Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola

ISSN 1516-635X 2022 / v.24 / n.4 / 001-008

http://dx.doi.org/10.1590/1806-9061-2021-1589

Technical Note

Knockdown of CPT1A Induce Chicken Adipocyte Differentiation to Form Lipid Droplets

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■Keywords

Adipocyte differentiation, CPT1A, lipid droplets.

Submitted: 11/February/2022
Approved: 09/May/2022

ABSTRACT

Lipid metabolism dysfunction is closely related to obesity, inflammation, diabetes, lipodystrophy, cardiovascular disease. Along with having a positive effect on energy homeostasis during fasting or prolonged exercise through mitochondrial fatty acid oxidation (FAO), more than two dozen enzymes and transport proteins are involved in the activation and transport of fatty acids into the mitochondrial, providing insights into their critical roles in metabolism. CPT1A has been reported to be expressed ubiquitously in the body and associated with dire consequences affecting fat deposition as the key rate-limiting enzyme of FAO. However, there is a dearth of data on the specific role of CPT1A on adipogenic differentiation and adipocyte lipolysis on chicken. This study assessed CPT1A's function in adipocyte differentiation andadipocyte lipolysis, and the mechanisms were investigated. We found that CPT1A knockdown (KD) promotes the differentiation of chicken preadipocytes into mature adipocytes. CPT1A KD increased PPARγ protein expression level. Expression levels of lipid synthesis-related genes were increased, and lipolysis genes were reduced.

Also, CPT1A KD can encourage the formation of lipid droplets. So our results confirmed that knockdown of CPT1A induced the lipid differentiation and inhibited the β-oxidation process to promote the formation of lipid droplets. These findings may deepen our understanding on CPT1A function, especially its regulatory role in adipocyte biology.

INTRODUCTION

As a high quality protein resource, chicken provides rich nutrients for human beings (Jayasena *et al.*, 2013). In poultry production, on one hand disorders of lipid metabolism can affect poultry health and thus meat and egg quality (Ni *et al.*, 2018; Nakamura *et al.*, 2020; Mir *et al.*, 2021; Ren *et al.*, 2021). On the other hand, differences in the distribution and composition of lipids can also affect the flavor of chicken (Aaslyng *et al.*, 2017). Therefore, studying the lipid metabolism and intermuscular fat is an entry point for poultry research. This study serves as a reference and theoretical basis for poultry production.

Lipid metabolism has been identified as an important biological process in living organisms, penetrating every aspect of physiological activity. When energy intake is scarce, this fat can be released as fatty acids into the bloodstream, where it is taken up by other tissues and used as an alternative energy source. When energy intake constantly exceeds the energy expenditure, the adipose tissue expands as a result of hypertrophy in particular but also due to hyperplasia of the adipocytes (Matsushita *et al.*, 2021). In addition to having a positive effect on energy homeostasis during fasting or prolonged exercise through mitochondrial fatty acid oxidation (FAO), more than two

dozen enzymes and transport proteins are involved in the activation and transport of fatty acids into the mitochondrial, providing further insights into their critical in metabolism (Kohjima *et al.*, 2007).

Carnitine palmitoyl transferase 1A(CPT1A), an enzyme present in the outer membrane of mitochondria, functions to transport fatty acid from the cytosol into mitochondria (Lin *et al.*, 2020). It also plays a vital role in regulating the oxidation process of fatty acids in vivo to a higher degree and affecting the normal life activities of the body (Park *et al.*, 1998; Gobin *et al.*, 2002; Zammit *et al.*, 2008). As the key rate-limiting enzyme of FAO, CPT1A has been reported to be expressed ubiquitously in the body and associated with dire consequences affecting fat deposition (Morash *et al.*, 2011). However, there is a dearth of data on the specific role of CPT1A on adipogenic differentiation and adipocyte lipolysis on chicken.

In our previous studies, we found significant differences in the gene expression of CPT1A in intramuscular adipocytes at different development stages on chickens (Li *et al.*, 2021). Changes in CPT1A activity occur in response to animal or cell's nutritional and hormonal status (Shin *et al.*, 2006). Intramuscular adipocyte deposition can greatly affect the quality and taste of chicken meat and the health of broilers, which is also of great significance in the production practice of broilers. Recent studies have shown that the production of flavor and aroma compounds is associated with intramuscular fat development, fatty acid deposition, and intramuscular lipid metabolism (Song *et al.*, 2013; Sohaib *et al.*, 2017). Hence, in this study, CPT1A's function in adipocyte differentiation and adipocyte lipolysis was assessed, and the mechanisms were investigated.

MATERIALS AND METHODS

Ethics Statement

All the procedure was approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University (Certification No. YCS-2020202024). All experiments were performed under the SAU Laboratory Animal Welfare and Ethics guidelines.

