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Article

Alpha-Linolenic Acid from *Zanthoxylum* Seed Powder Regulates Fatty Acid Metabolism and Influences Meat Quality of Pekin Duck via the ADIPOQ/AMPK/CPT-1 Pathway

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ABSTRACT: *Zanthoxylum* seed powder contains dominant α-linolenic acid (ALA). Its regulatory mechanism as a novel feed additive for the livestock field is not clear. In this study, RNA-seq was used to identify the differential gene expression in breast muscle of Pekin duck supplemented with different doses of *Zanthoxylum* seed powder, and *Adiponectin (ADIPOQ)* was found to be an important factor. Functional validation was performed in duck primary myoblasts. Our results revealed that *ADIPOQ* overexpression could promote myoblast myotube fusion, that is, myogenic differentiation. On the other hand, ALA inhibited lipid deposition in myoblasts. *SiADIPOQ* inhibited fatty acid oxidation, but stimulated fatty acid synthesis and transport. Furthermore, ALA promoted the up-regulation of ADIPOQ, AMPK, p-AMPK and CPT-1 protein levels. It was concluded that ALA regulates lipid deposition through the ADIPOQ/AMPK/CPT-1 pathway in myoblasts. These results may provide theoretical basis for the development and utilization of *Zanthoxylum* seed powder in duck production.

KEYWORDS: *Zanthoxylum, α-linolenic acid, lipid deposition, Pekin duck, ADIPOQ*

INTRODUCTION

China is the world's largest producer and consumer of meat ducks, with 75% of global duck production. Duck meat occupies an important position in China's food consumption.[1,2] Skeletal muscle is one of the major organs involved in the regulation of lipid metabolism in poultry, and myoblasts contain a variety of lipid droplets called intramyocellular lipids (IMCL), which are smaller than those found in adipocytes. Intramuscular fat (IMF) is an important substance affecting meat quality and flavor.[3] IMCL affects IMF quality, and then matters muscle meat quality and texture.[4−6] Therefore, it is important to explore the mechanisms of fatty acid metabolism regulation to improve the quality of duck meat.

Fatty acid deposition is regulated by a complex set of processes, and *Adiponectin (ADIPOQ)* is an adipokine that may be anti-inflammatory and regulate fatty acid metabolism.[7] It has multiple metabolic pathways in vivo, such as exerting metabolic effects by activating two homologous receptors, *ADIPOR1* and *ADIPOR2*, independently of AMPK.[8] Meanwhile, it was discovered that *ADIPOQ* may affect pork flavor by influencing IMF deposition in pigs.[9] The researchers also found *ADIPOQ* expression affect fat deposition in different parts of the pig.[10] It was indicated that *ADIPOQ* is an important candidate gene for thyroid-regulated Tianfu Nonghua speckled duck egg-laying performance, but there was no mention of the effect on fatty acid metabolism.[11] Currently, studies on the mechanisms by which *ADIPOQ* regulates fatty acid metabolism have focused on humans and rodents,[12,13] so it is necessary to study the role and mechanism of fatty acid regulation by *ADIPOQ* in the muscle tissue of poultry.

Zanthoxylum(*Zanthoxylum bungeanum Maxim*)belongs to the genus *Zanthoxylum* of the Rutaceae deciduous small trees, *Zanthoxylum* seed powder from the mature seeds produced by *Zanthoxylum*, has a high economic value and medicinal value.[14,15] *Zanthoxylum* seed powder consists of 27%−31% oil, of which 90% are polyunsaturated fatty acids (PUFAs) including α-linolenic acid (ALA) and linoleic acid (LA).[16] In recent years, *Zanthoxylum* seed powder has functioned in improving nonalcoholic fatty liver disease (NAFLD)[17,18] and antiobesity.[19] It also has been used as a feed additive, previous study revealed that the ratio of ALA and PUFAs in pork were increased by reducing the ratio of n-6/n-3 PUFAs in the pig ration.[20] Similarly, it was found that low dietary ratios of n-6/n-3 PUFAs enhanced the deposition of n-3 PUFAs in longissimus dorsi muscle (LDM) and subcutaneous adipose tissue (SAT) in Heigai Pigs.[21] PUFA supplementation in diets can increase IMF content, for example, conjugated linoleic acid (CLA) can be used as a body fat regulator, and studies have shown that the addition of 1% CLA to diets can significantly increase IMF content and fat metabolism in pork.[22,23] A significant relationship of fatty acids between the ration and the muscle was found in the Chinese crested white ducks. The fatty acids of the ration could promote the deposition of n-3

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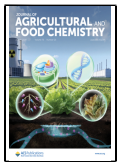

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PUFAs in duck muscles.[24] Similar results were found in pigs and quail.[25,26] Our previous study showed that the addition of *Zanthoxylum* seed powder to the ration promoted a significant increase in the ALA of the Pekin duck breast muscle (unpublished data), while the regulatory mechanism of *Zanthoxylum* seed powder is not clear. With the price of feed ingredients rising, the development of new low-opportunity-cost-feed is becoming a hot topic.[27] So, we can hypothesize whether dietary supplementation of *Zanthoxylum* seed powder affects the expression of genes related to fatty acid metabolism regulation. Whether these genes could promote muscle growth and development and thus affect muscle yield. Whether ALA from *Zanthoxylum* seed powder could mediate the regulation of fatty acid metabolism and thus have an effect on the fatty acid deposition in the muscle.

The aim of this study was to identify candidate genes and fatty acid metabolism mechanism in Pekin duck breast muscle by adding *Zanthoxylum* seed powder to the ration. After obtaining the major gene *ADIPOQ* based on RNA-seq, Pekin duck primary myoblasts were isolated for in vitro validation to investigate the role of ADIPOQ in early muscle development and regeneration. In view of the previous findings that ALA was significantly up-regulated in the *Zanthoxylum* seed powder-added group, this study analyzed the mechanisms of fatty acid metabolism regulation of ALA and ADIPOQ, and thus obtained the mechanism of their effects on IMF. All results can provide a theoretical basis for the in-depth development and utilization of *Zanthoxylum* seed powder in duck production and the selection and breeding of duck meat quality traits.

MATERIALS AND METHODS

Animals and Sample Collection. A total of 240 uniformly sized and healthy Pekin ducks were randomly divided into 4 groups with 6 replicates in each group and 10 ducks in each replicate. The control group (CG) was fed with a basal ration, and experimental groups were added with 1.5% (LG), 3.0% (MG) and 4.5% (HG) *Zanthoxylum* seed powder, respectively. The same feeding condition was continued until 35 days old during the experiment. Breast muscle tissues were collected quickly after slaughter and placed in liquid nitrogen for use, after which it could be stored at −80 °C for long periods of time. Duck eggs used in the experiment were incubated under prescribed procedures. Pekin ducks and eggs were purchased from Shaanxi Fuqiang Hongtu Husbandry Co Ltd. (Shaanxi, China). The composition of the basal diet is shown in Table 1. All birds used in this study were sanctioned by the Institute of Animal Care and Use Committee, Northwest Agriculture and Forestry University.

RNA-seq and Data Analysis. Total RNA was extracted from breast muscle tissues of Pekin ducks and RNA quality was assessed using Nanodrop 2000 and 1% agarose gel electrophoresis by TRIzol (AGbio, Hunan, China) reagent. Then the RNA integrity number was detected on an Agilent 2100 Bioanalyzer. Afterward, mRNA was enriched using Oligo dT, mRNA was randomly interrupted by adding fragmentation buffer and reverse transcribed into cDNA by adding random primers, and sequenced using the second-generation high-throughput sequencing platform of Shaanxi Xuanchen Biological Technology Co., Ltd. (Yangling, China). After filtering the raw data, checking the sequencing error rate, and examining the distribution of GC content, the clean reads were obtained for subsequent analyses. The clean reads were aligned to *Anas platyrhynchos* genome sequence using HISAT2 software. All the genes were aligned to GO and KEGG databases by BLAST. The new genes and their annotation information were obtained by sequence comparison. Sequencing depth and gene length were corrected via FPKM, followed by differential gene expression analysis as well as GO and KEGG analyses using Fold Change ≥2 and Q value <0.05 as criteria. String (https://cn.string-db.org/) was used for protein PPI network analysis.

