Original Article

Molecular Characterization of Goose Phosphoenolpyruvate Carboxylase Kinase 1 (Pepck) Gene and Its Potential Role in Hepatic Steatosis Induced by Overfeeding

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ABSTRACT

Over-accumulation of triglycerides (TGs) in goose hepatocytes leads to the formation of fatty acid liver. Phosphoenolpyruvate carboxylase kinase 1 (*PEPCK*) is regarded as the rate-limiting enzyme for gluconeogenesis, and there is evidence that *PEPCK* is involved in regulating hepatic glucolipid metabolism. Hence, we proposed that *PEPCK* may have a role in goose hepatic steatosis. To test our hypothesis, the present study was conducted to firstly determine the sequence characteristics of goose *PEPCK* and then to explore its role in overfeeding-induced fatty liver. Our results showed that goose *PEPCK* encodes a 622-amino-acids protein that contains highly conserved oxaloacetate-binding domain, kinase-1 and kinase-2 motifs. *PEPCK* had higher mRNA levels in goose liver, and overfeeding markedly increased its expression in livers of both Sichuan White and Landes geese ($p<0.05$). Besides, expression of *PEPCK* was positively correlated with hepatic TG levels as well as plasma glucose and insulin concentrations. Additionally, in cultured goose primary hepatocyte, treatment with either oleic acid (0.8, 1.2 or 1.6 mM) or linoleic acid (0.125 or 0.25 mM) significantly ($p<0.05$) enhanced the expression of *PEPCK*. Taken together, these data suggested a role for *PEPCK* in the occurrence of overfeeding-induced goose hepatic steatosis.

INTRODUCTION

Gluconeogenesis is a process through which non-carbohydrate sources, such as lactic acid, allyl alcohol, amino acids, and glycerol, can be converted into glucose or glycogen (Chakravarty *et al.*, 2007). In vertebrates, the liver is generally considered as the primary site of gluconeogenesis, which enables the organism to maintain relatively steady levels of circulating glucose. Phosphoenolpyruvate carboxylase kinase 1 is regarded as the key rate-limiting enzyme of gluconeogenesis because the amount of its transcription directly determines the rate of gluconeogenesis (Herzig *et al.*, 2001). Noticeably, gluconeogenesis is also involved in hepatic triglyceride (TG) synthesis by providing the essential raw materials glyceraldehyde 3-phosphate (Nye *et al.*, 2008). Following the conversion of non-carbohydrate sources to oxaloacetate (OAA) during the process of tricarboxylic acid cycle (TCA), PEPCK catalyzes the conversion of OAA into phosphoenolpyruvate (PEP) (Montal *et al.*, 2015), indicating that *PEPCK* may play a role in regulating hepatic glucose and lipid metabolism.

Increasing evidence has shown that *PEPCK* is crucial for hepatic glyceroneogenesis. A previous study reported that downregulation of *PEPCK* mRNA in diabetic (db/db) mice by RNAi led to decrease plasma glucose, insulin, and TG concentrations (Gómez-Valadés *et al.*, 2008). Liver-specific knockout of mice *PEPCK* significantly promoted gluconeogenesis and increased hepatic glycogen output, but resulted

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in the loss of oxaloacetate metabolic capacity in the TCA cycle, eventually leading to fatty liver (Beale *et al.*, 2007). Moreover, there was evidence that *PEPCK* functioned as a factor involving the oxaloacetate replenishment pathway (Owen *et al.*, 2002). In contrast to the situation in experimental animals, much less is known about the role of *PEPCK* in hepatic glycolipid metabolism of domestic animals.

It has been reported that overfeeding goose with corn enhanced the ability of *de novo* synthesis of fatty acids and decreased the secretion of VLDL-TG, resulted in the over-accumulation of TGs in the hepatocytes and formation of goose fatty liver (Hermier, 1997; Hermier *et al.*, 1999; Davail *et al.*, 2000; Davail *et al.*, 2003), but the underlying mechanisms remain largely unknown. Since hepatic gluconeogenesis constitutes the core part of hepatic glycolipid metabolism and *PEPCK* is considered as the key rate-limiting enzyme in the gluconeogenesis pathway, we postulated that *PEPCK* might be involved in the regulation of goose hepatic steatosis although its exact role has not been described.

Therefore, in this study, the full-length cDNA sequence of goose *PEPCK* gene was obtained using the rapid-amplification of cDNA ends (RACE) method. Then, the effects of overfeeding on the mRNA expression of *PEPCK* in the livers of both Sichuan white (SW) and Landes (LD) geese were examined. Finally, goose primary hepatocytes were isolated and cultured for studying the effects of palmitic acid, oleic acid, and linoleic acid on the mRNA levels of *PEPCK*. These data may provide a better understanding of the molecular mechanisms underlying goose hepatic steatosis.

MATERIALS AND METHODS

Ethics Statement

All experimental protocols involving animal manipulation were approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University (Ya'an, Sichuan, China) under the approved number: DKY-B201000805.

Animals and Sample Collection

A total of thirty male geese were used in this study, including eighteen SW geese (*Anser cygnoides*) and twelve LD geese (*Anser anser*). They were hatched at the same time and raised under the condition of natural light and temperature at the Experimental Farm for Waterfowl Breeding of Sichuan Agricultural University (Sichuan, China).

