression of FAS mRNA during adipogenesis was established. Results showed that duck preadipocytes began to differentiate at 12 h when treated with 300 μM oleic acid (Fig. 5a–d). After 48 h of oleic acid treatment, most preadipocytes differentiated into mature adipocytes containing multiple lipid droplets (Fig. 5e).

As shown in Fig. 6, expression level of FAS mRNA gradually increased for 24 h when treated with oleic acid ($P < 0.05$). However, expression level of FAS surprisingly decreased to a lower level after 24 h. There is a slight increase of FAS expression during 36–48 h post-oleic acid treatment ($P < 0.05$).

4. Discussion

Little is known about the role of FAS in duck. This study is the first to report the structures, expression, and possible functions of FAS during duck adipogenesis. Currently, we obtained the full-length coding sequence of duck FAS, which consists of 7515 nucleotides and encodes a 2505 amino acid protein. BLAST analysis revealed that the deduced amino acid sequence of FAS shared high identity with other known FAS sequences (65–92% identity in all the matches), especially with the *G. gallus* and *M. gallopavo* sequences. Based on the deduced amino acid sequence, we found that duck FAS, consistent with early reports [2,23], contained seven functional domains. The order of these functional domains is as follows: KS, MAT, DH, ER, KR, ACP, and TE. This gene has a molecular mass of 275.0667 kDa and a theoretical

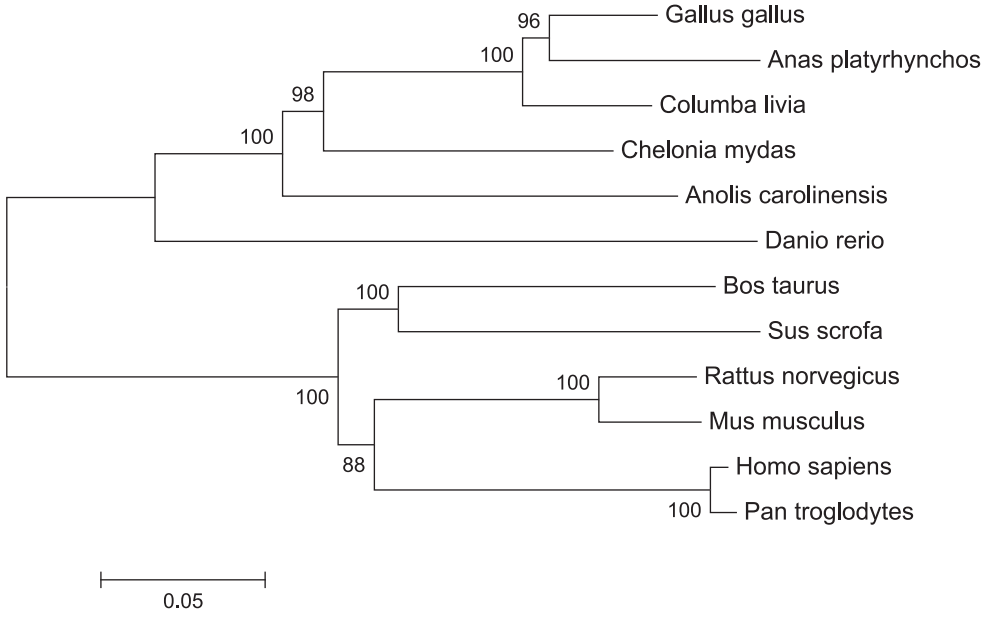


Fig. 1. A phylogenetic tree of fatty acid synthase family members constructed using the neighbor-joining method. Numbers at each branch indicate the percentage of times a node was supported in 1000 bootstrap pseudoreplications by neighbor joining. The species names and the Genbank accession numbers of FAS are as follows: *Gallus gallus*, NP_990486; *Homo sapiens*, AAA73576; *Mus musculus*, NP_032014; *Pan troglodytes*, XP_511758; *Bos taurus*, XP_005221054; *Sus scrofa*, NP_001093400; *Danio rerio*, XP_001923643; *Rattus norvegicus*, NP_059028; *Columba livia*, EMC83573; *Chelonia mydas*, EMP32461; and *Anolis carolinensis*, XP_003217337.