Table 1. Basal Diet Composition for Pekin Ducks

items	contents (%)
Ingredient	
corn	57.20
soybean meal	25.90
wheat bran	7.80
soybean oil	3.20
$CaHPO_4$	1.50
stone powder	1.25
NaCl	0.55
premix[a]	1.30
L-lysine	0.95
DL-methionine	0.35
total	100
Nutrient Level[b]	
AME, MJ/kg	12.32
crude protein	15.74
crude fiber	2.20
coarse ash	4.40
crude fat	8.00
calcium	0.82
total phosphorus	0.54
water content	12.20

[a]The premix provided the following for per kilogram of the diet: vitamin A 10,000 IU, vitamin D 32,500 IU, vitamin E 30 mg, vitamin K 12.5 mg, vitamin B_1 2 mg, vitamin B_6 3.8 mg, vitamin B_{12} 10 μg, D-pantothenic acid 12 mg, Folic acid 0.80 mg, Biotin 110 μg, Nicotinic acid 40 mg, choline 460 mg, Mn 60 mg, Fe 40 mg, Cu 10 mg, Zn 55 mg, I 1.6 mg, Se 0.35 mg. [b]Nutrient components were calculated values.

Cell Isolation and Cell Culture. Pekin duck primary myoblasts were isolated from the muscle tissues taken from 14-day-old duck embryos according to the method described previously.[28,29] The hind limb muscles of duck embryos were minced, digested with 0.1% collagenase I and trypsin until the muscle tissues were flocculent, sieved, centrifuged and resuspended, and then the fibroblasts were finally removed by the differential apposition method to isolate the duck primary myoblasts. It was successfully identified by Immunofluorescence (IF). The cells were cultured with DMEM (Cytiva, Uppsala, Sweden), 20% FBS (Biochannel, Nanjing, China) and 1% penicillin/streptomycin (Biosharp, Beijing, China), at 37 °C with 5% CO_2. Primary myoblasts differentiation was induced using DMEM complete medium containing 2% horse serum (Solarbio, Beijing, China).

RNA Interference and Overexpression. Negative control siRNA (siNC) and three Pekin duck *ADIPOQ* siRNAs (siADIPOQs) in the Table 2 were purchased from Beijing Zixi Biotechnology Co., Ltd. (Beijing, China). The *ADIPOQ* expression vector was made by inserting the CDS region of the Pekin duck *ADIPOQ* gene into the pcDNA3.1 vector. Then it was verified by sequencing successfully and

Table 2. Sequences of ADIPOQ-Targeting siRNAs

siRNA	primer sequence (5′-3′)
siADIPOQ-1	F: 5′-GGGAGAGAAAGGAGAGCAA-3′
	R: 5′-UUGCUCUCCUUUCUCUCCC-3′
siADIPOQ-2	F: 5′-GCAAGAUCUUCUACAACGA-3′
	R: 5′-UCGUUGUAGAAGAUCUUGC-3′
siADIPOQ-3	F: 5′-AGGUCAGCCUCUACAAGAA-3′
	R: 5′-UUCUUGUAGAGGCUGACCU-3′

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can be used for the further study. SiRNA or pcDNA3.1 vector was transfected by Lipo8000 (Beyotime, Jiangsu, China).

RNA Extraction and Real-Time Quantitative PCR. RNA was extracted from cultured duck primary myoblasts and muscle tissues fed with different doses of *Zanthoxylum* seed powder. cDNA was synthesized using 5× Evo M-MLV RT Reaction Mix Ver.2 (Agbio, Hunan, China), and analyzed on a real-time fluorescence quantitative PCR instrument (Bioer, FQD-96A, Zhejiang, China) by SYBR Green qPCR mixture (Agbio, Hunan, China). Meanwhile, qRT-PCR was used to verify the transcriptome sequencing results. GAPDH was used as an internal reference gene, and the information on primers for these experiments were listed in the Table 3.

Table 3. Primers for qRT-PCR

gene	primer sequence (5′-3′)
ACSL6	F: 5′-ATGGTGGTGGTTCCCCTCTA-3′
	R: 5′-GATGATGGAGCTGAGACCCG-3′
AMPD1	F: 5′-CACGGGTGAAAATTCCAGCA-3′
	R: 5′-CTCTTGCCTGATCGCCTCAT-3′
VEGFA	F: 5′-AAGTCTACGAGCGCAGTTTCT-3′
	R: 5′-TCATCAGAGGCACACAGGATG-3′
DUSP1	F: 5′-GAGCCCCGCTATGTCAGTAT-3′
	R: 5′-GGAAGTGGTGATGGGACTCTG-3′
NR1H3	F: 5′-CAGCAGCGTTTTGCTCACTT-3′
	R: 5′-ATCTTCCCTGGTGAGGTCCA-3′
ADIPOQ	F: 5′-AAGGGGCCTATGTTTACCGC-3′
	R: 5′-GTCGTAGTGGTTCTGCTCGT-3′
CDKN1B	F: 5′-GCCAAACCGTGATTTTTGCC-3′
	R: 5′-TTGCTCCACTGAACTGGCAC-3′
CYTB	F: 5′-ACATCGGACAGACCCTGGTA-3′
	R: 5′-GGACTAGGGTGATTCCTGCG-3′
ND4	F: 5′-GGCACCCTACACCTACCAAC-3′
	R: 5′-GTATTGAGCCTGCGATGGGA-3′
COX2	F: 5′-TTCCAAGACGCCTCATCACC-3′
	R: 5′-CGATGGCGGGTAGGATTGTT-3′
SCD1	F: 5′-ACCATCGTGTCCACCACAAA-3′
	R: 5′-GAGGGCTTGTAGTATTTCCGCT-3′
ACC1	F: 5′-CAGGGAGTTCACACAGAGCA-3′
	R: 5′-ATCCACCTCACAGTTGACCT-3′
ACC2	F: 5′-GAACATCCGTGAGGAGCCAA-3′
	R: 5′-CCGGAGACCACAGTCAACAA-3′
CD36	F: 5′-TGGTTAACGGCACAGATGGG-3′
	R: 5′-ACGAGGAACTGCGAAACGAT-3′
FABP5	F: 5′-TACATGAAGGAGCTGGGTGTG-3′
	R: 5′-GCGTCTGAGTTTTTCTGCCA-3′
FATP4	F: 5′-CCAGTCAGCCACCAACAAGA-3′
	R: 5′-GTCCCCTCCACCTCTGTAGT-3′
CPT-1	F: 5′-GCTACGGCGTCTCCTACATC-3′
	R: 5′-GATGTTCCTCCCGAAACGCT-3′
LPL	F: 5′-GGCTCCACACTATGGCTTTGA-3′
	R: 5′-AAGGACGAAGCGTTGATGGG-3′
PPARα	F: 5′-TGCTTGTGAAGGTTGTAAGGG-3′
	R: 5′-CAGACCTTGGCATTCGTCCA-3′
GAPDH	F: 5′-ATGCTGGTGCTGAATACG-3′
	R: 5′-GGAGATGATGACACGCTTA-3′

Cell Counting Kit-8 (CCK-8). Primary myoblasts were inoculated in 96-well plates, transfected with siRNA or pcDNA3.1 vectors and cultured for 24 h. CCK-8 solution (10 μL, Beyotime, Jiangsu, China) was added to each well and incubated for 2 h. Absorbance of each well was measured at 450 nm via a multifunctional enzyme label instrument (Bio Tek).

Flow Cytometry. Duck primary myoblasts transfecting with siRNA or pcDNA3.1 vectors were fixed in 70% ethanol overnight at 4 °C. Cells were permeabilized using Triton-100 (Accuref Scientific, Shaanxi, China) and incubated with propidium iodide staining solution (Beyotime, Jiangsu, China) for 30 min at 37 °C. Red fluorescence was detected by flow cytometry at excitation wavelength 488 nm and cellular DNA content was analyzed using Modfit LT 5.0.

5-Ethynyl-2′-deoxyuridine (EdU) Assay. Primary myoblasts were inoculated in 24-well plates. The samples were transfected with siRNA or pcDNA3.1 vectors and cultured for 24 h. After that, 2× EdU (20 mM) working solution was added to the medium and incubated for 2 h. Subsequently, fixation was carried out using 4% paraformaldehyde, and cells were permeabilized using Triton-100 (Accuref Scientific, Shaanxi, China) and stained for EdU using HyperFluor 594 azide (APExBIO, Houston, USA). And the nuclei of the cells were stained with Hoechst 33342 (APE × BIO, Houston, USA). Observations were made by an inverted fluorescence microscope and the percentage of EdU-positive cells was analyzed through Image-J.

