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Regulation and Expression of ELOVL Fatty Acid Elongase-5 Genes with Overfeeding in Goose Fatty Liver

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Abstract
Goose liver was an important economic trait which can be affected by high carbohydrate diet contents. So, fatty acid elongase ELOVL plays an important role in the synthesis of long-chain polyunsaturated fatty acids (LCPUFA). We hypothesized that ELOVL5 are involved in goose fatty liver development. To address this, we determined the response of goose ELOVL5 gene to overfeeding and their expression in goose liver and primary hepatocytes with related factors (glucose, fatty acid and insulin). Overfeeding expression data indicated that ELOVL5 was significantly reduction after two days of overfed. In primary hepatocytes data expression by quantities PCR was not affected by glucose and palmitate treatment while reduction expression by high level of insulin. Bioinformatics analysis of the sequence gene was indicated considerably conserved among avian species.
Keyword: Fatty liver, Goose, ELOVL fatty acid elongase-5, Overfeeding
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Introduction

Fatty acids one of the important sources of energy in the cell biology and playing vital role in the regulation of cellular signaling by the reflecting the cellular homeostasis. The cell metabolic state is influences the lipid pool in by two ways; on one hand it alters the lipid storage lipid and on the other hand it alters fatty acid oxidation in order to generate energy.

Seven different types of ELOVL proteins were found in mammals, which includes both enzymes ubiquitously expressed and some which are tissues specific enzymes. They are characterized as monosaturated and polysaturated fatty acid elongases. Elovl3, Elovl6, Elovl1 and Elovl7 belong to the saturated and monounsaturated fatty acid elongases while the Elovl2, Elovl4 and Elovl5 are classified into polysaturated fatty acid elongases (Leonard *et al.* 2004).

The Elovl5 was extremely expressed in testis, adrenal glands and liver, it has been found to be expressed, to some extent, in all tissues tested (LEONARD *et al.* 2000). The enzyme has been suggested to play an important role in liver development during the postnatal stage in rat (Wang *et al.* 2005). Elovl5 is suggested to be involved in elongation of polyunsaturated fatty Acyl-CoA substrates of 18 and 20 carbons in length (Parker-Barnes *et al.* 2000; Moon *et al.* 2001; Inagaki *et al.* 2002; Wang *et al.* 2005). In rat primary hepatocytes cell the Elovl5 expression an increased elongation of arachidonic acid (20:4, n-6) and eicosapentaenoic acid (20:5, n-3) into adrenic acid (22:4, n-6) and docosapentaenoic acid (22:5, n-3) (Wang *et al.* 2008). The highly expression gave rise to changed fatty acid content, which in turn affected the lipid and carbohydrate composition. Elovl5 deletion lead to develops hepatic steatosis in mouse with increased hepatic cholesterol and triglyceride levels by increasing activation of sterol regulatory element-binding protein-1c (SREBP-1c) and its target genes (Moon *et al.* 2009). The levels of arachidonic acid (20:4, n-6) and docosahexaenoic acid (22:6, n-3) were reduced in this mouse model. However, there was an increased elongation activity of ELOVL2 and ELOVL6, pointing towards compensation of these enzymes in the Elovl5-ablated mice.

In this study, we are used diet content high carbohydrate for fed Landes geese for 19 days, at which time the average weight of a fatty liver could reach 800g, approximately more than 10 time than the normal weight. We hypothesized that goose liver Elovl5 was involved in the development of fatty liver in a different way. To test this, Landes geese were normally fed or overfed, and their livers were used for transcriptome analysis with RNA-sequencing (RNA-seq) technology. The differentially expressed gene resulted from this analysis was subjected to further bioinformatics analysis. Indeed, Elovl5 was extremely differentially expressed gene, and a majority of Elovl5 was down-regulated in overfed geese compared to normally fed geese. This result was verified by quantitative PCR. To understand how the Elovl5 was down-regulated in the context of fatty liver, we treated goose primary hepatocytes with fatty liver-related factors, including high levels of glucose, fatty acids and insulin. Together, the present study suggests that the reduction of Elovl5 was expression is required for the development of goose fatty liver.