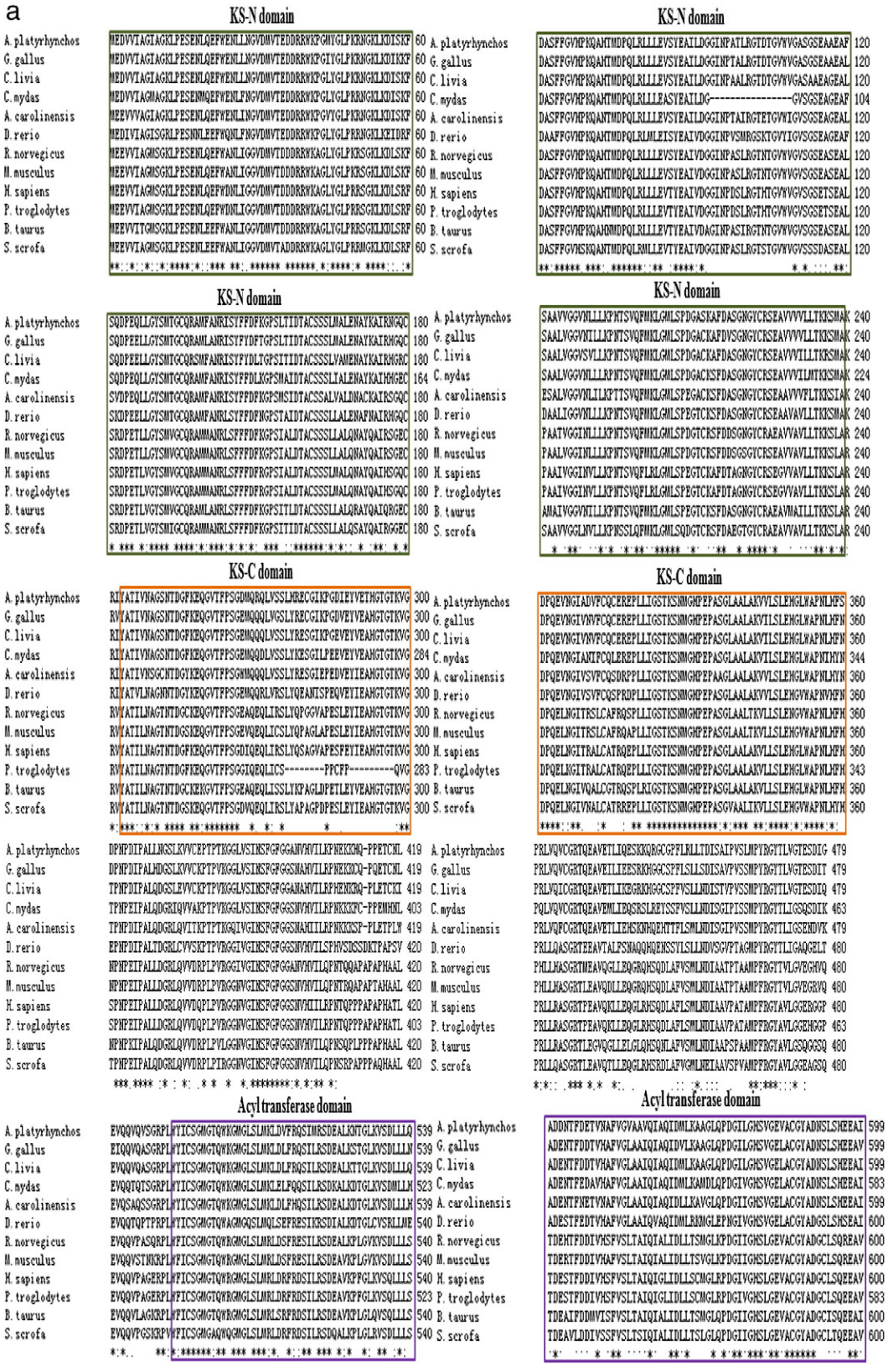


Fig. 2. (a) Multiple alignments of the deduced amino acid sequences of duck FAS with other species. The sequences were compared by ClustalX Multiple Sequence Alignment Program software. The numbers shown indicate the residue positions. Asterisks denote completely conserved residues; dashes indicate gaps introduced into the sequences to optimize the alignment, colons indicate conservative substitutions, and points indicate non-conservative substitutions. The species names and the GenBank accession numbers of FAS are as follows: *Gallus gallus*, NP_990486; *Homo sapiens*, AAA73576; *Mus musculus*, NP_032014; *Pan troglodytes*, XP_511758; *Bos taurus*, XP_005221054; *Sus scrofa*, NP_001093400; *Danio rerio*, XP_001923643; *Rattus norvegicus*, NP_059028; *Columba livia*, EMC83573; *Chelonia mydas*, EMP32461; and *Anolis carolinensis*, XP_003217337. (b) Linear domain map of duck FAS. The number of residues in each domain was indicated above the map. The specific locations of each domain were shown below. KS-N: β-ketoacyl synthase, N-terminal domain; KS-C: β-ketoacyl synthase, C-terminal domain; AT: Acyl transferase domain; DH: Dehydratase domain; ER: Enoyl reductase domain; KR: β-ketoacyl reductase domain; ACP: Acyl carrier protein; TE: Thioesterase domain.