Immunofluorescence (IF). Duck primary myoblasts were cultured in 12-well plates, transfected with siRNA or pcDNA3.1 vectors and incubated for 24 h, and then differentiated for 48 h by replacing complete medium containing 2% horse serum for IF detection. After fixation and permeabilization, the cells were incubated overnight at 4 °C with Desmin antibody (Beyotime, Jiangsu, China), then added FITC-labeled goat anti-rabbit IgG secondary antibody (Beyotime, Jiangsu, China) and incubated for 2 h at room temperature, protected from light. The nuclei of the cells were stained with DAPI.

Addition of ALA. ALA was added 0, 20, 40, and 60 μmol/L to the complete cell culture medium and induced for 24, 48, and 72 h, respectively. The effects of ALA on cell activity were observed by CCK-8. Then the amount of ALA added and the induction time were determined. Subsequently, IMCL was detected using Oil Red O (ORO) Staining and Triglyceride (TG) Content Assay Kit with the addition of ALA. And the total protein was extracted from these samples.

Primary myoblasts were added the ALA (A/+ group), transfected with ADIPOQ overexpression vector (A group) and siADIPOQ vector with addition of ALA (siA/+ group). ORO staining and TG assay were used to test lipid deposition in duck primary myoblasts.

Oil Red O (ORO) Staining. Primary myoblasts were cultured in 12-well plates, fixed with ORO fixative for 30 min at ambient temperature, stained with ORO staining solution for 15 min, rinsed with 60% isopropyl alcohol. The nuclei of the cells were restained for 2 min by adding Mayer's hematoxylin staining solution. Finally, ORO buffer was added for 1 min and then discarded. The fixed cells were covered with distilled water and observed under a CKX53 microscope (Olympus, Japan).

Triglyceride (TG) Content Assay Kit. Duck primary myoblasts were centrifuged and removed the supernatant. The extraction solution (1 mL) were added to the cells (about 5×10^6), sonicated for 3 s, 10 s intervals, repeated 30 times to break the cells, and then centrifuged for 10 min, 12000 rpm The absorbance value of the supernatant was measured at the wavelength of 510 nm, and then calculated the TG content.

Western Blotting (WB). Primary myoblasts were cultured in 6-well plates. The samples were transfected with siRNA or pcDNA3.1 vectors and cultured for 24 h. The total protein was extracted using RIPA lysis buffer (Solarbio, Beijing, China) with the addition of phosphatase inhibitors, protease inhibitors and PMSF (Solarbio, Beijing, China). Total protein was separated by SDS-PAGE and transferred onto PVDF member (Millipore, Massachusetts), immunoblotted using specific antibodies (listed in Table 4), and finally visualized by a chemiluminescence imaging system (ChampChemi 610 Plus, Beijing, China). The grayscale statistics were quantified via Image-J.

Statistical Analysis. All data were presented as mean ±SD. All experiments in this study were performed at least in triplicate. The statistical differences were analyzed with Student's *t* test for comparison of two groups or ANOVA for comparison of more than

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J. Agric. Food Chem. 2025, 73, 14651−14665

Table 4. Antibodies in This Study

antibody name	brand name
anti-ADIPOQ antibody	Proteintech
anti-AMPKα-2 antibody	Wanleibio
anti-p-AMPK(Thr 183/172) antibody	Immunoway
anti-CPT-1 antibody	Immunoway
anti-GAPDH antibody	Huabio

two groups using IBM SPSS 26.0. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Identification of Differentially Expressed Genes (DEGs) and GO Function Annotation and KEGG Enrichment Analysis. In this project, the screening criteria were Fold Change ≥2 and Q value ≤ 0.05 was used for differential expression analysis. HG vs CG showed a total of 566 differentially expressed genes (DEGs) including 243 up-regulated ones and 323 down-regulated ones (Figure 1A). There are 252 DEGs in MG vs CG, of which 126 were up-regulated and 126 were down-regulated (Figure 1B). 85 DEGs were found in LG vs CG, of which 25 were up-regulated genes and 60 were down-regulated genes (Figure 1C).

In order to evaluate the gene expression differences among different additive groups, the three groups of breast muscle DEGs were compared. 144, 57, 56 common DEGs were found in HG vs CG and MG vs CG, HG vs CG and LG vs CG, MG vs CG and LG vs CG, respectively. A total of 44 common DEGs were obtained in the three groups, among which DEGs with functional annotations included *ADIPOQ*, *ATP6*, *COX1*, *ND1*, *FGF*, *VEGFA*, and so on (Figure 1J).

GO functional enrichment analysis included Molecular Function (MF), Biological Process (BP) and Cellular Component (CC). GO enrichment analysis showed that the main pathways significantly enriched in HG vs CG include Oxidative phosphorylation, ATP synthesis coupled electron transport. In MG vs CG, the main pathways significantly enriched include Ribonucleotide metabolic process, Purine ribonucleotide metabolic process, Generation of precursor metabolites and energy, Oxidative phosphorylation and Energy derivation by oxidation of organic compounds. Meanwhile, Ribose phosphate metabolic process, Purine nucleotide metabolic process and Oxidative phosphorylation were significantly enriched in LG vs CG significantly enriched (Figure 1D−F). KEGG analysis revealed that the significantly enriched pathways in three comparison groups included Oxidative phosphorylation, PPAR signaling pathway, Cardiac muscle contraction, Arachidonic acid metabolism, Adipocytokine signaling pathway and NAFLD, etc. (Figure 1G−I).

PPI analysis showed that ADIPOQ plays a major regulatory role in fatty acid metabolism-related proteins such as PPARγ, CPT1A, CD36, FABP4, FASN, PPARα and others (Figure 1M). Meanwhile, *ADIPOQ* mRNA expression levels were significantly changed in between the control group and three additive groups, so ADIPOQ was used as the main research gene for subsequent steps.

Ten DEGs related to fatty acid metabolism were selected for testing. The results showed that the qRT-PCR results were basically consistent with the sequencing results, indicating that the reliability of the RNA-seq results was high (Figure 2).

ADIPOQ Has a Significant Effect on Myogenic Differentiation but Little Effect on Cell Proliferation. In order to determine the inhibitory effect of the three *ADIPOQ*-targeting siRNAs on *ADIPOQ* expression in duck primary myoblasts, the transcription levels of *ADIPOQ* in primary myoblasts were examined after transfection with each of the three siRNAs for 24 h. The results showed that three siRNAs all reduced the transcription level of *ADIPOQ* very significantly, with *siADIPOQ-1* having the most significant inhibitory effect ($P < 0.001$). Thus, *siADIPOQ-1* (abbreviated as *siADIPOQ*) was used for followed steps (Figure 3A).

To investigate whether *ADIPOQ* affected the proliferation of primary myoblasts or not, CCK-8, EdU and Flow cytometry were used to detect the proliferation of cells in the case of knockdown and overexpression of ADIPOQ. CCK-8 results indicated no significant difference between siNC and *siADIPOQ*. The results of *ADIPOQ* overexpression were the same as *siADIPOQ* groups (Figure 3B). Compared with the control group, Flow cytometry assay cell cycle results showed no significant changes in the proportion of cells during whole periods when *ADIPOQ* was knocked down or overexpressed (Figure 3C−F). EdU results revealed that no significant differences in the proportion of EdU-positive cells between *siNC* and *siADIPOQ*, or pcDNA3.1 and *ADIPOQ* (Figure 3G,H). Therefore, the expression level of *ADIPOQ* had no significant effect on the proliferation of duck primary myoblasts.

To determine the effect of *ADIPOQ* on the myogenic differentiation of primary myoblasts, IF was used to detect myotube differentiation of cells with the overexpression and knockdown of *ADIPOQ* for 24 h and change of differentiation medium to induce differentiation for 48 h. It showed that the myotubular differentiation of cells was lower in *siADIPOQ* groups than *siNC* groups, whereas *ADIPOQ* overexpression groups showed the opposite trend, demonstrating that overexpression of *ADIPOQ* could significantly promote myotubular differentiation (Figure 3I,J).

ALA Inhibits Lipid Droplet Accumulation and TG Synthesis in Primary Myoblasts. To determine the effect of *Zanthoxylum* seed powder on lipid deposition in ducks, different doses of ALA were added to primary myoblasts. The cell viability was detected by CCK-8, which showed that there was a significant down-regulation at 72 h of induction in each addition group. However, there was no significant effect on cell viability at 24 and 48 h of induction. The addition of 60 μmol/L ALA and induction for 48h was chosen for the subsequent steps (Figure 4A).