Materials and methods

Animals Experiment

Healthy Landes geese one-day-old were purchased from Wu Wang Farm (Chuzhou, China) and randomly divided into two groups, i.e., a control and an overfeeding group. The geese were raised under the condition of natural light and temperature. The control geese were allowed *ad libitum* feeding of cooked maize, while the overfed geese were provided with a diet that contains cooked corn, 1% plant oil and 1% salt. All the geese were kept in a cage with free access to water. For the overfeeding group, a 5-day-long pre-overfeeding was performed to prepare the geese for formal overfeeding, which lasted 19 days. During the pre-overfeeding period, the feed intake of the geese was gradually increased from 100 g to 300 g per day. The formal overfeeding began at 70-day-old of the geese. The following protocol was applied: in the first 5 d the daily feed intake (3 meals a day) reached 500 g, followed by 800 g of daily feed (4 meals a day) for the following week and 1,200 g of daily feed intake (5 meals a day) for the remaining days. The geese were sacrificed at 70, 77, 84 and 89 days of age. The livers from the geese were snap-frozen in liquid nitrogen and stored at -70 °C until use. All animal protocols were approved by the Yangzhou University Animal Ethics Committee.

Preparation of goose primary hepatocytes

Hepatocytes were isolated from Landes goose embryos at 23 days' post-hatch. The preparation was performed as previously described (Osman *et al.* 2016b).

After the primary hepatocytes were obtained, the cells were dilute with culture medium to 1×10^6 cells/ml, plate 1×10^6 cells per well in 12-well dishes, followed by incubation in 5% CO_2 incubator at 38 ℃ until treatment. The media was renewed at first 6 h of incubation and every 24 h for later incubation.

Treatment of cultured goose primary hepatocytes with glucose, fatty acids and insulin

The isolated hepatocytes were cultured for at least 24 h before any treatments. The cell culture treatment was performed as previously described (Osman *et al.* 2016a). The control cells were treated with serum free culture media containing 11.6lL/mL of HCl. All the primary hepatocytes were rinsed with PBS twice, followed by harvesting the cells at the end of treatment with 1 mL TRIzol Reagent (Cat. No. 15596026, Life, USA) per well.

Isolation of total RNA and amplification to cDNA:

Total RNA was extracted by TRIzol (TIANGEN BIOTECH (Beijing) CO., LTD) from the liver of the overfeeding and control group, according to manufacturer's instructions. RNA was eluted in nuclease-free water and was subjected to DNAase treatment to remove genomic DNA. Both the quality and quantity of total RNA were assessed at OD A260/A280 values were ranged between 1.8 to 2, indicating high quality RNA using a NanoDrop® Spectrophotometer (Nano Drop Technologies, Inc. Wilmington, DE, USA). Samples were stored at −70 ºC before reverse transcription was performed. We reverse-transcribed 2 µg of RNA per sample following the manufacturer's instructions SuperQuickRT cDNA kit (CWBIO). The cDNA was stored at −20 ºC until analysis.

Determination of mRNA abundance by quantitative PCR

Quantitative analysis on gene expression was previously described (Zhang *et al.* 2013). Briefly, expression levels of ELOVL fatty acid elongase5 gene in the liver of the overfed and normally fed geese as well as the treated goose primary hepatocytes was performed with SYBR® Green Master Mix kit (Vazyme Biotech Co., Ltd). The primer for quantitative PCR was listed in **Table1**. The glyceraldehyde-3-phosphate dehydrogenase gene (*Gapdh*) gene was used as an internal control gene for normalization. Cycle threshold (Ct) was determined with the supplied software. The relative mRNA abundance of genes of interest was calculated using $2^{-\Delta\Delta Ct}$ and presented as fold change over control using the method previously described (Livak and Schmittgen 2001).

Table 1: primers used in this study

Gene	Forward 5'-3'	Reverse 5'-3'	Product size
ELOVL5	ATTCTGATACTCTTTCCTCCTC	TCCAGCAATGCGTCCTTA	235 bp
GAPDH	GCCATCAATGATCCCTTCAT	CTGGGGTCACGCTCCTG	200 bp

Bioinformatics analysis:

ELOVL fatty acid elongase5 and GAPDH genes primers were designed by Premier5® Software (PremierBiosoft, Palo Alto, CA, USA) and figures were constructed by Graph pad version 5 software.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 (SPSS China, Shanghai, China) for Windows. The statistical significance of differences among the means of the control and different treatments was determined by one-way analysis of variance. A $p<0.05$ was considered statistically significant.