Six male SW geese were used to analyze the expression level of goose *PEPCK* gene in different

tissues. They were provided with free access to feed and water until they were sacrificed at 10-days of age. Various tissues, including the liver, subcutaneous fat, abdominal fat, intestine, breast muscle, leg muscle, heart, kidney, testis, and brain were collected from six male SW geese, snap-frozen in liquid nitrogen and finally stored at -80 °C until total RNA extraction.

From 14 to 16 week of age, twelve male LD geese and twelve male SW geese were overfed with a carbohydrate-rich diet consisting of boiled and salted maize (14 MJ/kg, 9% protein and 0.45% fat) with 0.4% waterfowl fat and water added for four meals a day. During the overfeeding period, the geese were housed in individual cages with continuous free access to water. The temperature was 15-18 °C, and the hygrometry was 70-80%. On the last day of overfeeding (16 weeks), the geese were provided with water but deprived of food overnight. The next day, the geese were sacrificed by exsanguination within 10 min of capture, and a sample about 60g was immediately taken from each liver. A part of each sample snap-frozen in liquid nitrogen and finally stored at -80°C until RNA extraction, and the remaining samples were characterized for total TG content.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from each sample using TRIzol Reagent (Invitrogen) according to the manufacturer's instruction. The quality and quantity of total RNA were checked by electrophoresis on a 1.5% agarose gel. The cDNA was obtained by a cDNA Synthesis Kit (Takara, Japan) under the manufacturer's protocol with 1 ug of total RNA as a template.

Rapid Amplification of the *PEPCK* cDNA Ends

Primers (shown in Table 1) were designed according to the EST sequence of goose *PEPCK* obtained from the suppression subtractive hybridization (SSH) library (Pan *et al.*, 2010). 5'-RACE and 3'-RACE PCR were performed with the SMART™ RACE cDNA Amplification Kit (Clontech Laboratories, CA, USA), according to the manufacturer's instructions. The conditions of the Touchdown PCR reactions were: pre-denaturation at 94°C for 30 seconds; annealing at 72°C for 30 seconds; 5 cycles of pre-denaturation at 94°C for 30 seconds, annealing at 70°C for 30 seconds and extension at 72°C for 3min; 5 cycles of pre-denaturation at 94°C for 30 seconds, annealing at 68°C for 30 seconds and extension at 72°C for 3min; 27 cycles with a final extension at 72°C for 10 min, then stored at 4°C. The PCR products were separated

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Table 1 – RACE primer of PEPCK.

Gene	Primer name	Primer sequence (5'-3')	Annealing temperature(°C)
PEPCK	PF1	5'GGAGCAGCCATGAGATCTGAAGCAACAGC3'	71.0
	PR1	5' ATTTCCCTTGGCTGTCTTTCCGGAACC 3'	73.1

by electrophoresis on a 1.0% agarose gel and purified using E.Z.N.A.TM Gel Extraction Kit (Bioleaf Biotech Co., Ltd, Shanghai, China). The purified PCR products were ligated into the pMD-18T vector (Bio-Engineering, Dalian, China) and transformed into E. coli JM109 cells. Finally, the single and positive clones were selected and sequenced by the Shanghai Yingjun Biology Company (Shanghai, China).

Isolation and Culture of Goose Primary Hepatocytes

Hepatocytes were isolated from three 30-day-old SW geese according to the methods introduced by Seglen (Seglen, 1973), and were then cultured with Dulbecco's modified Eagle's medium (DMEM; Hyclone, Utah, USA) containing 10% fetal bovine serum (Gibco, USA). The cells were incubated at 40°C in a humidified atmosphere containing 5% CO_2, and the medium was renewed after 3 hours of culture. 24 hours later, the medium was replaced with serum-free DMEM medium. In order to induce hepatic steatosis of goose primary hepatocytes, the culture media were supplemented with palmitic acid (0.3, 0.6, 0.9 or 1.2 mM), oleic acid (0.4, 0.8, 1.2 or 1.6 mM) or linoleic acid (0.125, 0.250, 0.500 or 1.000 mM) cultured for 24 hours.

Quantitative Real-time PCR (qRT-PCR)

The primers used for qRT-PCR were designed according to goose *PEPCK* sequence obtained in this study. *18S rRNA* (GenBank NO. L21170.1) and β-*actin* (GenBank NO. M26111.1) were used as the internal controls. All primers (shown in Table 2) were designed using the Primer Premier 5 software (Premier Biosoft International, USA). The mRNA expression levels of *PEPCK* were measured by qRT-PCR. The qRT-PCR was performed in a 96-well Bio-Rad iQ5 (Bio-Rad Laboratories, USA) using a Takara ExTaq RT-PCR Kit and SYBR Green as the detection dye (Takara, Japan). Real-time PCR was carried out under the following condition: 1 cycle of pre-denaturation at 95°C for 10 seconds; 40 cycles of 95°C for 5 seconds, and 60°C for 40 seconds, and starting at a temperature of 55°C and increasing by 0.5°C every 10 seconds to determine primer specificity. All cDNA samples were tested three times, and the results were normalized to the levels of goose *18S rRNA* and β-*actin* expression.