Lipid deposition in primary myoblasts was detected by ORO staining and TG assay with 60 μmol/L ALA added and induced for 48 h. ORO results indicated that lipid droplets were significantly reduced compared with the control group (Figure 4B). TG assay results showed that the TG content of ALA-induced primary myoblasts was significantly lower than that of the control group (Figure 4C). Thus, ALA inhibited lipid droplet accumulation and TG synthesis.

ORO staining and TG assay were used to detect lipid deposition in primary myoblasts in the case of A/+ group, A group and siA/+ group. ORO results showed a gradual increase in lipid droplet accumulation among three groups (Figure 4D). TG assay results indicated that the TG content of siA/+ group was significantly higher than that of A/+ and A groups ($P < 0.001$). The TG content of A/+ group was not significantly different from that of A group, while there was an upward trend for the TG content in group A compared to group A/+ (Figure 4F). Therefore, it can be concluded that

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J. Agric. Food Chem. 2025, 73, 14651−14665

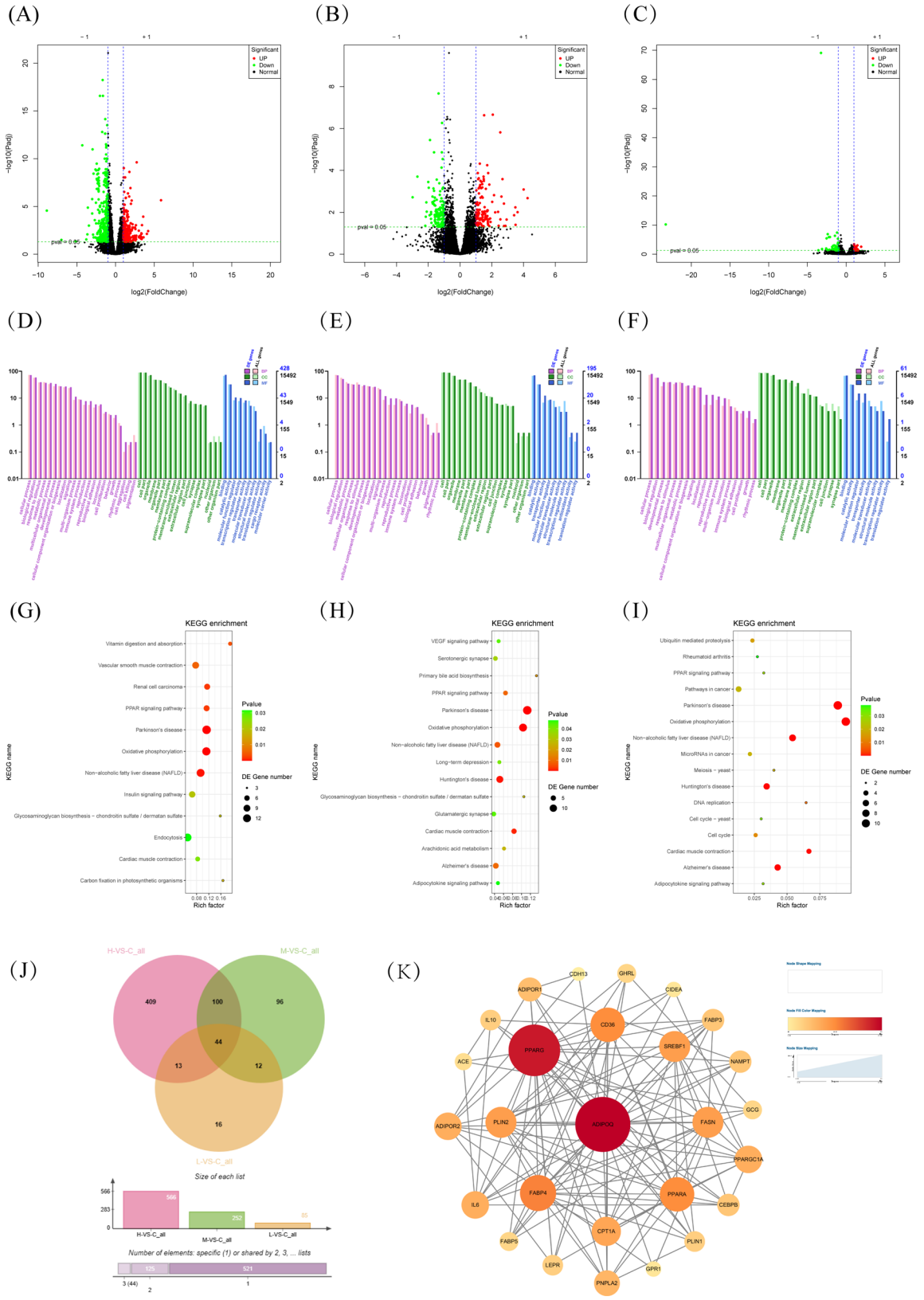

Figure 1. Identification of differentially expressed genes and GO Function Annotation and KEGG Enrichment Analysis. (A) The volcano plot of DEGs in HG vs CG. (B) The volcano plot of DEGs in MG vs CG. (C) The volcano plot of DEGs in LG vs CG. (D) Histogram analysis of GO enrichment of DEGs in breast muscles of HG vs CG. (E) Histogram analysis of GO enrichment of DEGs in breast muscles of MG vs CG. (F) Histogram analysis of GO enrichment of DEGs in breast muscles of LG vs CG. (G) KEGG functional analysis of DEGs in breast muscles of HG vs CG. (H) KEGG functional analysis of DEGs in breast muscles of MG vs CG. (I) KEGG functional analysis of DEGs in breast muscles of LG vs CG. (J) Venn diagram of DEGs in three addition groups. (K) PPI network diagram of ADIPOQ.

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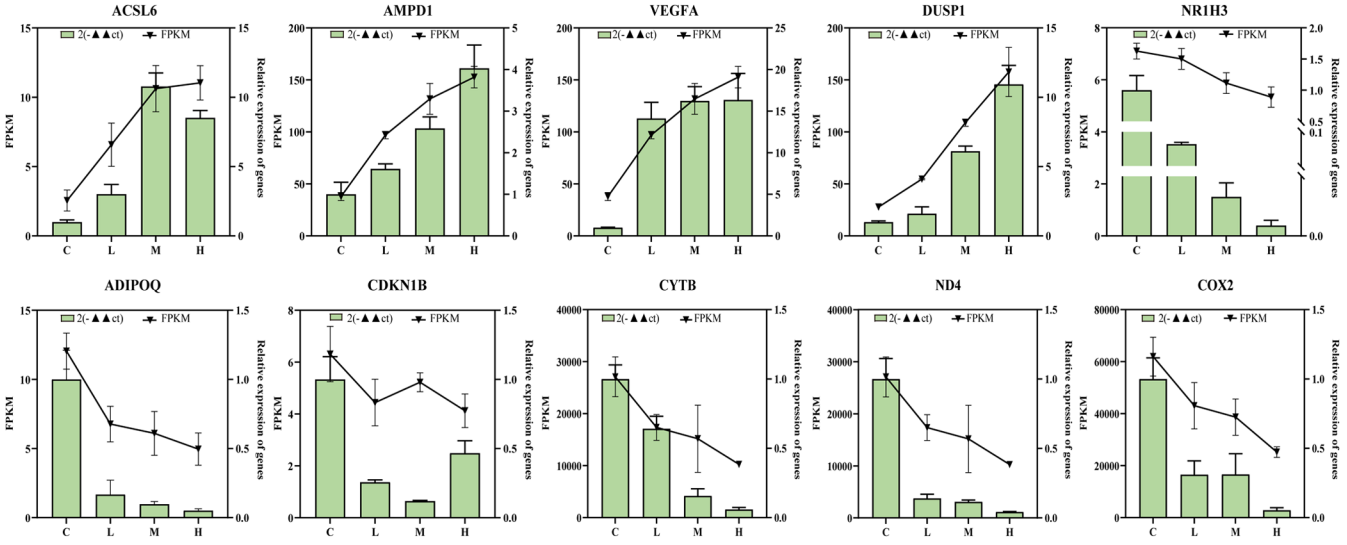

Figure 2. Validation of the DEGs from sequencing. The histogram showed the results of qRT-PCR, and the height of the columns are the values of $2(-\triangle\triangle Ct)$. Inflection points of the broken lines represented the FPKM from the sequencing results. The left ordinate is FKPM, and the right ordinate the values of $2(-\triangle\triangle Ct)$.