Result

Induction of ELOVL fatty acid elongase5 gene in goose fatty liver by overfeeding

ELOVLs play an important role in mammalian LCPUFA and EFA metabolisms, which may have involved in regulate some biological processes and several metabolic disorders. As the gene has not been cloned and sequenced yet in goose, cDNA was synthesized from liver total RNA as template to specifically amplify the complete CDSs of the gene with the primers designed based on the sequences of their duck counterparts. The

amplicons were subsequently cloned, and several clones were sequenced. The sequence result was also validated by RNA-seq analysis of goose liver transcriptome that we performed. Bioinformatics analysis indicated that the sequences of the amplicons shared 98% identity to duck sequence, which confirmed the cloned amplicons was indeed goose ELOVL5 genes. Moreover, the predicted amino acid sequence of goose ELOVL5 shared 98, 94 and 93% homology to the counterparts of other animals, including duck (A. platyrhynchos), turkey (Meleagris gallopvo) and chicken (Gallus gallus), respectively (Table 2), suggesting the gene was considerably conserved among avian species.

Table2. Comparisons of goose ELOVL fatty acid elongase5 nucleotide sequences with their counterparts of some other animals

Species	GenBank accession number	Length (pb)	Nucleotide identity(%)
Duck	XM_005009190.2	888	98
Turkey	XM_003204431.3	888	94
Chicken	XM_015284840.1	887	93

Regulation of ELOVL 5 by-related factors in goose primary hepatocytes

To identify whether the differentially expressed of ELOVL fatty acid elongase5 gene was regulated by fatty liver-associated factors, we treated goose primary hepatocytes with different levels of glucose, free fatty acids (palmitate & oleate) and insulin. Data indicated that ELOVL5 has not been affected by glucose treatment (5.5, 25 and 50mM) Fig 2, while in Insulin 50mM and 100 was significantly inhibited ($p<0.05$) the expression of ELOVL5 gene Fig 3, 0.25mM but not 0.5mM Oleate has significantly ($p<0.05$) reduced only the expression of the gene (Fig. 4), and 0.25mM and 0.5mM Palmitate also did not significantly induced the expression of the genes, though the expression of the gene was slightly increased in the cells treated with 0.25mM oleate vs. control (Fig. 5).

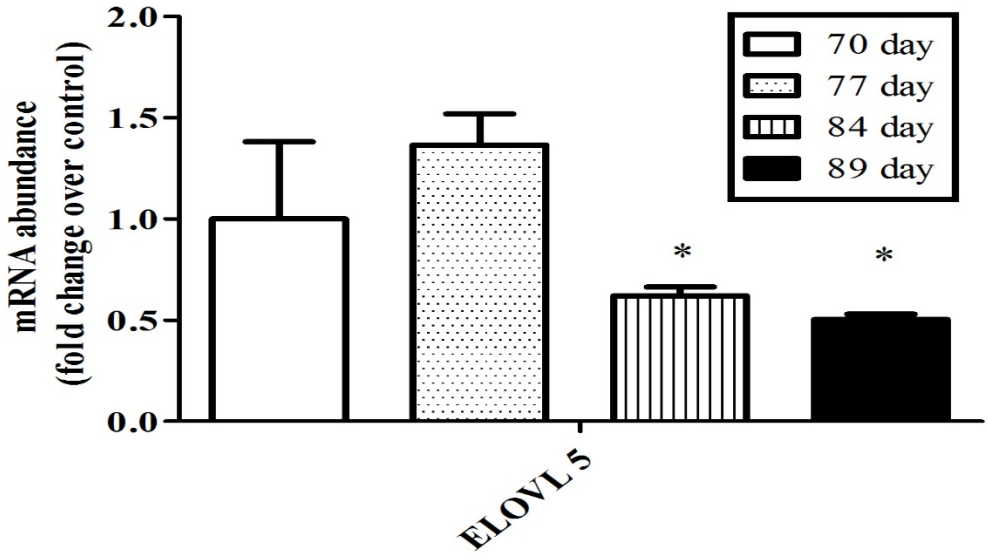

Fig. 1. Regulation of ELOVL fatty acid elongase5 gene in the livers of the overfed vs. normally fed geese. The messenger RNA abundance was determined by quantitative PCR. The control group consists of the geese that were normally fed, while overfeeding group consists of the geese that were overfed for 0, 7, 14, and 19 days (i.e., the overfed geese at 70, 77, 84 and 89 days of age). The messenger RNA abundance in the overfeeding group was presented as fold change over the control group. N=6. * denote $P<0.05$ vs. control, all data are presented as means ±SEM.