Animals and Sample Collection

Daheng broiler chicken were used in the current research, provided by the Daheng Poultry Breeding Company (Sichuan, China). Five chickens were sacrificed at 150 days for tissue expression profile testing. The tissues were collected, including heart, liver, spleen, lung, kidney, breast muscle, leg muscle, subcutaneous fat, abdominal fat, gizzard, glandular stomach, ovary, and pituitary.

Broiler Intramuscular adipocyte Cell Isolation

Primary chicken intramuscular preadipocytes were isolated from pectoral major breast muscle tissue of 15-day-old chickens following the methods Zhang M at al. (2018) described previously. Breast muscles were aseptically isolated, and all visible connective tissue was removed. The isolated muscle tissues were washed three times in pH 7.4 phosphate-buffered saline (PBS) with 200 U/mL penicillin-streptomycin. Excess fascia and connective tissue were removed. Then the tissue was minced to 1 mm^3 to be digested by collagenase I and II. Finally, adipocyte was isolated by differential attachment method.

Cell Culture and Treatment

Intramuscular adipocytes cells were seeded in 6-well plates, cultured in DMEM, and transfected si-CPT1A and Negative Control (NC). The sequences are listed in Table1. Small interfering RNAs (siRNAs) were synthesized by GenePharma Co. Ltd. (Shanghai, China). The Lipofectamine 3000 Reagent (Invitrogen, USA) was used as a transfection reagent, and cells were harvested after 48h, subsequent WB experiment, RNA extraction, and oil red O staining, and triglyceride extraction. The appropriate test methods are according to Li *et al.* (2020)'s described previously.

Table1 – The RNA oligonucleotides used for cell transfection.

Name	siRNAsequence (5'-3')
siRNA-CPT1A-gallus-879	GCAAUGGACUUCCUUCAUUTT AAUGAAGGAAGUCCAUUGCTT
Negative siRNA	UUCUCCGAACGUGUCACGUTT ACGUGACACGUUCGGAGAATT

RNA Extraction and Quantitative Real-Time PCR

According to the manufacturer's instructions, total RNA was extracted from the tissues and cells using TRIzol reagent (Takara, Tokyo, Japan). Quantitative real-time PCR (qRT-PCR) analysis was performed in reaction volumes of 10 μL containing 1 μL of cDNA, 0.5 μL of forward and reverse primers, 5 μL of TB GreenTM Premix (Takara), and 3 μL of DNase/RNase-Free Deionized Water (Tiangen, Beijing, China). The qPCR amplification procedure was as follows: preincubation at 95 for 5 min, followed by 40 cycles of 30 s at 95 °C,

30 s at 60 °C, and 30 s at 72 °C. Reaction conditions were based on the manufacturer's instructions, and the $2^{-\Delta\Delta Ct}$ method was used to calculate fold changes in gene expression (Livak *et al.*, 2001). The GAPDH genes and β-actin genes were used as internal controls, and primer sequences are shown in Table 2.

Western Blotting

Western Blotting was performed as Luo *et al.* (2016), as previously described. Intramuscular adipocytes were seeded in 6-well plates, cultured in DMEM, and transfected si-CPT1A and Negative Control. The concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China); after 72 h, the proteins of transfected cells were extracted using lysis buffer. Total proteins (10μg) were separated by 12% SDS-PAGE and transferred to a 0.2mm polyvinylidene fluoride (PVDF) membrane that was soaked in formaldehyde. The membrane was blocked with blocking buffer (Beyotime) for approximately one h at room temperature before incubation overnight at 4 °C with direct detection antibodies [anti-PPARγ, ABclonal Technology Co., Ltd; 1:1,000) and anti-β-tubulin (ZenBio, Chengdu, China; 1:2,000)]. The PVDF membrane was washed three times with western wash Buffer (Beyotime) and then incubated for one h at room temperature with goat anti-mouse immunoglobulin G (IgG) H & L (ZenBio; 1:5,000) and Rabbit Anti-Rabbit IgG H&L (ZenBio; 1:5,000). Finally, antibody reactive bands were noticed using enhanced chemiluminescence (ECL) (Beyotime). Image J software was used to measure the relative expression of the protein. Image Lab5.2 was used for densitometric analysis of the expressed protein bands.

Oil Red Staining and Cellular TG Content Measurement

Harvested Broiler Intramuscular adipocytes were washed three times with PBS and fixed with 4% formaldehyde for 30 min at 25 °C. After washing with PBS twice, cells were stained with diluted Oil Red O for 30 min at 25 °C in a dark environment. Then, Oil Red O was removed, adipocytes were washed five times with distilled water. In the end, adipocytes were studied with microscopy (Pang *et al.*, 2013). The manufacturer's instructions measured TG content in a triglyceride content detection kit (APPLYGEN, Beijing, China).