ADIPOQ overexpression can inhibit lipid accumulation, but *siADIPOQ* can promote lipid accumulation. Overexpression of *ADIPOQ* has the same effect as addition of ALA, and cotransfection of *siADIPOQ* and addition of ALA can produce a 'neutralizing' effect.

The expression of ADIPOQ was examined by qRT-PCR and WB after the addition of 60 μmol/L ALA and induction for 48 h. qRT-PCR results showed that the mRNA level of *ADIPOQ* was significantly up-regulated by the addition of ALA compared with the control group (Figure 4F). WB confirmed that the protein level of ADIPOQ also saw a significant upward trend after the addition of ALA (Figure 4G). Therefore, the addition of ALA to duck primary myoblasts can promote the expression of ADIPOQ.

SiADIPOQ Significantly Stimulates Fatty Acid Synthesis and Inhibits Lipid Accumulation. To verify the role of *ADIPOQ* in Pekin ducks related to fatty acid metabolism, *ADIPOQ* was knocked down or overexpressed in primary myoblasts, and then the mRNA expression levels of fatty acid synthesis, catabolism, and transport-related cytokines were detected by qRT-PCR. The results showed that among the fatty acid synthesis-related factors: *SCD1* ($P < 0.001$), *ACC1* ($P < 0.05$), and *ACC2* ($P < 0.05$) were significantly down-regulated in the case of *ADIPOQ* overexpression (Figure 5A). When compared with siNC groups, *SCD1* ($P < 0.05$), *ACC1* ($P < 0.05$), *ACC2* ($P < 0.05$) were significantly up-regulated in *siADIPOQ* groups (Figure 5B). In terms of fatty acid transport, the mRNA levels of *CD36* ($P < 0.05$), *FABP5* ($P < 0.05$), *FATP4* ($P < 0.05$) were all significantly decreased under the effect of *ADIPOQ* overexpression (Figure 5C). *SiADIPOQ* also had a highly significant up-regulation effect on the mRNA level of *FATP4* ($P < 0.001$) (Figure 5D). Inhibition of *ADIPOQ* expression significantly reduced the mRNA levels of *CPT-1* ($P < 0.05$), *LPL* ($P < 0.05$), and *PPARα* ($P < 0.001$) among the fatty acid catabolism-related factors (Figure 5F). Overexpression of *ADIPOQ* showed the opposite trend (Figure 5E). The results indicated that *ADIPOQ* overexpression inhibited fatty acid synthesis and transport, and promoted fatty acid catabolism.

WB was used to detect the expression of ADIPOQ, AMPK, p-AMPK, and CPT-1 proteins. The results showed that AMPK, p-AMPK, and CPT-1 protein levels were significantly increased when ADIPOQ protein levels were increased in primary myoblasts (Figure 5G). With ADIPOQ protein level decreasing, the opposite trend was observed (Figure 5H). After the addition of ALA, the ADIPOQ protein level was increased, suggesting that ALA can regulate lipid accumulation through the ADIPOQ/AMPK/CPT-1 pathway in myoblasts (Figure 6).

DISCUSSION

Dietary Addition of *Zanthoxylum* Seed Powder Can Affect Genes Related to Fatty Acid Metabolism in the Breast Muscles of Pekin Duck. In this study, there were 566, 252, 85 DEGs identified in the HG vs CG, MG vs CG, and LG vs CG, respectively. DEGs increased gradually as the level of *Zanthoxylum* seed powder in the diet rose. The GO enrichment results showed that DEGs were significantly enriched in Oxidative phosphorylation, Ribonucleotide metabolic process, Purine ribonucleotide metabolic process and Ribose phosphate metabolic process, which indicated that the metabolic level of Pekin duck was significantly enhanced after adding *Zanthoxylum* seed powder to the diet. Although no transcriptomic data related to the addition of *Zanthoxylum* seed powder to the diet have been reported before, the metabolic process is very important for the production performance of ducks, such as egg production, which is consistent with the results of previous reports.[11,30] KEGG functional analysis showed that the DEGs were significantly enriched to fatty acid metabolic pathways such as Oxidative phosphorylation, PPAR signaling pathway, Adipocytokine signaling pathway, same as in previous study.[31]

GO enrichment and KEGG analysis included functionally annotated genes such as *ADIPOQ*, *ACSL6*, *COX1*, *ND4*, *CYTB*. According to previous reports, these genes can be involved in fatty acid metabolism. *ND4* belongs to NADH-related genes. *COX1* and *CYTB* belong to cytochrome-related genes.[32] *ND4* encodes NADH dehydrogenase, which is used to maintain ATP synthesis in fatty acid metabolism. It was shown

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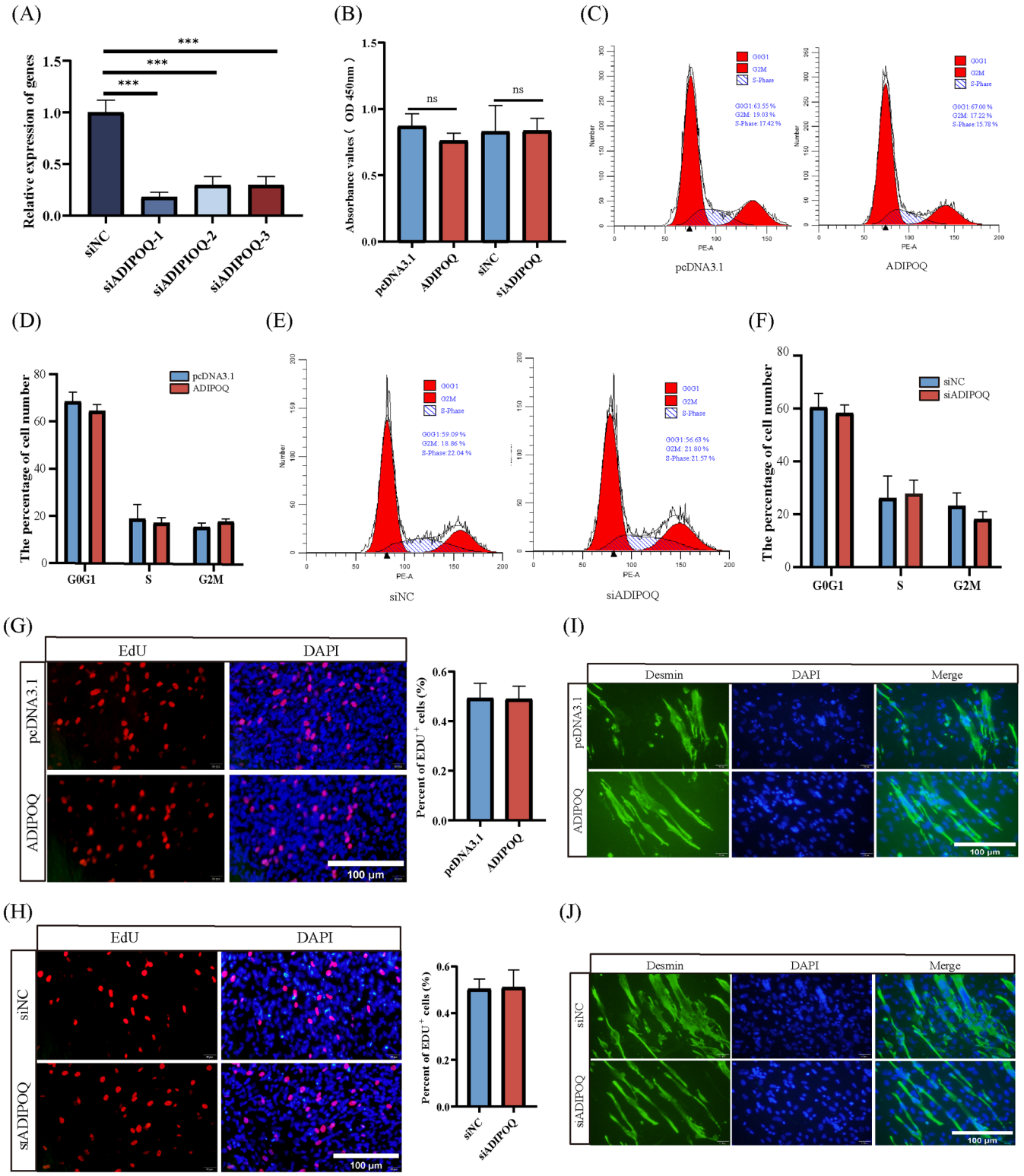

