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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).