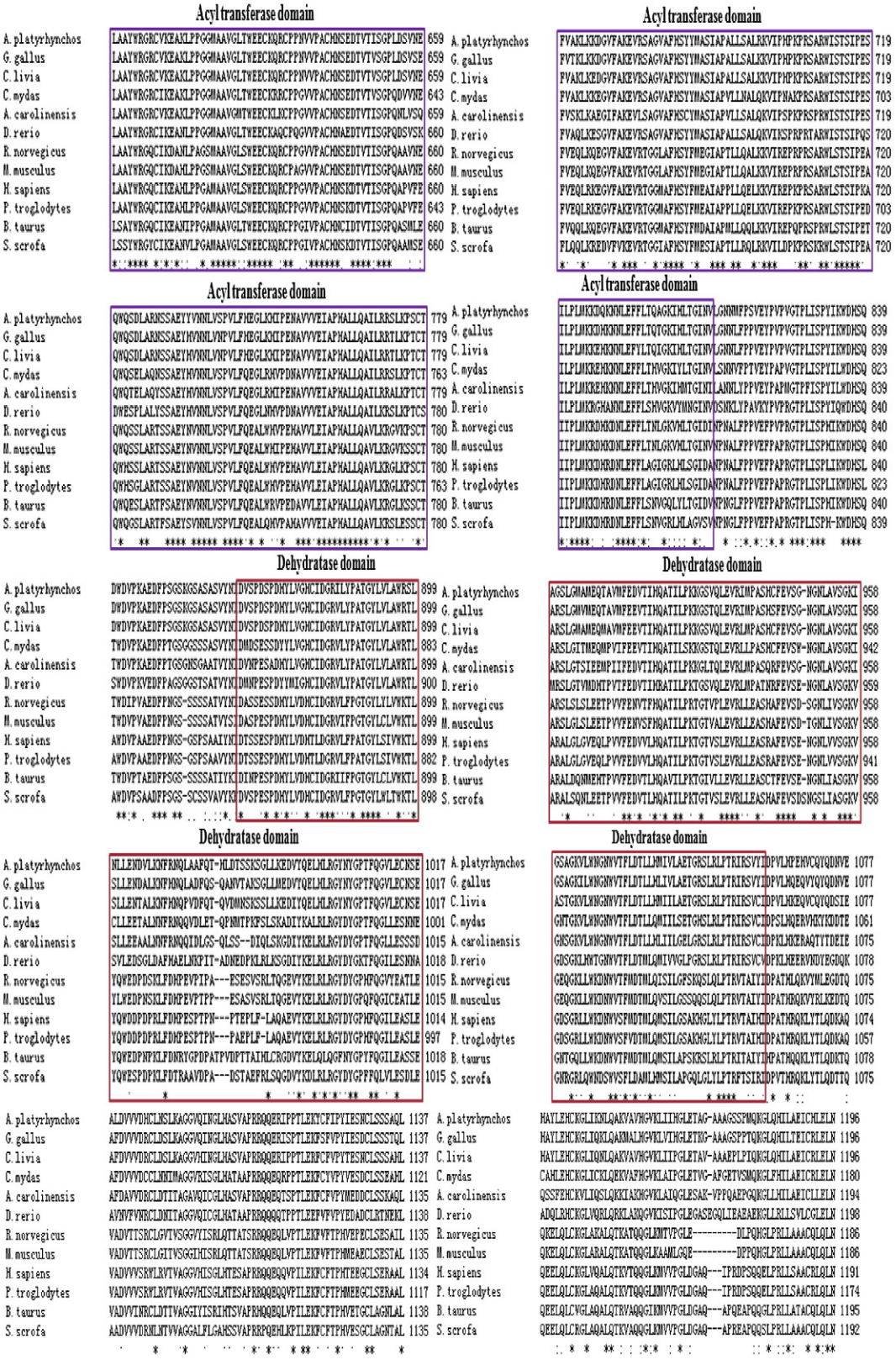