Table 2 – Primers sequence of qRT-PCR.

gene	Primer Sequences (5'-3')	Fragment (bp)	Annealing temperature(°C)
PEPCK	F:5'GGGCAATCAATCCAGAAAATG3' R:5'CAGCTACGTTGGTAAAGATGGTG3'	114	60
18S	F:5'TTGGTGGAGCGATTTGTC3' R:5'ATCTCGGGTGGCTGAACG3'	129	60
β-*actin*	F:5'CAACGAGCGGTTCAGGTGT3' R:5'TGGAGTTGAAGGTGGTCTCG3'	92	60

Bioinformatic Analysis

The data of *PEPCK* sequence were edited using Editseq. Sequence alignment was performed with Megalign software. The NCBI BLASTn program on NCBI was used to identify homologous genes (https://blast.ncbi.nlm.nih.gov/Blast.cgi). ORFs of *PEPCK* gene was searched using NCBI ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/). The predicted amino acid sequence was analyzed with the ExPASy ProtParam System (http://web.expasy.org/protparam/). The amino acid sequence structure was analyzed by NCBI CDD (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Phosphorylation sites were predicted by NetPhos 3.1 Server (http://www.cbs.dtu.dk/services/NetPhos/). Protein subcellular localization was predicted by CELLO (http://cello.life.nctu.edu.tw/). Homology analysis of amino acid sequences was performed with DNAMAN software. Phylogenetic trees of different vertebrate *PEPCK* protein were constructed by using the neighbor-joining method with the software of Molecular Evolution Genetics Analyses (MEGA) version 7.0.

Data Analysis

The relative mRNA expression of target gene was calculated by the comparative Ct method ($2^{-\Delta\Delta Ct}$ methods) (Livak and Schmittgen, 2001). All data were subjected to analysis of variance (ANOVA), and the means were compared for significance using Tukey's test. The ANOVA and t-tests were performed using the SAS software (SAS Institute Inc., Cary, NC). Differences were considered statistically significant at $p<0.05$.

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RESULTS

Sequence Analysis of Goose PEPCK Gene

After sequencing and assembling the fragments, the full-length 2675bp of goose *PEPCK* (GenBank NO.MF767270) was obtained. The longest ORF of goose *PEPCK* was 1869bp encoding 622 amino acids. Subcellular localization prediction suggested that goose PEPCK protein was mainly located in the cytoplasm. The modified sites of residues prediction showed that goose PEPCK had 21 phosphorylation sites, including 12 Ser sites, 6 Thr sites, and 3 Tyr sites (Figure 1). Analysis of goose PEPCK protein showed its predicted molecular mass is 69.71 kDa, and the theoretical isoelectric point is 5.87 (pI = 5.87).