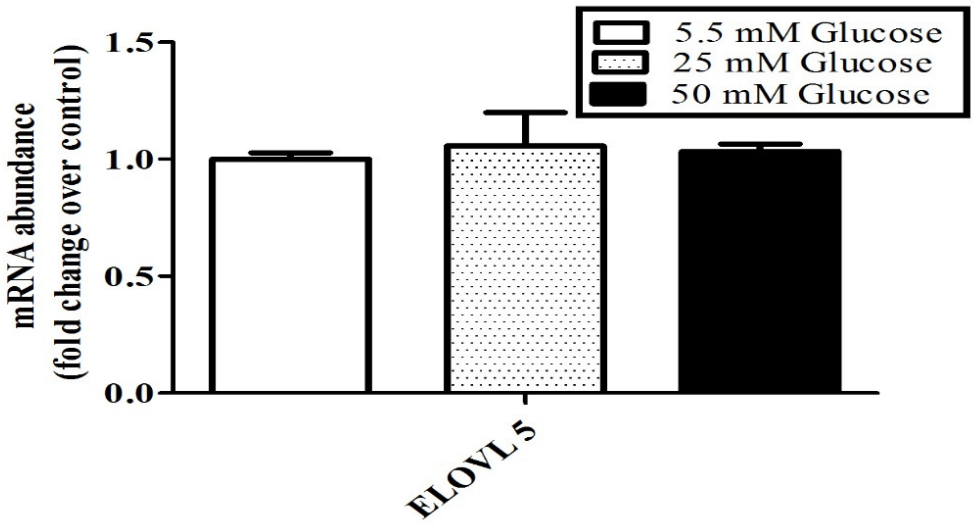

Fig.2. Messenger RNA expression of ELOVL fatty acid elongase5 gene in goose primary hepatocytes treated with vs. without glucose. The expression of the genes was determined by quantitative PCR. Primary hepatocytes isolated from goose embryos at 21 days of hatch were treated with 25 mM, and 50 mM glucose in serum-free media, while primary hepatocytes untreated with glucose were used as control. N=3. The average mRNA abundances of the genes in the hepatocytes treated with different levels of glucose are presented as fold change over the control. N=3. All data are presented as the means ± SEM.

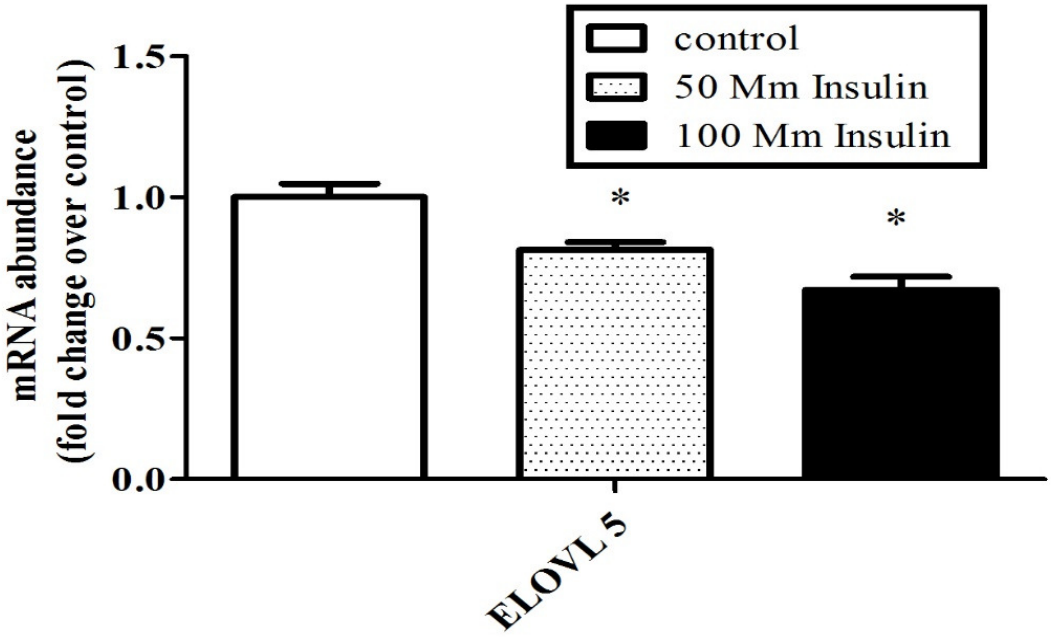

Fig.3. Messenger RNA expression of ELOVL fatty acid elongase5 in goose primary hepatocytes treated with vs. without insulin. The expression of the genes was determined by quantitative PCR. Primary hepatocytes isolated from goose embryos at 21 days of hatch were treated with 50 mM, and 100 mM insulin in serum-free media, while primary hepatocytes untreated with insulin were used as control. N=3. The average mRNA abundances of the genes in the hepatocytes treated with different levels of insulin are presented as fold change over the control. N=3. * denote $P<0.05$ vs. control. All data are presented as the means ± SEM.