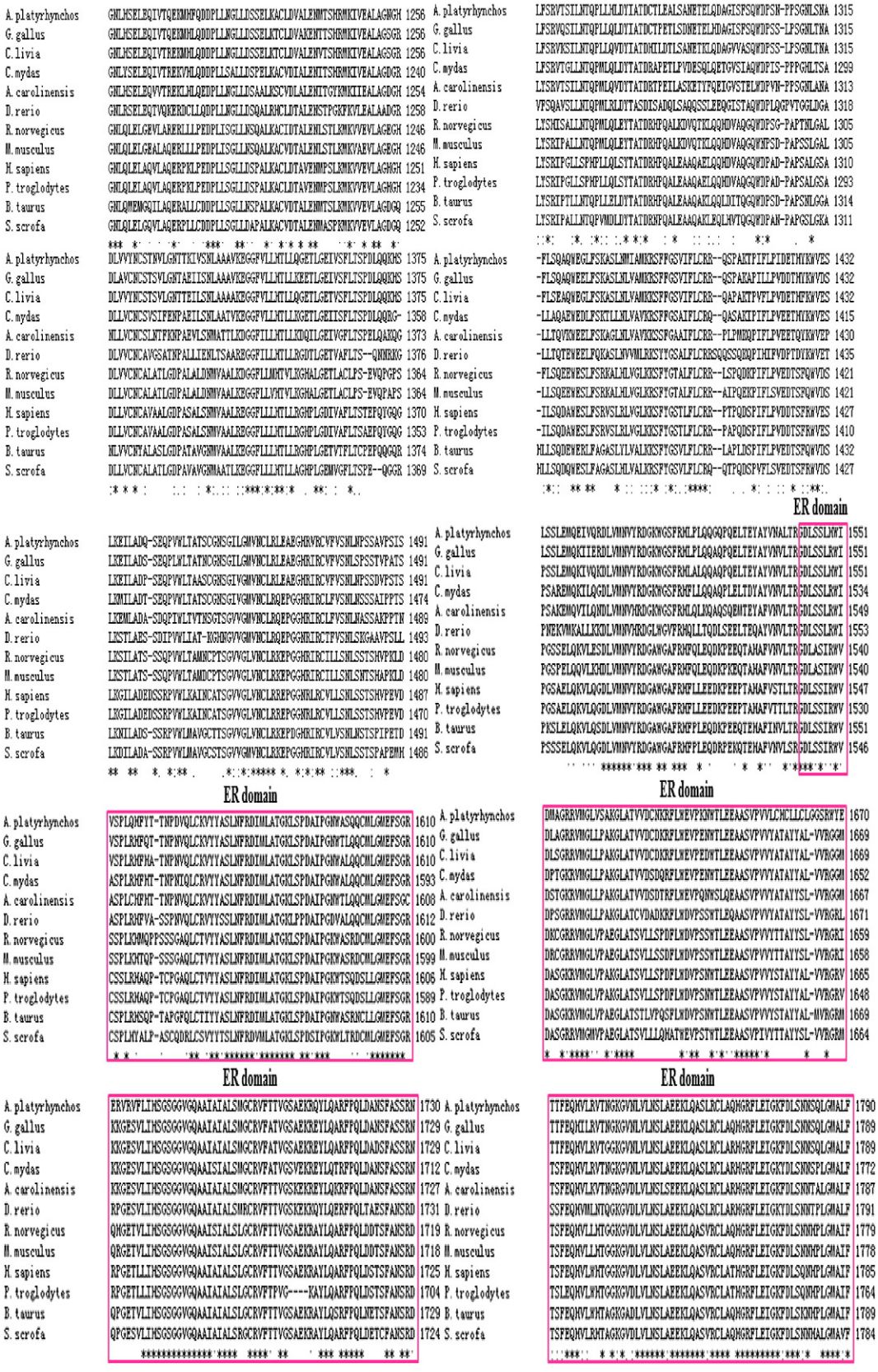