The amino acid sequence of goose PEPCK was compared with those of other species. Similar to other species, there was PEPCK-specific domain, kinase-
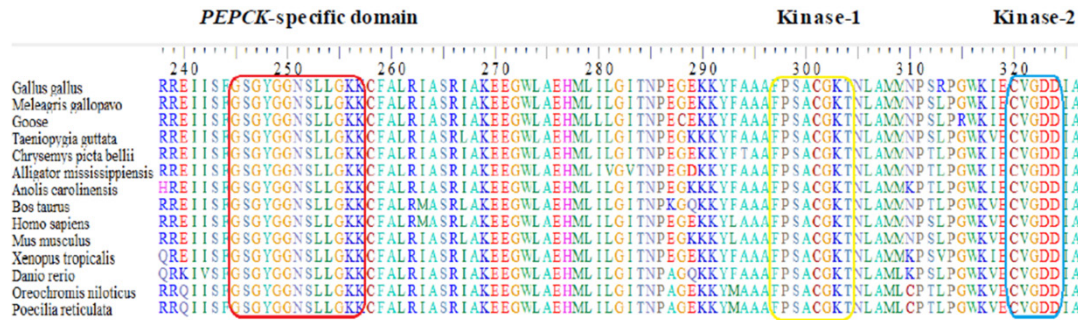
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1    acatggggaatgatcacaagagcactccaaagcaagtgaaaccagaccaacagaaaggggagccaaatacttgctgacatccacccaactcaa    93
94   tcttccaaagacaattgtgatcagtatttcaaatccctcagctagatccactgcaggctgcaagaATGCCCCCAGAGTTGAAAGCTGAAGTA    185
1                                                                        M  P  P  E  L  K  A  E  V      9
186  AACATCGTGCCCAAGGTTACCCAGGGGGATCTGGAGAGCCTGCCTCCAGAAGCGAGGGAGTTCATTGAAAGTAATGCCAAGCTGTGCCAA     275
10   N  I  V  P  K  V  T  Q  G  D  L  E  S  L  P  P  E  A  R  E  F  I  E  S  N  A  K  L  C  Q       39
276  CCTGAGAACATTCACATCTGCGATGGCTCAGAAGAAGAAAACAAAAAAATTCTGGACATCATGGTAGAGCAAGGCATGATTAAGAAGCTG     365
40   P  E  N  I  H  I  C  D  G  [S] E  E  E  N  K  K  I  L  D  I  M  V  E  Q  G  M  I  K  K  L      69
366  AGCAAGTATGAGAACTGCTGGTTGGCCCTCACCGACCCAAGAGATGTAGCAAGAATTGAGAGCAAAACTGTCATTATCACTCAAGAACAG     455
70   [S] K  Y  E  N  C  W  L  A  L  T  D  P  R  D  V  A  R  I  E  [S] K  T  V  I  I  T  Q  E  Q     99
456  AGAGATACCACTCCAATCCCTAAAACTGGAACTAGCCAGCTGGGACGCTGGATGTCAGAAGAAGATTTTGAGAAGGCCTTCAATACCAGA    545
100  R  D  T (T) P  I  P  K  T  G  T  S  Q  L  G  R  W  M [S] E  E  D  F  E  K  A  F  N  T  R     129
546  TTCCCAGGCTGCATGCAAGGACGCACAATGTACGTCATCCCCTTCAGCATGGGGCCTATTGGGTCTCCTTTGTCCAAGATTGGGATTGAG    635
130  F  P  G  C  M  Q  G  R  T  M  Y  V  I  P  F  S  M  G  P  I  G [S] P  L  S  K  I  G  I  E     159
636  CTGACAGATTCACCATACGTGGTGGCCAGCATGAGGATCATGACGCGGATGGGAACAGCTGCTTTGAAAGCCCTGAGCAATGGAGAGTTT    725
160  L  T  D  S  P /Y\ V  V  A [S] M  R  I  M (T) R  M  G  T  A  A  L  K  A  L [S] N  G  E  F     189
726  GTAAAATGCCTTCACTCGGTTGGATGTCCTCTACCACTAAAAGAACCATTAATCAACAACTGGCCGTGCAACCCAGAGTTAACGCTGATT    815
190  V  K  C  L  H  S  V  G  C  P  L  P  L  K  E  P  L  I  N  N  W  P  C  N  P  E  L  T  L  I     219
816  GCTCATCTCCCGGATCGCAGAGAGATCATTTCATTTGGCAGCGGTTATGGAGGAAACTCCTTACTGGGGAAAAAATGCTTTGCTCTCAGA    905
220  A  H  L  P  D  R  R  E  I  I [S] F  G  S  G  Y  G  G  N  S  L  L  G  K  K  C  F  A  L  R     249
906  ATTGCCAGCAGAATTGCCAAGGAAGAGGGCTGGTTAGCAGAGCACATGCTGATCCTGGGCATTACAAATCCAGAATGTGAGAAGAAGTAT    995
250  I  A  S  R  I  A  K  E  E  G  W  L  A  E  H  M  L  I  L  G  I  T  N  P  E  C  E  K  K /Y\   279
996  TTTGCTGCAGCATTCCCTAGTGCCTGTGGAAAAACTAACTTGGCCATGATGAACCCAAGCCTGCCAAGATGGAAGATTGAGTGCGTGGGT    1085
280  F  A  A  A  F  P  S  A  C  G  K  T  N  L  A  M  M  N  P  S  L  P  R  W  K  I  E  C  V  G     309
1086 GATGATATCGCCTGGATGAAATTTGATGAACAAGGCCACTTAAGGGCAATCAATCCAGAAAATGGCTTTTTTGGAGTTGCCCCTGGAACC    1175
310  D  D  I  A  W  M  K  F  D  E  Q  G  H  L  R  A  I  N  P  E  N  G  F  F  G  V  A  P  G (T)   339
1176 TCAGTCAAAACAAACCCCAATGCTATTAAAAACCATATTCAAGAACACCATCTTTACCAACGTAGCTGAAACCAGTGATGGCGGTGTCTAT   1265
340  [S] V  K  T  N  P  N  A  I  K (T) I  F  K  N  T  I  F  T  N  V  A  E  T  S  D  G  G  V  Y    369
1266 TGGGAAGGCATTGACGAGCAATTGCCGCCAGGAGTAACCCTGACTTCATGGAAGAACAAGGACTGGACCCCAGATAATGGGAACCTTGT    1355
370  W  E  G  I  D  E  Q  L  P  P  G  V  T  L (T)[S] W  K  N  K  D  W  T  P  D  N  G  E  P  C     399
1356 GCTCATCCCAACTCACGATTCTGCACTCCAGCCAGCCAGTGCCCCATCATGGACCCTGCCTGGGAATCACCCGAAGGTGTCCCCATTGAA   1445
400  A  H  P  N  S  R  F  C (T) P  A  S  Q  C  P  I  M  D  P  A  W  E [S] P  E  G  V  P  I  E    429
1446 GGGATAATATTTGGAGGCCGCAGACCTGCTGGTGTGCCTCTTGTATATGAGGCTTTTAACTGGCAGCACGGAGTATTTATAGGAGCAGCC   1535
430  G  I  I  F  G  G  R  R  P  A  G  V  P  L  V  Y  E  A  F  N  W  Q  H  G  V  F  I  G  A  A    459
1536 ATGAGATCTGAAGCAACAGCAGCTGCTGAGCACAAAGGCAAAATCATTATGCACGATCCATTTGCCATGAGACCTTTCTTTGGCTACAAT   1625
460  M  R  S  E  A  T  A  A  A  E  H  K  G  K  I  I  M  H  D  P  F  A  M  R  P  F  F  G  Y  N    489
1626 TTTGGCAAATACTTAGCCCACTGGCTTAGCATGGCACATCGCCCAGCGGCAAGACTACCAAGGATCTTTCATGTTAACTGGTTCCGGAAA   1715
490  F  G  K  Y  L  A  H  W  L  S  M  A  H  R  P  A  A  R  L  P  R  I  F  H  V  N  W  F  R  K    519
1716 GACAGCCAAGGGAAATTCCTGTGGCCTGGCTATGGAGAAAATTCCCGTGTGCTGGAGTGGATGTTCAACAGAATTCAAGGGAAGGCCTCT   1805
520  D  S  Q  G  K  F  L  W  P  G  Y  G  E  N  S  R  V  L  E  W  M  F  N  R  I  Q  G  K  A [S]   549
1806 GCCAAGCCGACTGCTATAGGTTACATCCCTGCTGACGCTGCTTTGAACCTGAAGGGTTTAGAAGACGTTAACTTGACTGAACTGTTTGAT   1895
550  A  K  P  T  A  I  G  Y  I  P  A  D  A  A  L  N  L  K  G  L  E  D  V  N  L  T  E  L  F  D    579
1896 ATCTCCAAAGAGTTCTGGGAAAAGGAGGTGGAAGAAATCAAACAATACTTTGAAGGGCAAGTTAATGCTGACCTTCCCTACGAAATAGAA   1985
580  I  S  K  E  F  W  E  K  E  V  E  E  I  K  Q  Y  F  E  G  Q  V  N  A  D  L  P /Y\ E  I  E    609
1986 AGAGAAATGCTTGCCTTGGAGATGAGGATAAAACAGTTGTAGctgatcagaaaaacaattatttatcaatccacatatacaggacaaaa     2075
610  R  E  M  L  A  L  E  M  R  I  K  Q  L  *                                                    622
2076 cattttaccaaatcaccactggaagtacaacagcacactgatgaacaggggttataatgaaagtaggtcttagtttggctgggctataga    2165
2166 taagggtatcctaaagtaactccagacctaatgattataactgcatcagttgttgtgaattggtgagcagctgtgagaatgtatgtgca    2255
2256 cggtcttcagaactctgatctgtcactcacatgcacaactctgatgatgtatttaaattacttgctgacagttctgcagat            2345
2346 acgatctaaaatgatccaccccgcaaggtgtagcacgttgttcctatcacccaagccactagagtttcagactaatttttttcatacttca    2435
2436 gcagtatcgcttacaggaaagaatgaaaattaaatgttgtatatatgtattatctcaaggatatttcatgcttaaatatttttttagaaaaa   2525
2526 atgaagttgtaactttttaccagagtgaaagatgactttgtattactaatgcatatttaagatatcgctattatatatgcctcatgttcct    2615
2616 gtgatttccagatgttttgcatggcttgttctttaaactattgaaataaatgtttatata                                 2675
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Figure 1 – Nucleotide and deduced amino acid sequence of goose PEPCK gene. Numbers on the right and left in each row represent nucleotide or amino acid position. The start codon ATG and stop codon TAG are marked by an underline. The Ser, Thr and Tyr phosphorylation sites were labeled with the boxed, circled and triangle, respectively, when their scores above the threshold of 0.5.