Figure 3. ADIPOQ promotes myoblasts myogenic differentiation but little effect on cell proliferation. (A) Selection of siADIPOQ. (B) CCK-8. (C, D) detecting cell proliferation using Flow cytometry after ADIPOQ overexpression. (E, F) detecting cell proliferation using Flow cytometry after transfecting with siADIPOQ. (G) detecting cell proliferation using EdU after ADIPOQ overexpression. (H) detecting cell proliferation using EdU after transfecting with siADIPOQ. (I) Detecting cell differentiation using IF after ADIPOQ overexpression. (J) Detecting cell differentiation using IF after transfecting with siADIPOQ. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

that mutations in *ND4* were associated with type 2 diabetes mellitus (T2DM).[33] COXs are key enzymes in the biosynthesis of prostaglandins (PG) from arachidonic acid. One study showed that the COX1-WIPI2 axis prevents nonalcoholic steatohepatitis (NASH).[34,35] *CYTB* is similar to *ND4*, which encodes Cytochrome b and is also essential for the proper functioning of the electron transport chain during fatty acid metabolism. The researchers tested mice exercised and fed a

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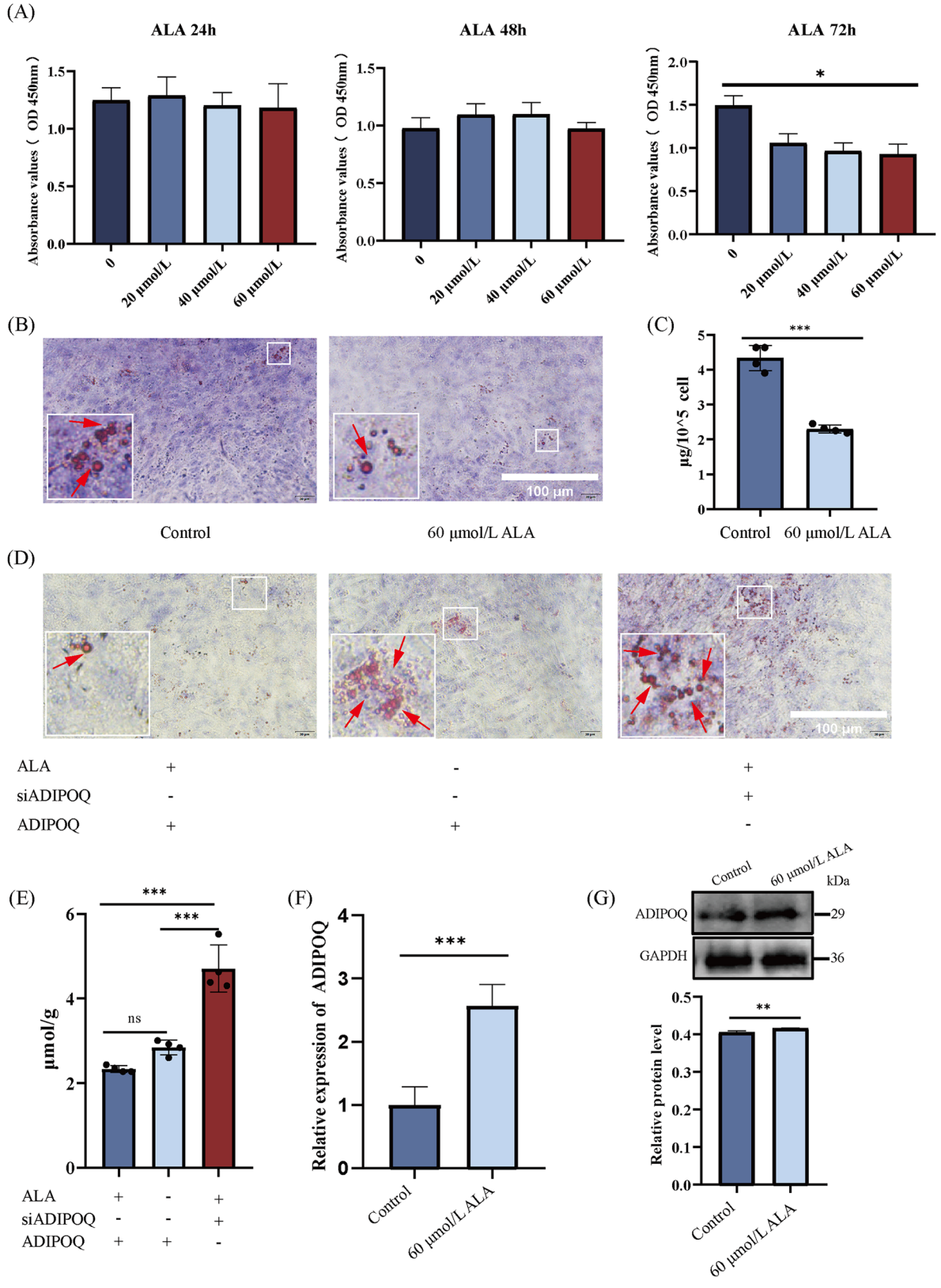

Figure 4. ALA inhibits lipid droplet accumulation and TG synthesis. (A) Selection of ALA addition amount and induction time using CCK-8. (B) Detecting lipid droplets using ORO staining after addition of ALA. (C) Detecting the TG content after addition of ALA. (D) Detecting lipid droplets using ORO staining after overexpression of ADIPOQ with addition of ALA (A/+ group), overexpression of ADIPOQ (A group), and siADIPOQ with addition of ALA (siA/+ group). (E) Detecting the TG content after overexpression of ADIPOQ with addition of ALA (A/+ group), overexpression of ADIPOQ (A group), and siADIPOQ with addition of ALA (siA/+ group). (F) Detecting the relative expression of *ADIPOQ* using qRT-PCR after addition of ALA. (G) Detecting the protein level of ADIPOQ using WB after addition of ALA. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

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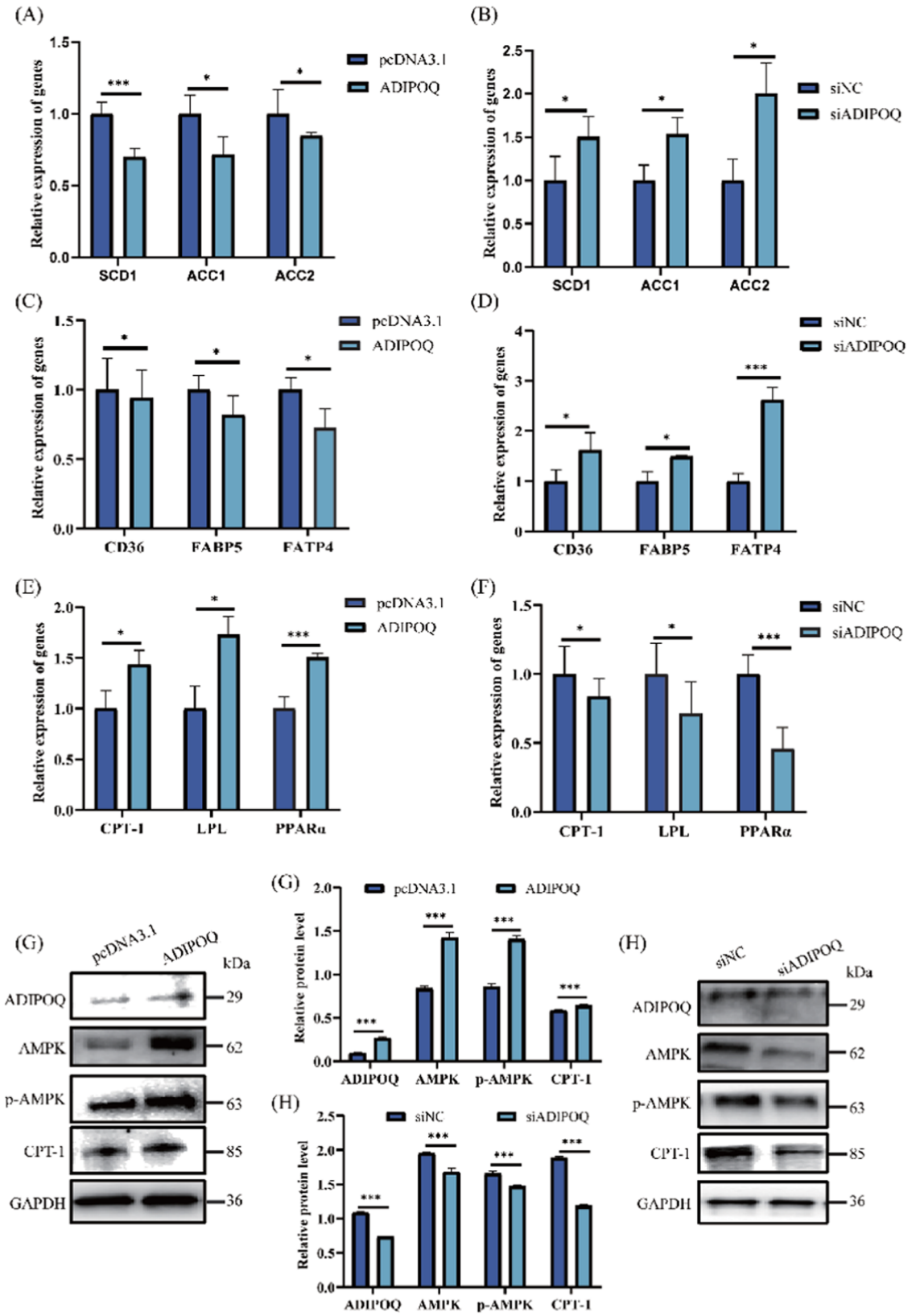