Fig. 2 (*continued*).

isoelectric point of 6.07. From this, we deduce that the sequence we cloned is the gene for Peking duck FAS.

Early studies have identified that expression of the FAS gene is controlled primarily at the level of transcription and is responsive to both nutritional and hormonal signals [24,25,26]. In diabetic mice, insulin administration causes a significant and rapid induction of FAS mRNA expression. The dramatic induction of hepatic FAS mRNA by fasting/refeeding is prevented by cAMP and by streptozotocin induced diabetes [27]. Moreover, FAS mRNA expression decreased dramatically after fasting and rapidly restored to a level equal to or exceeding the



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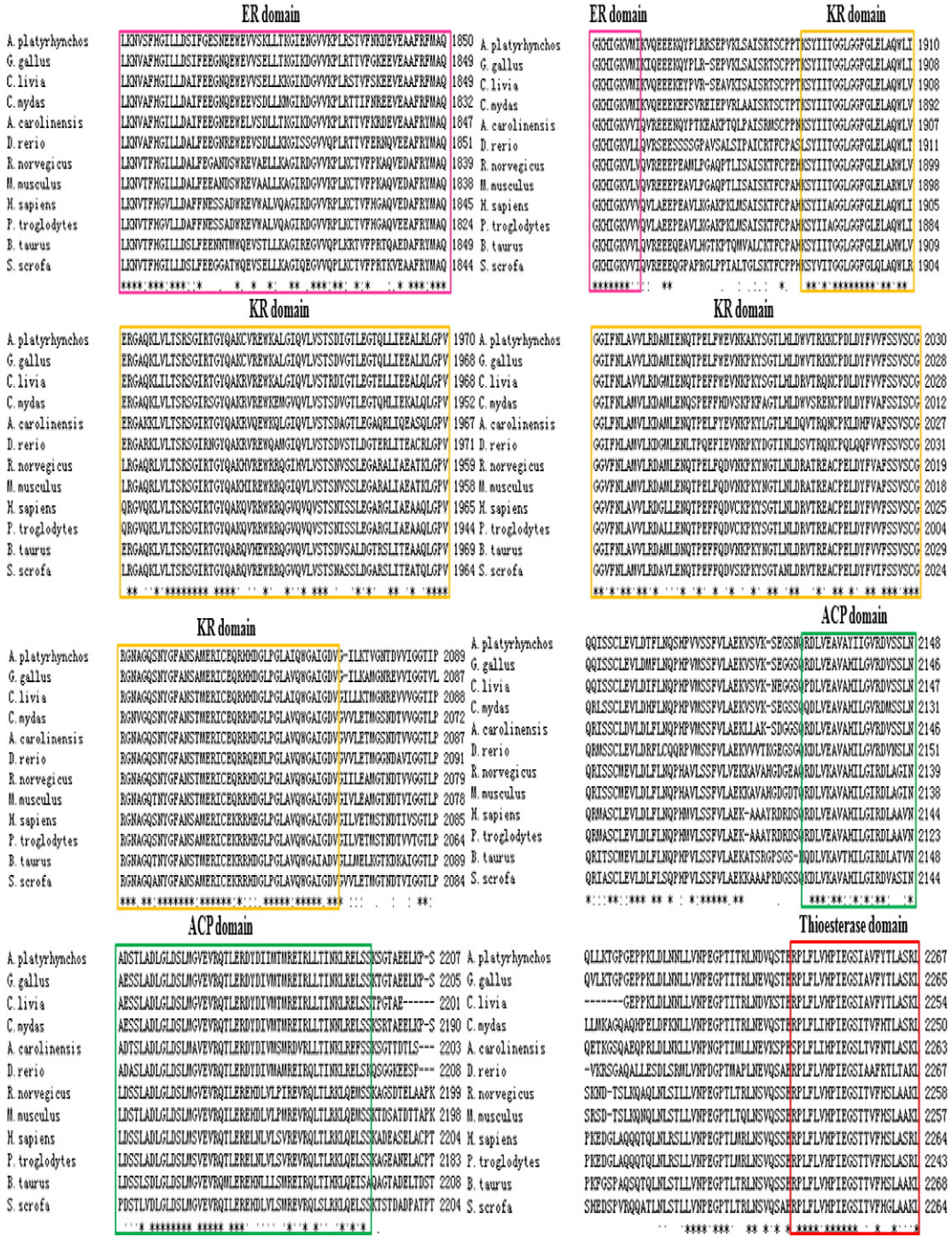
original fed state by refeeding [28]. In goose primary hepatocytes, both insulin and glucose increase FAS activity, gene expression, and TG accumulation [29]. Also, it should be emphasized that FAS can be activated by a combined effect of glucose and insulin in activating sterol regulatory element-binding protein-1c (SREBP-1c), which is an important transcription factor regulating lipid metabolic related gene