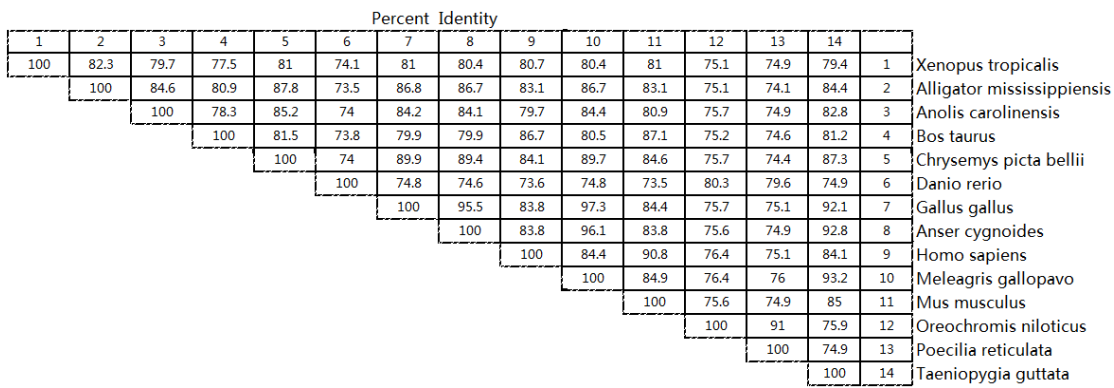1and kinase-2 motifs in goose *PEPCK* (Figure 2). Homology analysis showed that goose *PEPCK* shared high homology with turkey, chicken, and other birds, but had a low homology with that of mammals and

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Figure 2 – Comparison of the deduced amino acid sequence of goose PEPCK with other species. The amino acid residues in the box indicated the sequence oxaloacetate-binding domain, kinase-1 and kinase-2 motifs.