Statistical analysis

All experiments were conducted at least three times. GraphPad Prism 8.0 was used to graph the results. A two-sample t-test was used to perform the statistical significance test between groups, where all experimental data were presented as Mean ±SEM. Differences were considered statistically significant at $p<0.05$ or $p<0.01$.

RESULTS

Expression Profiles of CPT1A in Broiler Tissues and intramuscular adipocytes during adipogenesis

The total RNAs from thirteen tissues of 150-day-old Daheng broiler chickens were extracted, and real-

Table 2 – The specific primers used for qPCR.

Gene	AccessionNumber	Forward Primer Sequence 5′-3′	Reverse Primer Sequence 5′-3′
β-actin	NM_205518.1	AATGGCTCCGGTATGTGCAA	GGCCCATACCAACCATCACA
GAPDH	NM_204305.1	TCCTCCACCTTTGATGCG	GTGCCTGGCTCACTCCTT
CDK2	NM_001199857.1	CCAGAACCTCCTCATCAAC	CAGATGTCCACAGCAGTC
CCND1	NM_205381.1	CAGAAGTGCGAAGAGGAAGT	CTGATGGAGTTGTCGGTGTA
CCND2	NM_204213.1	CCCACTCGAAAGTGCCATCT	TGCTGCAAGGTTCCACTTCA
PCNA	NM_204170.2	GAGACCTCAGCCACATTGGT	AGTCAGCTGGACTGGCTCAT
MKI67	XM_004942359.4	TAGCACCAGCAGACACCTTG	GCCATCAGCCTACTCAGACC
Caspase-3	NM_204725.1	TGGCCCTCTTGAACTGAAAG	TCCACTGTCTGCTTCAATACC
Caspase-8	XM_040703262.1	AGCTGTAATGCAGGGGTTCT	GGCCTCACGATCCTTCTGAC
Caspase-9	XM_424580.5	TCCCGGGCTGTTTCAACTT	CCTCATCTTGCAGCTTGTGC
BCL2	NM_205339.1	ATCGTCGCCTTCTTCGAGTT	ATCCCATCCTCCGTTGTCCT
PPARG	XM_015292931.2	GTGCAATCAAAATGGAGCC	CTTACAACCTTCACATGCAT
FASN	NM_205155.3	AGCCCAAGTATTCAGGCACC	TTCAGGATGCCCACATCACC
ACSL1	NM_001012578.1	TCAATGCCTGGTGACTGGAC	CCTCACCTTCTCCTTTGGCA
ME1	NM_204303.1	AAGAGGGGCTACGAGGTGCT	CAGTTGTTGCCTTTCCTCCAAG
ACOX1	NM_001006205.1	AAGGAGATCGAGGCCTTAGTG	GCCGTCCACGATGAACAAAG
ACOX2	XM_004944646.4	ATGTAAGGCGTGGGTCACAG	GCTTCTTCTTCCCCAGGCTT
ACOX3	XM_420814.6	AAAGAAGACAGTGGCAACCG	TCACCATCGAGAAACACTGC

time PCR (RT-PCR) was performed to determine the tissue expression profile of CPT1A. The results showed (Figure 1) that CPT1A was highly expressed in liver, subcutaneous fat, and abdominal fat.

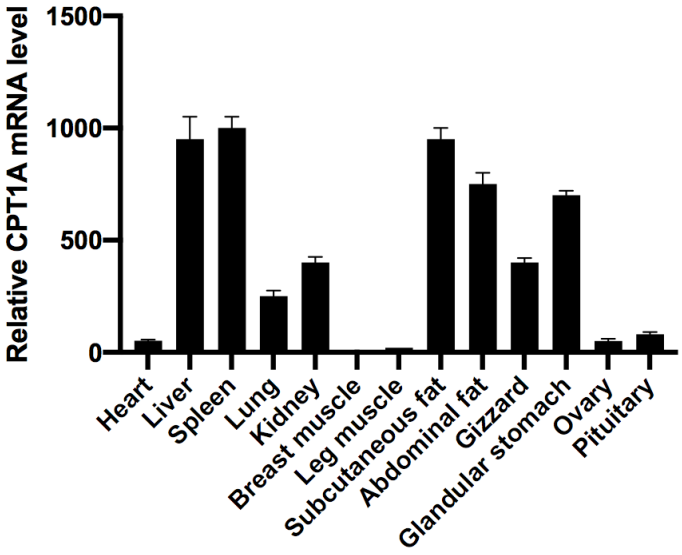

Figure 1 – The expression profile of CPT1A in thirteen tissues.

The broiler intramuscular adipocytes were isolated to study the role of CPT1A in broiler intramuscular adipogenesis. The intramuscular adipocytes were fully differentiated and fulfilled with large lipid droplets (Figure 2) after eight days of induction. Then RNA was removed from adipogenic cells at 0, 2, 4, 6, 8, and10 days of differentiation to spot the CPT1A expression pattern during intramuscular adipocyte differentiation (Figure 3). The expression level of CPT1A in all stages of induced differentiation was higher than that in the period without induced differentiation (0d).