Figure 5. SiADIPOQ promotes fatty acid synthesis and inhibits lipid accumulation. (A, B) mRNA levels of fatty acid synthesis marker genes. (C, D) mRNA levels of fatty acid transportation marker genes. (E, F) mRNA levels of fatty acid oxidation marker genes. (G) Detecting the protein level of pathway proteins using WB after an upward trend of ADIPOQ protein. (H) Detecting the protein level of pathway proteins using WB after a downward trend of ADIPOQ protein. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

caffeine-free green tea extract for significant increases in the mitochondria-related genes *CYTB*, *ND5*, and *ACOX1*. Meanwhile, the weight of mice lost significantly, demonstrating that the expression of these genes were associated with the

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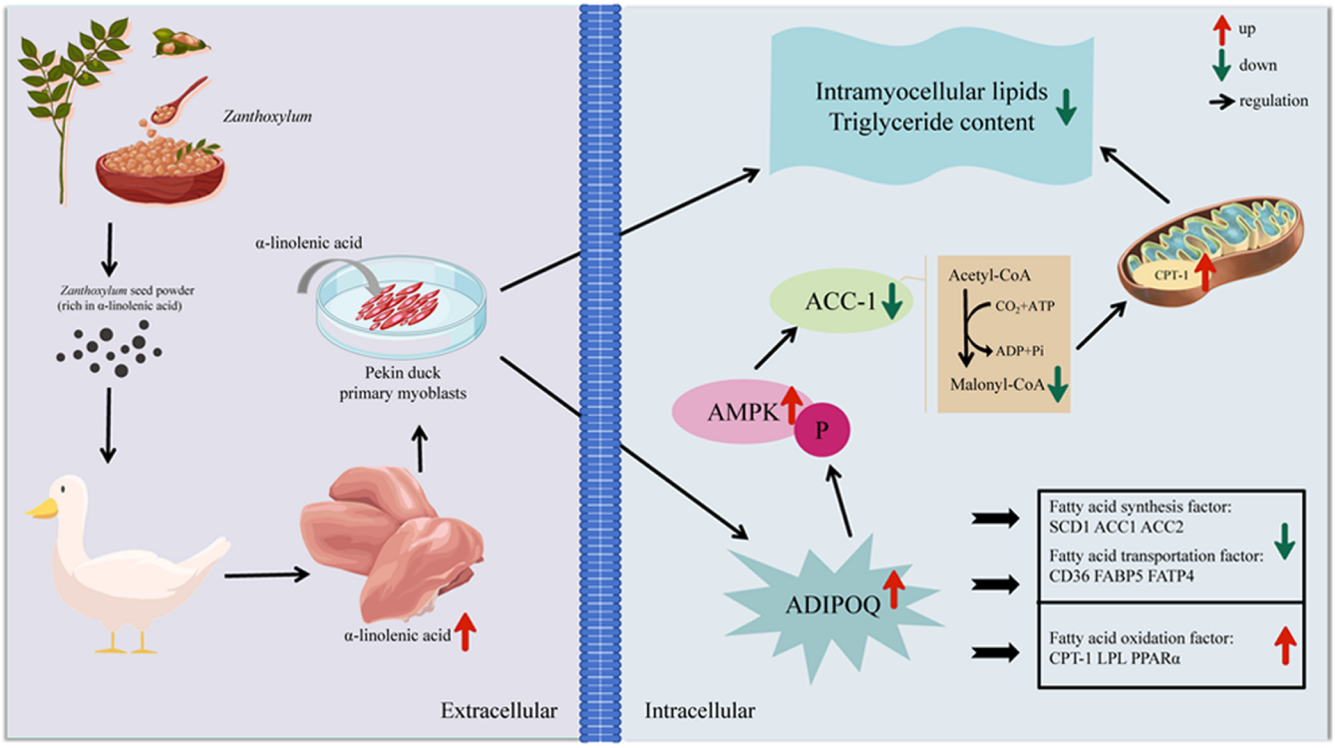

Figure 6. Signaling pathway map of ALA from *Zanthoxylum* seed powder regulating duck fatty acid metabolism.

oxidative capacity of mitochondria for fatty acid oxidation in skeletal muscle.[36]

The *ACSL* family is required for fatty acid degradation, phospholipid remodeling and the production of long-chain acyl cofactor esters that regulate a variety of physiological processes. *ACSL1* can accelerate the initial steps of fatty acid metabolism and is an important enzyme in lipid synthesis and catabolism.[37] It was shown that *ACSL1* positively regulates PUFA synthesis in bovine adipocytes and *ACSL1* overexpression promotes lipid droplet aggregation.[38] *ACSL6* is the only isoform of the *ACSL* family expressed in skeletal muscle. One study found that *ACSL6* gene repression in rat primary myotubes reduces lipid accumulation and activates a higher mitochondrial oxidative capacity program via the AMPK/PGC1-α pathway, which has also been demonstrated in other studies.[39−41] *ADIPOQ* is a hormone secreted by adipose tissue. It has been demonstrated that *ADIPOQ* is closely related to fatty acid metabolism processes in humans,[42,43] sheep,[44] pigs.[9,10,45] It has also been reported that *ADIPOQ* plays an important role in the pathogenesis of NAFLD.[46,47]

The PPI network analysis in this study showed that ADIPOQ plays a major regulatory role in fatty acid metabolism-related proteins such as PPARγ, CPT-1, CD36, FABP4, PPARα. CPT-1 is considered to be a key regulator of fatty acid oxidation.[24,48] PPARs regulate the processes of lipid metabolism, cell growth, differentiation, as well as immune and inflammatory responses.[49,50] FABP4 is an intracellular transporter protein, which is mainly used to transport lipids and is related to the transportation of PUFA.[51] CD36 also plays an important role in fatty acid metabolism, which can uptake long-chain fatty acids in muscle tissues.[52] Thus, ADIPOQ may be a key regulatory factor affecting fatty acid metabolism in duck breast muscle.

ADIPOQ Can Promote the Differentiation of Myoblasts. Skeletal muscle development is a complex biological process that depends on the proliferation of myofibroblasts, while these cells differentiate and fuse to form multinucleated myotubes. The process is mainly orchestrated by myogenic regulators, such as *MyoD*, *MyoG*, and *MRF4*, and ultimately produces mature muscle fibers.[53,54] *Cyclin D1*, *PCNA* and *mTOR* play important functions in the proliferation of myoblasts. The key role of *mTOR* in muscle is achieved through mTORC1, which can promote muscle hypertrophy in the short term.[55]

There is evidence that *ADIPOQ* is expressed by skeletal muscle fibers and can influence muscle fiber type, suggesting that *ADIPOQ* plays an important role in the regulation of muscle fiber type.[56] Leucine may increase *ADIPOQ* levels and then activate the expression of *AMPK* and *PGC-1α*, leading to the transformation of muscle fibers from a fast to a slow muscle type in pigs ultimately.[57] It was found that resveratrol (RES) treatment increased plasma levels of ADIPOQ and upregulated MyHC1 expression via the AdipoR1-AMPK-PGC-1α pathway, which is consistent with the results of the leucine assay described above.[58] This is also consistent with previous report that the *ADIPOQ* gene increases oxidized type I muscle fibers by regulating AMPK and PGC-1α expression.[59] In the present study, it was shown that when *ADIPOQ* was transfected in duck primary myoblasts, the rate of myotube fusion of the cells could be significantly promoted, and the opposite result was found when *siADIPOQ* was transfected. However, *ADIPOQ* had no significant function in cell proliferation, which may be due to the fact that *ADIPOQ* mainly acts on the process of muscle maintenance and regeneration by promoting cell differentiation and regulating the transformation of myofibre types in Pekin duck, thus promoting muscle production. But it has no significant effect on the process of early muscle development.