fishes (Figure 3). A phylogenetic tree was constructed based on the amino acid sequences of goose *PEPCK* and other species including mammals, reptiles, birds, amphibians, and fishes. The overall topology of the

Percent Identity

1	2	3	4	5	6	7	8	9	10	11	12	13	14		
100	82.3	79.7	77.5	81	74.1	81	80.4	80.7	80.4	81	75.1	74.9	79.4	1	Xenopus tropicalis
	100	84.6	80.9	87.8	73.5	86.8	86.7	83.1	86.7	83.1	75.1	74.1	84.4	2	Alligator mississippiensis
		100	78.3	85.2	74	84.2	84.1	79.7	84.4	80.9	75.7	74.9	82.8	3	Anolis carolinensis
			100	81.5	73.8	79.9	79.9	86.7	80.5	87.1	75.2	74.6	81.2	4	Bos taurus
				100	74	89.9	89.4	84.1	89.7	84.6	75.7	74.4	87.3	5	Chrysemys picta bellii
					100	74.8	74.6	73.6	74.8	73.5	80.3	79.6	74.9	6	Danio rerio
						100	95.5	83.8	97.3	84.4	75.7	75.1	92.1	7	Gallus gallus
							100	83.8	96.1	83.8	75.6	74.9	92.8	8	Anser cygnoides
								100	84.4	90.8	76.4	75.1	84.1	9	Homo sapiens
									100	84.9	76.4	76	93.2	10	Meleagris gallopavo
										100	75.6	74.9	85	11	Mus musculus
											100	91	75.9	12	Oreochromis niloticus
												100	74.9	13	Poecilia reticulata
													100	14	Taeniopygia guttata

Figure 3 – Homology of goose PEPCK with other vertebrates. Homology analysis of amino acid sequences were performed with DNAMAN software.

phylogenetic tree revealed that goose *PEPCK* was closely related to other birds (Figure 4).
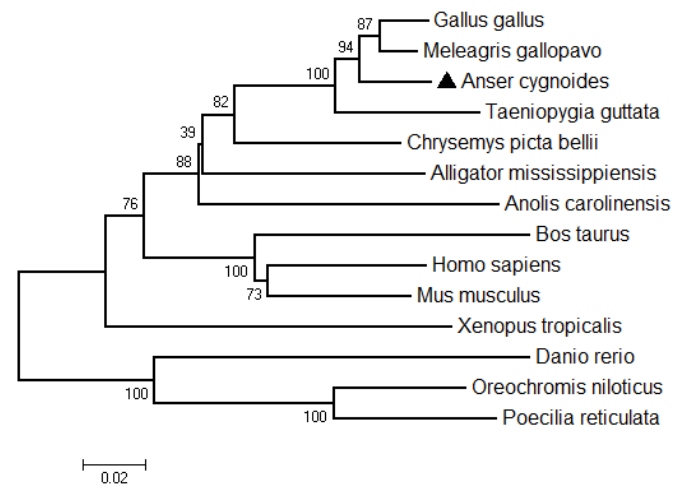
Figure 4 – Phylogenetic tree of *PEPCK* gene constructed using the neighbor-joining method in MEGA7. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The scale bar represents amino acid substitutions per site. GenBank accession numbers of the proteins as follow: Gallus gallus (NP_990802.1); Meleagris gallopavo (XP_003212171.1); Taeniopygia guttata (XP_002199208.1); Chrysemys picta bellii (XP_005305891.1); Alligator mississippiensis (XP_014465195.1); Anolis carolinensis (XP_003223771.1); Bos taurus (NP_777162.1); Homo sapiens (NP_002582.3); Mus musculus (NP_035174.1); Xenopus tropicalis (NP_001073036.1); Danio rerio (NP_999916.1); Oreochromis niloticus (XP_003448423.1); Poecilia reticulate (XP_017160082.1).

Tissue Distribution of Goose PEPCK mRNA

As shown in Figure 5, *PEPCK* was expressed at the highest levels in goose liver and kidney, followed by small intestine, leg muscles, and brain. Additionally, it was also present in other tissues with very low levels.
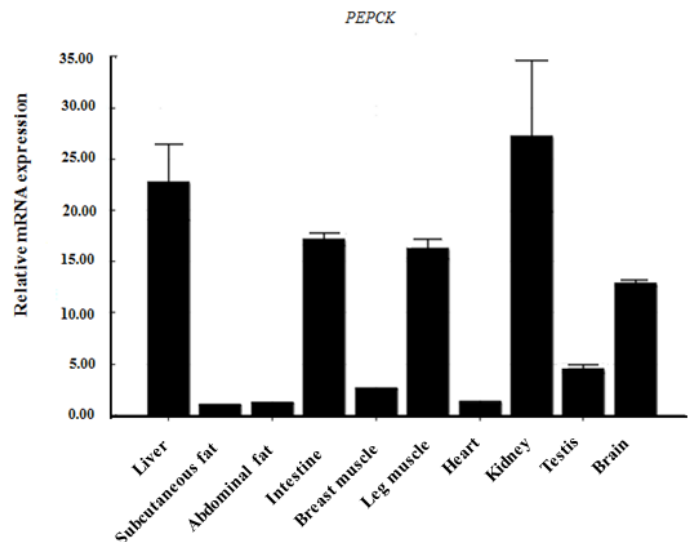

Figure 5 – The relative mRNA expression levels of *PEPCK* in different tissues from Sichuan white goose. The tissues include liver, subcutaneous fat, abdominal fat, intestine, breast muscle, leg muscle, heart, kidney, testis and brain.