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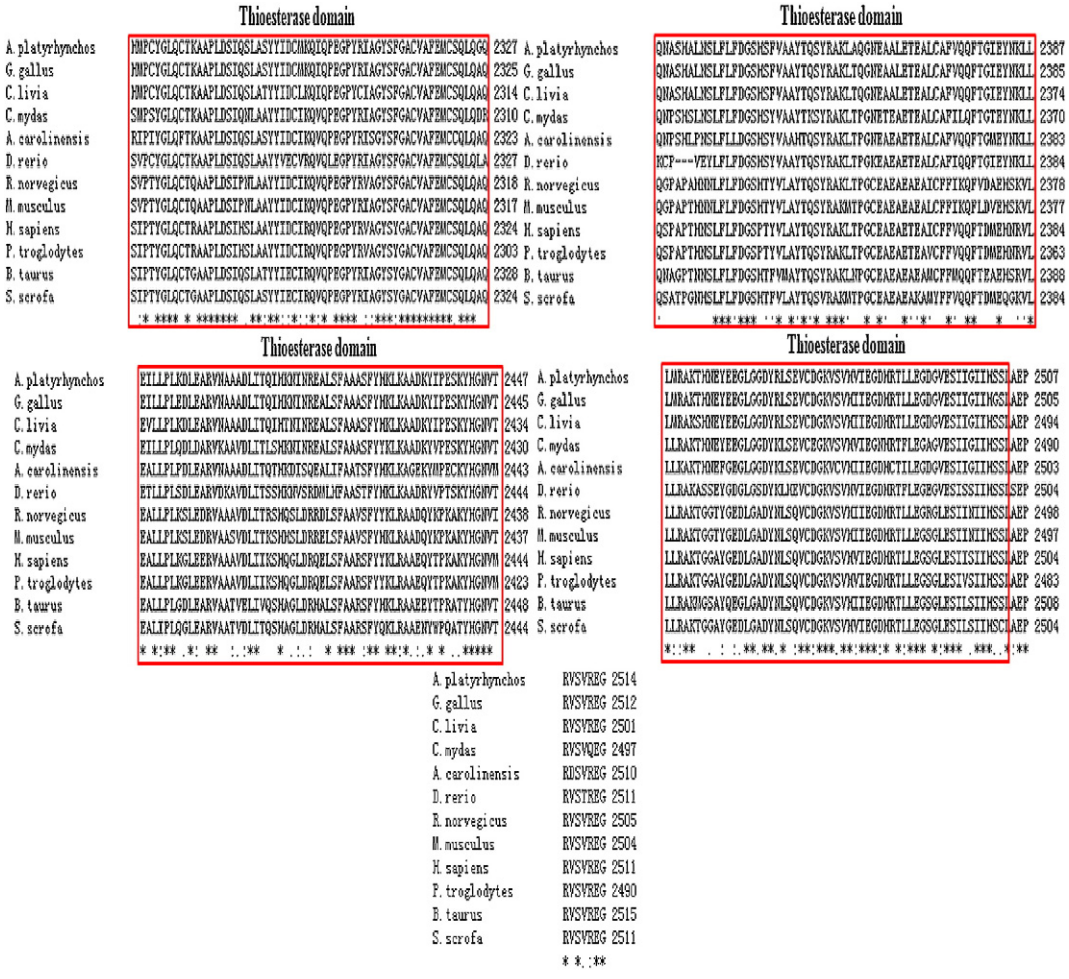
expression [29,30]. In addition, dietary polyunsaturated fatty acids (PUFA) could suppress hepatic FAS gene expression, which is mediated by a pathway independent of peroxisome proliferator-activated receptor-α (PPARα) [31]. In goose preadipocytes, expression of FAS can be up-regulated by a high concentration of oleic acid [16]. Similarly, in our study, we found that duck FAS gene expression was also up-regulated with increasing concentrations of oleic acid ($P < 0.05$) (Fig. 3). Additionally, our results showed that oleic acid enhanced duck preadipocyte viability, but at high concentrations, such as 1000 μM, oleic acid decreased cell viability due to its lipotoxic effects ($P < 0.05$) (Fig. 4). These results demonstrate that oleic acid would stimulate FAS mRNA expression in duck preadipocytes. However, high concentrations of oleic acid might decrease FAS gene expression due to its toxic effect on cell viability.

In poultry exogenous fatty acids are essential for adipogenesis. Compelling evidence has shown that oleic acid may promote adipogenic gene expression and lipid accumulation [16,17,32]. As a key gene in the DNL, FAS is of great importance for adipogenesis. In chicken, adipogenesis is induced by oleic acid and the expression level of FAS gradually increases for the first 24 h and remains high



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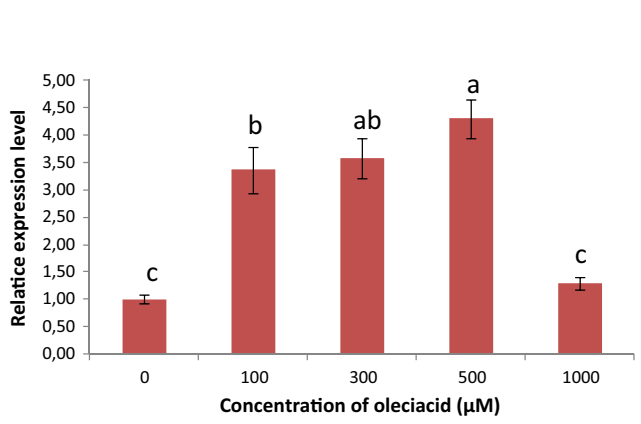

Fig. 3. Relative expression level of FAS gene in duck preadipocytes treated with different concentrations of oleic acid for 12 h. The data shown are mean ± S.D. The mRNA expression level in the cells without oleic acid treatment was assigned as control. The different lowercase letters at the top of each bar indicate significant differences between the treatments ($P < 0.05$).