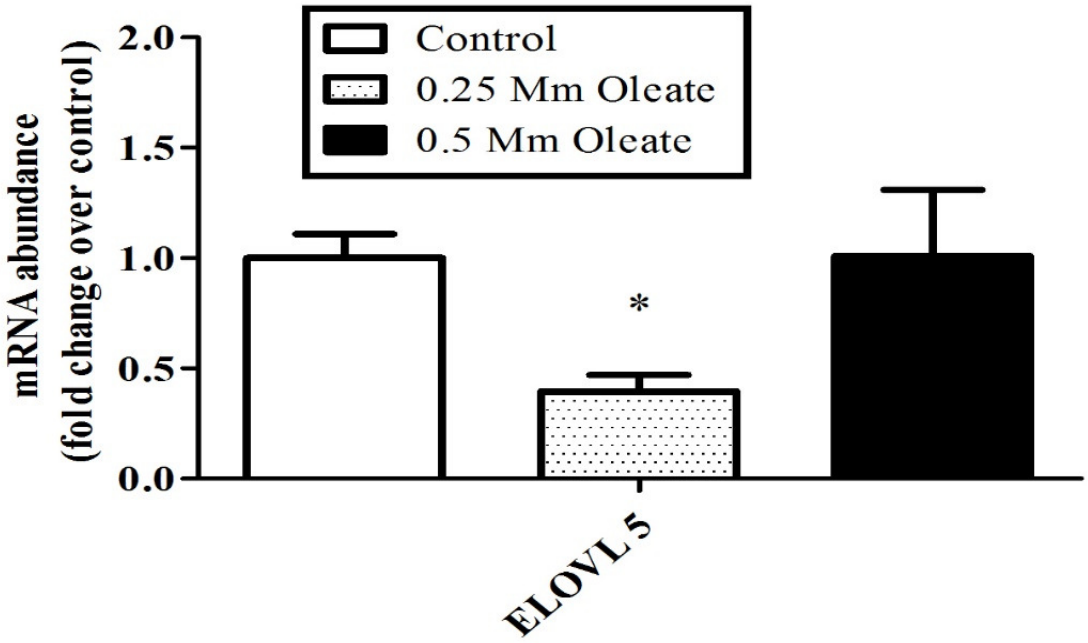

Fig. 5. Messenger RNA expression of ELOVL fatty acid elongase 1/2 in goose primary hepatocytes treated with vs. without Oleate. The expression of the genes was determined by quantitative PCR. Primary hepatocytes isolated from goose embryos at 21 days of hatch were treated with .25 Mm and .50 mM Oleate in complete cell culture media, while primary hepatocytes untreated with oleate were used as control. N=3. The average mRNA abundances of the genes in the hepatocytes treated with different levels of oleate are presented as fold change over the control. N=3. * denote $P< 0.005$ vs. control. All data are presented as the means ± SEM.