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Effect of Overfeeding on the Expression of Goose PEPCK

As shown in Figure 6, compared to respective control group levels of *PEPCK* mRNA were increased by 13.1- and 1.6-fold in the overfed SW and LD geese, respectively (*p*<0.05).
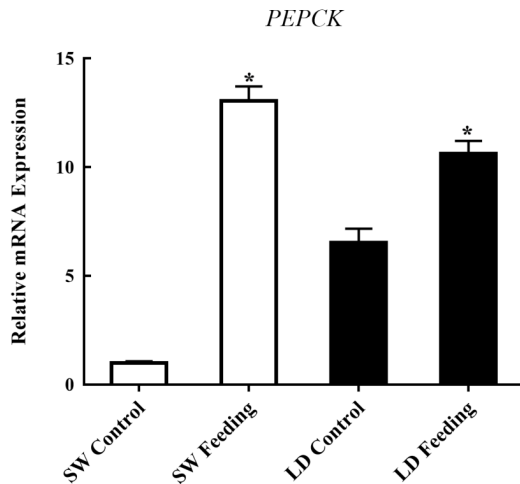
Figure 6 – Influence of overfeeding geese on the expression levels of *PEPCK*. SW represents Sichuan white goose; LD represents Landes goose. Marking "*" on the column indicates significant difference compared with the control group (*p*<0.05).

Correlation Analysis

As shown in Table 3, hepatic TG content, plasma glucose, and insulin concentrations were markedly increased in the overfeeding group of SW and LD geese. Meanwhile, the expression of *PEPCK* mRNA in the liver was positively correlated with hepatic TG levels as well as plasma glucose and insulin concentrations.

Table 3 – Correlation analysis between *PEPCK* mRNA abundance and TG content, plasma insulin and glucose concentrations.

	Insulin(mmol/L)	TG(%BW)	Glucose(mmol/L)
Sichuan White geese (n=3)	0.646*	0.514*	0.723*
Landes geese (n=3)	0.623*	0.478*	0.711*

Effects of Fatty Acids Treatment on the mRNA Levels of PEPCK in Goose Primary Hepatocytes

Goose primary hepatocytes were treated without or with palmitic acid, oleic acid or linoleic acid of different concentrations. As shown in Figure 7, palmitic acid had no significant effect on the *PEPCK* mRNA levels (*p*>0.05). Oleic acid can promote the mRNA expression of *PEPCK*, among which oleic acid at the concentration of 0.8, 1.2 or 1.6 mM significantly increased its expression in goose hepatocytes (*p*<0.05). Besides, levels of *PEPCK* mRNA were markedly enhanced by addition of 0.125 or 0.25 mM of linoleic acid (*p*<0.05).
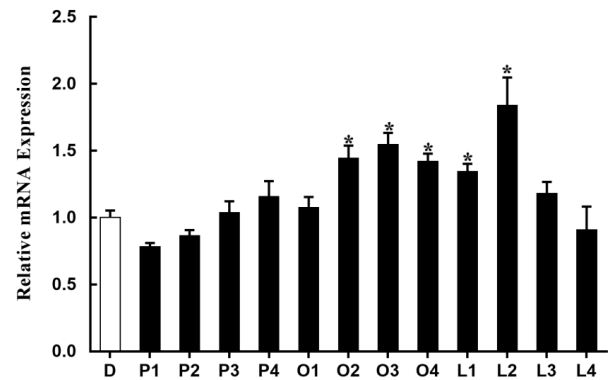
Figure 7 – Effects of different fatty acids treatments on the expression levels of *PEPCK* in goose hepatocytes *in vitro*. D represents the control group; P1, P2, P3 and P4 represent palmitic acid (0.3 mM), palmitic acid (0.6 mM), palmitic acid (0.9 mM) and palmitic acid (1.2 mM), respectively; O1, O2, O3 and O4 represent oleic acid (0.4 mM), oleic acid (0.8 mM), oleic acid (1.2 mM) and oleic acid (1.6 mM), respectively; L1, L2, L3 and L4 represent linoleic acid (0.125 mM), linoleic acid (0.25 mM), linoleic acid (0.5 mM) and linoleic acid (1.0 mM), respectively. Marking "*" on the column indicates significant difference compared with the control group (*p*<0.05).

DISCUSSION

In our previous study, a 636 bp EST of goose *PEPCK* gene was screened by constructing the SSH library of SW and LD geese hepatocytes, and the mRNA expression levels of *PEPCK* in the liver were shown to be higher in LD than in SW geese (Pan *et al.*, 2010). To further explore the function of *PEPCK* in goose hepatic steatosis, its full-length cDNA sequence was firstly obtained by RACE in this study. Homology analysis showed that goose *PEPCK* shared high homology with other avian species, which was in accordance with the animal evolutionary trend, suggesting that the sequence amplified in our study was the goose *PEPCK*. Based on the deduced amino acid sequence, conserved functional motifs were characterized in goose *PEPCK*, including oxaloacetate-binding domain, kinase-1 and kinase-2 motifs, which was similar to the results described by Matte *et al.* (1997), indicating that goose *PEPCK* was evolutionarily conserved. Thus, these data suggested that goose *PEPCK* may possess similar biological functions with other species.