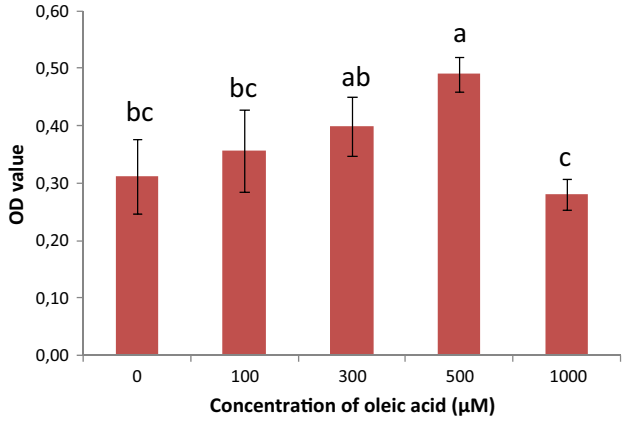

Fig. 4. Viability of duck preadipocytes cultured in the medium supplemented with different concentrations of oleic acid for 12 h. The data shown are mean ± S.D. Viability of the cells cultured in the medium in the absence of oleic acid was assigned as control. Different lowercase letters at the top of each bar indicate significant differences between the treatments ($P < 0.05$).

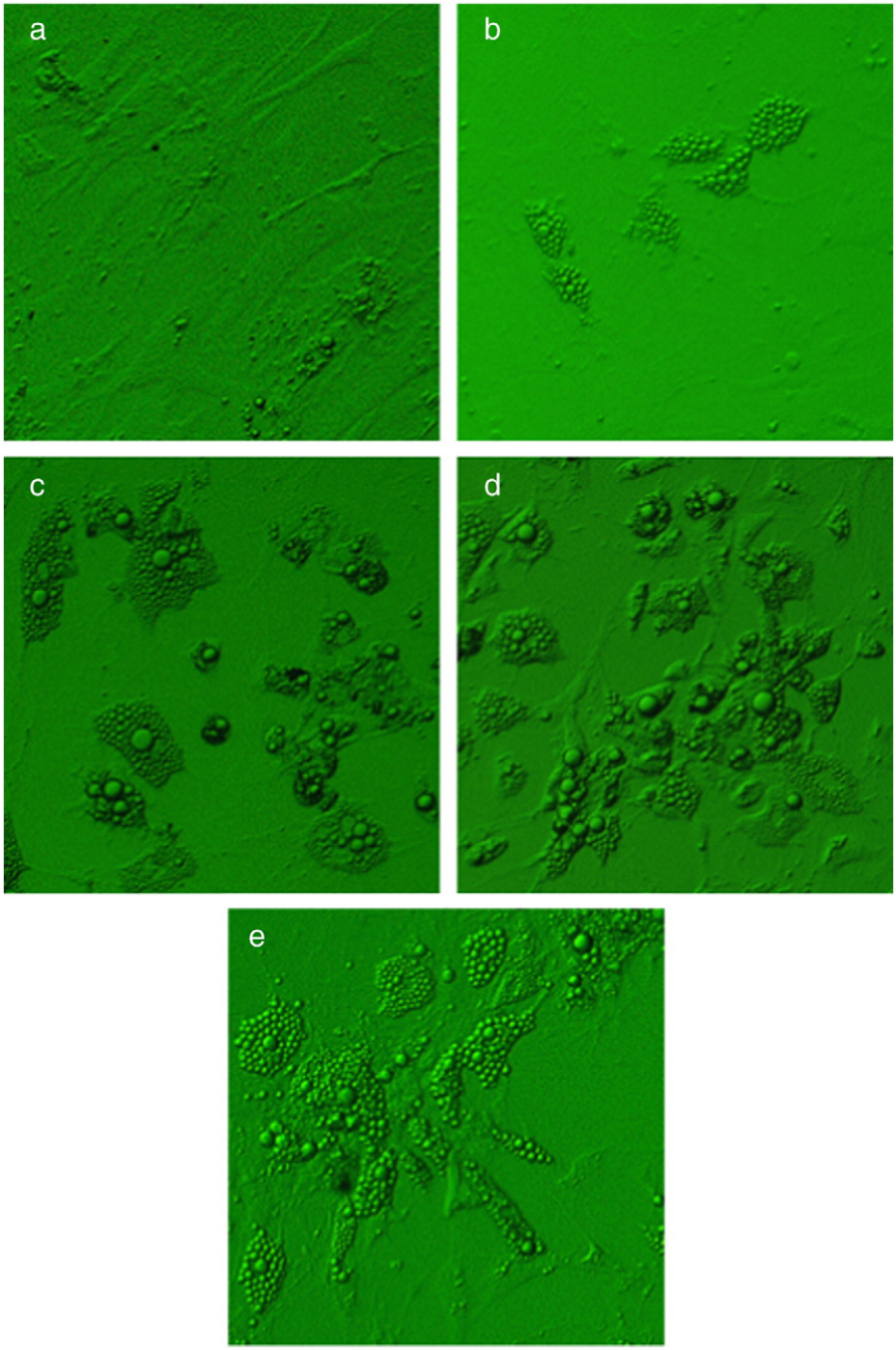

Fig. 5. Morphological changes of duck preadipocytes during differentiation. Duck preadipocytes were untreated (a), cultured with 300 μM oleic acid for 12 h (b), 24 h (c), 36 h (d), and 48 h (e). Cells were observed by phase contrast microscopy at 100 × magnification.

for 48 h [14]. Meanwhile, inhibition of FAS by standard inhibitors (such as cercumin or tannic acid) significantly suppresses the differentiation and lipid accumulation in 3T3-L1 preadipocytes [12,33]. These findings suggest that FAS is essential to promote adipocyte differentiation. In the present study, duck preadipocytes were induced to differentiate in the medium supplemented with 300 μM oleic acid. We found that most preadipocytes have differentiated and accumulated huge lipid droplets after administration of oleic acid for 48 h (Fig. 5). During adipogenesis, the expression of FAS mRNA was notably up-regulated for the first 24 h; however, it declined to a lower level over 36–48 h (Fig. 6). Interestingly, one of our early studies showed that during the early growth stages, FAS mRNA in duck subcutaneous adipose tissue exhibited a 'rise and decline' expression pattern. Moreover, the changes in FAS mRNA expression correlated to the changes in