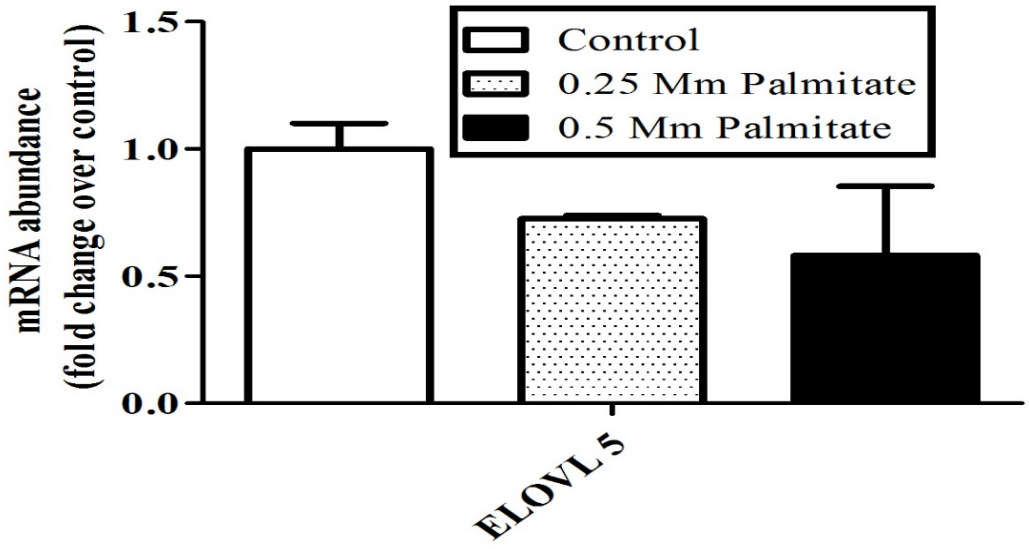

Fig. 4. Messenger RNA expression of ELOVL fatty acid elongase5 in goose primary hepatocytes treated with vs. without Palmitate. The expression of the genes was determined by quantitative PCR. Primary hepatocytes isolated from goose embryos at 21 days of hatch were treated with .25 and .5 mM Palmitate in complete cell culture media, while primary hepatocytes untreated with palmitate were used as control. N=3. The average mRNA abundances of the genes in the hepatocytes treated with different levels of palmitate are presented as fold change over the control. N=3. All data are presented as the means ± SEM.

Discussion

Goose, as the descendant of a migratory bird, has an excellent capacity to deposit fat in the liver (Hermier *et al.* 1991). In the goose industry, this capacity has been utilized for fatty liver (foie gras) production within 2–3 weeks of overfeeding. In this study, at 19 day of overfeeding, the pheno topic data (body and liver weights, and the ratio

of liver weight to body weight) of the overfed group was significantly ($p<0.01$) higher than the control group (Osman *et al.* 2016b).

Interestingly, the reduction of ELOVL5 was associated with the development goose fatty liver, which is opposite to the observation that the induction of hepatic ELOVL5 expression is associated with increased total hepatic fat content. ELOVL5was highly expressed in liver tissue and increased rapidly during the peak-laying period in laying hens (Zhang *et al.* 2017). The duck and turkey ELOVL5 activities were limited to C18 and C20 PUFA substrates, as found in most vertebrate (Agaba *et al.* 2004; Gregory *et al.* 2010; Gregory *et al.* 2011) and invertebrate ELOVL5 enzymes (Monroig *et al.* 2012).

In the present study, we found that ELOVL5 was decreased expressed in the goose fatty liver after two weeks from overfeeding period than control group. Pervious study suggested that the ducks and turkey ELOVL5 enzymes activities were different from chicken ELOVL5, which has unique DPA to 24:5n-3 activity (Gregory *et al.* 2013; Gregory and James 2014). Gregory et al (Gregory *et al.* 2013) found the relative abundance of the rats ELOVL5is lower of in the chickens. We speculated that the expression of goose ELOVL5 gene was regulated by different transcriptional factor(s). Indeed, we treated goose primary hepatocytes with higher dosages (25 or 50 mM) of glucose due to glucose transporter may pump a large amount of glucose into the hepatocytes in the liver when insulin resistance occurs in goose fatty liver, in addition to the diffusion of glucose, which may lead to intracellular glucose level much higher than blood glucose level (Han *et al.* 2009). The fatty liver-associated factors (i.e., hypoinsulinemia, hypoglycemia and hypolipidemia) was share in contribute to the reduction of the genes in goose fatty liver.

Moreover, the regulation expression of ELOVL5 gene by insulin and Oleate was different from that regulated by glucose and palmitate in goose hepatocytes cells. Transcription factors and several hormones (insulin, T3, glucocorticoids, and leptin) have no impact on hepatic Elovl-5 expression. Only PPARa, n-3 PUFA-enriched diets (Wang *et al.* 2005), high-fat diets and obesity affect Elovl-5 expression. suppression of Elovl-5 in high-fat-fed mice correlates with a decreased hepatic 20:4, n-6-to-18:2, n-6 ratio (Wang *et al.* 2006).

IN CONCLUSION: The findings suggest the Elovl5 gene is important to the development goose fatty Liver. Our data indicated that goose fatty liver was different from other animal organism fatty liver in some respects, which supports the notion that goose liver is a unique model for fatty liver study in birds.

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