Mammalian *PEPCK* gene is widely expressed in different types of tissues and organs, with high levels in the liver, kidney, and white and brown adipose tissues (Tilghman, 1976). In the present study, the mRNA levels of *PEPCK* in goose different tissues were determined. We observed that goose *PEPCK* was highly expressed in the liver and kidney, with low levels in the heart, subcutaneous fat and abdominal adipose tissue, which was consistent with the mentioned studies, suggesting that *PEPCK* may have a significant role in the liver. The liver is the primary organ to produce energy and lipid

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by utilizing free fatty acids (FFAs). A previous study has reported that *PEPCK* was expressed in a cell-type specific manner (Zimmer and Magnuson, 1990), and it was also showed that knockout *PEPCK* in mice liver would shunt hepatic lipid into triglyceride, which results in fatty liver (Hakimi *et al.*, 2005). Therefore, higher expression level of *PEPCK* in the liver indicated that *PEPCK* might have an essential role in regulating goose hepatic lipid metabolism.

To further investigate the role of *PEPCK* in goose fatty liver development, studies have been conducted to determine the expression characteristics of *PEPCK* gene during goose fatty liver development in overfed geese. In this study, after overfeeding, compared to respective control group levels of *PEPCK* mRNA were increased by 13.1- and 1.6-fold in SW and LD geese ($p<0.05$), respectively. Meanwhile, it should be noted that the concentrations of plasma glucose and insulin also increased. The above results suggested that *PEPCK* may play roles in regulating goose fatty liver formation. *PEPCK* is a vital gene related to glycolipid metabolism. Studies in mice have shown that liver-specific knockout of mice *PEPCK* results in impaired lipid metabolism (She *et al.*, 2000). Altogether, it could be summarized that *PEPCK* may be involved in the process of goose hepatic steatosis.

During the process of goose hepatic steatosis, FFAs play an essential role in hepatic glycolipid metabolism. Osman *et al.* (2016) found that the main types of fatty acids in LD goose liver included palmitic acid, stearic acid, and oleic acid. Moreover, recent studies have shown that palmitic acid, oleic acid, and linoleic acid promoted lipid deposition in goose hepatocytes (Pan *et al.*, 2009; Pan *et al.*, 2011a; Pan *et al.*, 2011b). Palmitic acid, as a long chain saturated fatty acid, is abundant in foods, and Molette *et al.* (2001) showed that the content of palmitic acid in goose liver is one-quarter of the total fatty acids. Pan *et al.* (2011a) provided evidence that low to moderate levels of palmitic acid can promote lipid deposition in goose hepatocytes, but higher concentrations decreased the activity of hepatocytes, suggesting that palmitic acid is involved in regulating goose hepatic steatosis. Our *in vitro* data showed that addition of palmitic acid had no significant effect on the mRNA expression of *PEPCK* ($p>0.05$). It was reported that oleic acid could induce *PEPCK* transcription and increase gluconeogenesis in mammals (Grasfeder, 2007). In this study, we found that treatment with 0.8, 1.2 or 1.6 mM of oleic acid increased the expression of *PEPCK* ($p<0.05$), indicating that oleic acid could promote gluconeogenesis in

goose primary hepatocytes. Linoleic acid is an essential fatty acid for humans and animals, and it has been previously shown that linoleic acid can promote lipid deposition and cell activity in goose hepatocytes (Pan *et al.*, 2011b). Moreover, one report pointed out that linoleic acid promoted gluconeogenesis in chicken hepatocytes through G protein-coupled receptor 40 (GPR_{40}) protein expressions (Suh *et al.*, 2008). Our results showed that 0.125 or 0.25 mM of linoleic acid significantly promoted the expression of *PEPCK* ($p<0.05$), while the expression of *PEPCK* was inhibited by adding 0.5 or 1.0 mM of linoleic acid, suggesting that low concentrations of linoleic acid may promote gluconeogenesis. In vitro, we demonstrated that three kinds of fatty acids differently regulated the transcription of *PEPCK* and among them oleic acid appeared to have the most significant effect on its mRNA expression*,* suggesting that *PEPCK* may play a much more important role in mediating oleic acid-induced goose hepatic steatosis.

In conclusion, we first obtained the full-length CDS of goose *PEPCK* gene, and bioinformatic analysis revealed that goose PEPCK contains the highly conserved oxaloacetate-binding domain, kinase-1, and kinase-2 motifs. Results of qRT-PCR showed that goose *PEPCK* had higher mRNA levels in the liver and kidneys, and overfeeding induced the mRNA expression of *PEPCK* in the livers of SW and LD geese. At the cellular level, it was observed that addition of fatty acids changed the mRNA expression of *PEPCK* in cultured goose primary hepatocytes. Taken together, our results suggested a role for goose *PEPCK* in regulating the process of goose hepatic steatosis.

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