subcutaneous adipose tissue lipid content [34]. Altogether, these results indicate that FAS plays an important role in the early stage of duck adipogenesis. However, the molecular mechanism of how FAS promote duck adipogenesis is currently unknown and further research is needed.

5. Concluding remarks

For the first time we have successfully isolated the whole-length coding sequence of Peking duck FAS. Duck FAS encodes a 2515 amino

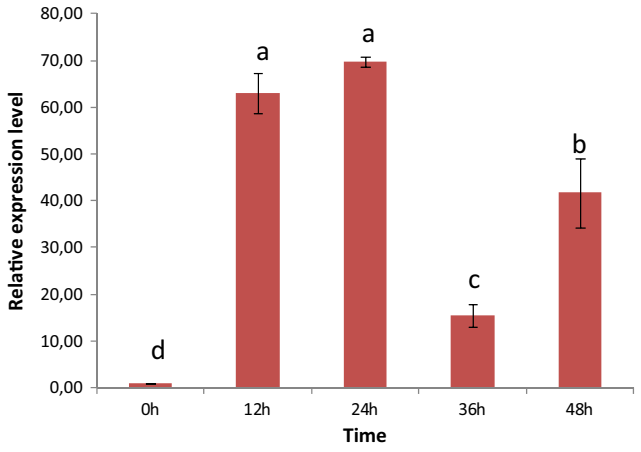

Fig. 6. Relative expression level of duck FAS gene in the preadipocytes during differentiation. The data are shown as mean ± S.D. The mRNA expression level in the cells without oleic acid treatment was assigned as control. The different lowercase letters at the top of each bar indicate significant differences between different time points ($P < 0.05$).

acid protein and shows high nucleotide and amino acid homology to other avian species. In addition, oleic acid was able to significantly stimulate FAS mRNA expression in duck preadipocytes. However, a high level of oleic acid decreased FAS expression due to its toxic effect on the cell viability. Furthermore, we found that FAS mRNA was highly expressed in the early stage of duck adipogenesis. Taken together, these results indicate that FAS plays an important role for duck adipocyte differentiation.

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Author contributions

Proposed the theoretical frame: JW, FD; Conceived and designed the experiments: FD, JW; Contributed materials and analysis tools: QL, WS, CG; Performed the experiments: QL, HH, CS; Analyzed the data: FD, XY; Wrote the paper: FD.

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