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J. Agric. Food Chem. 2025, 73, 14651−14665

ALA Can Significantly Affect Lipid Deposition in Pekin Duck. Fatty acids are synthesized in poultry mainly in the liver and can then be transported to other tissues such as fat, heart and muscle.[60] Many studies have shown that the addition of linseed oil to the ration significantly increases the n-3 PUFAs content of broiler muscle.[61,62] Addition of 10% flaxseed to the duck ration resulted in an overall increase in n-3 PUFAs levels in breast muscle and skin.[63] The results of our previous study showed a significant increase in ALA content in duck breast muscles after the addition of *Zanthoxylum* seed powder to the ration (unpublished data). Furthermore, we added ALA to duck primary myoblasts. The results showed that ALA significantly inhibited the accumulation of IMCL and TG content, and promoted an increase in ADIPOQ levels. This is consistent with the findings that flaxseed oil increased the expression of *ADIPOQ* in visceral fat and hypothesized that ALA helps to suppress obesity.[64] ALA can down-regulate *FASN* thereby inhibiting the de novo synthesis of fatty acids. This is consistent with previous results.[65] In contrast, in this study, ALA levels went up and the mRNA and protein levels of ADIPOQ subsequently decreased in duck muscle tissues with a higher dose of *Zanthoxylum* seed powder. The reason for analyzing this may be due to the dose effect, which may result in the abnormal mRNA and protein expression of *ADIPOQ* with the high intake of n-3 PUFAs. It has been suggested that the daily intake of ALA in the human body should be 1600−1800 mg.[66] One study presented that a high-fat diet (HFD)-induced model of Diabetic cardiomyopathy (DCM) showed a significant decrease in *ADIPOQ* expression, suggesting that *ADIPOQ* levels may be associated with diet-induced metabolic disorders.[67] Therefore, the reason for this analysis could also be due to the fact that *Zanthoxylum* seed powder is rich in both saturated fatty acids such as palmitic acid and stearic acid, as well as monounsaturated fatty acids. There is also an interaction of their intake on the *ADIPOQ*, which modulates ADIPOQ expression and thus the risk of obesity. The conjecture was also confirmed in the study.[68] Compared to other animals, the poultry has a greater ability to deposit n-3 PUFAs from the diet into their tissues,[69] which explains the ability to significantly increase the ALA content in duck muscle tissues after the addition of *Zanthoxylum* seed powder to the ration.

Lipids in the form of free fatty acids or lipoproteins in the blood can be obtained from dietary sources or synthesized in the liver, adipose tissue and mammary glands. Then they are transported into cells via *FABP5*. Fatty acids can be taken up from the extracellular environment through cell surface receptors, such as *CD36*.[70] Fatty acid biosynthesis takes place mainly in the cytoplasm and is subject to the combined action of three key enzymes-*ACLY*, *ACC*, and *FAS*. *PPARs* are transcription factors belonging to the lipid-activated transcriptional factors, including three forms: *PPARα*, *PPARβ*, and *PPARγ*. Among them, *PPARγ* is positively correlated with adipogenesis.[71] *PPARα* is mainly expressed in tissues with high fatty acid oxidation rate, regulating and participating in fatty acid catabolism. It was shown that PUFAs combining PPARα to participate in the transportation, synthesis and β-oxidation of fatty acid metabolism.[72] Another study found a significant increase in the expression of *PPARα* and *LPL* after the addition of LA and EPA to duck liver cells cultured in vitro.[73] In this study, transfection of *siADIPOQ* significantly up-regulated the levels of fatty acid transport-related factors *FABP5*, *CD36*, *FATP4*, and fatty acid synthesis factors *SCD1*, *ACC1*, and *ACC2*, while fatty acid catabolism-related factors *CPT-1*, *LPL*, and *PPARα* were down-regulated. These results proved that *ADIPOQ* could inhibit fatty acid transport and synthesis. Meanwhile, this study found that *ADIPOQ* overexpression had the same effect as addition of ALA. Cotransfection of *siADIPOQ* and addition of ALA produced a 'neutralizing' effect. It is proved that *ADIPOQ* could inhibit the accumulation of IMCL, which was in line with the results of the previous study.[74−77]

Fatty acids can be produced through anabolism and catabolism, and can also be obtained through diet or gut microbiota.[78] *AMPK* is a heterotrimer consisting of one catalytic subunit (α) and two regulatory subunits (β and γ), which plays an important role in regulating the body's material and energy metabolism.[79,80] Previous studies have shown that the phosphorylation site (Thr172) in the catalytic α-subunit can be phosphorylated by upstream protein kinases (LKB1, CaMKK, and TAK1) within the catalytic subunit to activate AMPK.[81] The LKB1-AMPK cascade reaction is a key signaling pathway that regulates changes in glucose uptake, fatty acid uptake, and fatty acid oxidation in contracting skeletal muscle.[82,83] It has been demonstrated that the absence of *LKB1* leads to a significant decrease in the phosphorylation level of AMPK and enhances lipid accumulation in muscle progenitor cells and mature muscles.[84] It was found that γ-linolenic acid (GLA) treatment of AML-12 up-regulated the mRNA and protein expression of LKB1, thereby preventing and treating NAFLD and regulating lipid metabolism by balancing autophagy and apoptosis through the LKB1-AMPK-mTOR pathway.[85] In the present study, the protein level of p-AMPK was up-regulated in ALA-treated myoblasts, so it is reasonable to hypothesize that the expression level of LKB1 was up-regulated, which may also be one of the reasons for the reduced content of lipid deposition in myoblasts. *CPT-1* is the rate-limiting enzyme for fatty acid β-oxidation. *ACC* can catalyze the carboxylation of acetyl coenzyme A to generate malonyl coenzyme A, which can inhibit the expression of *CPT-1*. The activation of *AMPK* phosphorylation inhibits its downstream target *ACC*, which in turn promotes the expression of *CPT-1*. Therefore, when fatty acid anabolism is enhanced, the increase in malonyl coenzyme A content will further inhibit *CPT-1* activity and reduce the level of fatty acid oxidative catabolism. In this study, the addition of ALA to primary myoblasts up-regulated the protein level of ADIPOQ, and then the protein levels of AMPK, p-AMPK, and CPT-1 all increased. Therefore, it is so clear that ALA can regulate lipid accumulation through the ADIPOQ/AMPK/CPT-1 pathway in myoblasts.

According to these results of discussion, *ADIPOQ*, *ACSL6*, *COX1*, *ND4* and *CYTB* played key roles in regulating fatty acids in duck breast muscles, among which ADIPOQ may be the most important regulatory gene. The fat content of meat is affected by species, muscle and tissue type, and production management. It can affect the nutritional, organoleptic, and overall quality of meat products.[86] *ADIPOQ* may improve muscle production by stimulating cell differentiation, regulate muscle fiber type and muscle quality. Addition of ALA to primary myoblasts revealed that ALA inhibited lipid droplet accumulation and TG synthesis, suggesting that ALA may have an antiobesity function. ALA can promote the transcription and protein levels of ADIPOQ, which is contrary to the RNA-seq results. The reasons may be due to dose effects or high-fat-induced eating disorders. It is suggested that when

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J. Agric. Food Chem. 2025, 73, 14651−14665

Zanthoxylum seed powder is added to the ration, LG (1.5%) may be the optimal level of addition among three addition groups in this study. Otherwise, it may lead to excessively abnormal ADIPOQ expression levels, which can result in the risk of obesity that affects the quality of the meat. It may also lead to the muscle yield to be affected in an adverse manner.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.5c01995.

Whole sequence of duck ADIPOQ; the specific primer sequences of duck ADIPOQ overexpression vector (Table S1) (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ALA: α-linolenic acid; ADIPOQ: adiponectin; IMF: intramuscular fat; IMCL: intramyocellular lipids; PUFAs: polyunsaturated fatty acids; LA: linoleic acid; NAFLD: nonalcoholic fatty liver disease; DEGs: differentially expressed genes; MF: molecular function; BP: biological process; CC: cellular component; T2DM: type 2 diabetes mellitus; PG: prostaglandins; NASH: nonalcoholic steatohepatitis; HFD: high-fat diet

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