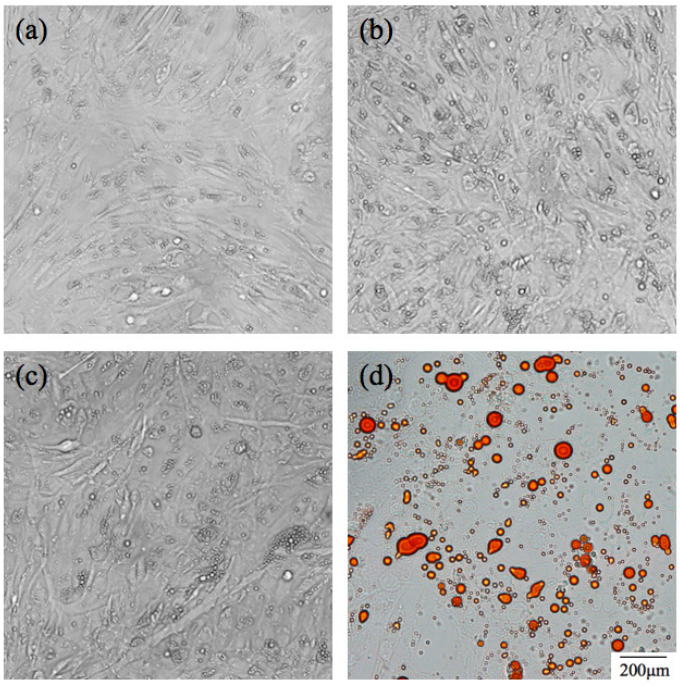

Figure 2 – Cell morphology change during broiler intramuscular adipocyte differentiation.

(a) broiler intramuscular adipocyte before 3-isobutyl-1-methylxanthine (IBMX)- Dexamethasone (DEX)- inslin (INS) induction; (b) broiler intramuscular adipocyte at 2 days after induction; (c) broiler intramuscular adipocyte at 6days after induction; (d) broiler intramuscular adipocyte 8days after induction staining with Oil Red O).

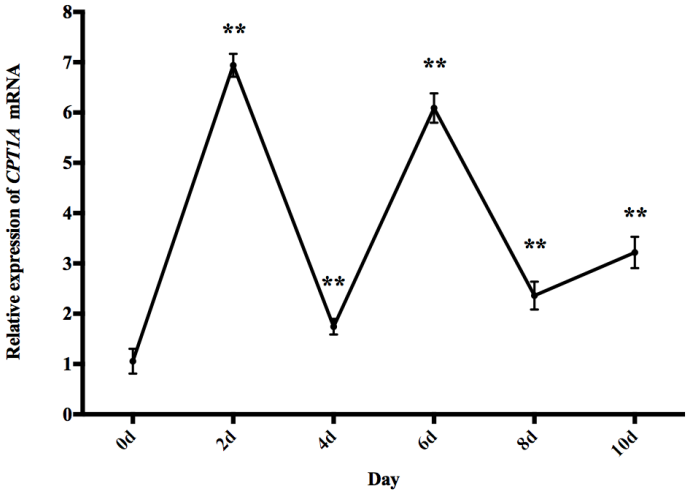

Figure 3 – Expression of CPT1A during broiler intramuscular adipocyte differentiation.

The expression at the day 0 as a control. GAPDH RNA was used as a reference gene. Results were presented as means ± SEM, n=3,$^{**}p<0.01$.

CPT1A inhibits lipid droplet synthesis

Si-CPT1A or Negative Control was transfected to intramuscular adipocytes to research the potential function of CPT1A for intramuscular adipogenesis. The RT-qPCR assay displayed that the knockdown efficiency is obvious, reaching 67% (Figure 4a). The adipocytes cell morphology changed after induction staining with Oil Red O (Figure 4b); The results of oil red O determination displayed that in the random visual field area, the area of fat droplets formed in the si-CPT1A transfected group was way higher than that in the Negative Control group. The corresponding absorbance measured the content of triglycerides at 510nm (Figure 4c) to verify the further comparison of triglyceride formation. The absorbance of the si-CPT1A treatment group after adsorption of triglyceride with isopropanol and transfection at 510nm was also higher than that of the Negative Control group.

Knockdown of CPT1A promoted lipid differentiation and inhibited lipid oxidation

We measured the downstream gene expression related to the lipid metabolism of the si-CPT1A and Negative Control group to explore these genetic evidence in cell proliferation, cell differation, cell apoptosis, lipid transport and β oxidation strongly suggested that after being transfected by si-CPT1A,

the triglyceride content was significantly elevated in the si-CPT1A group.

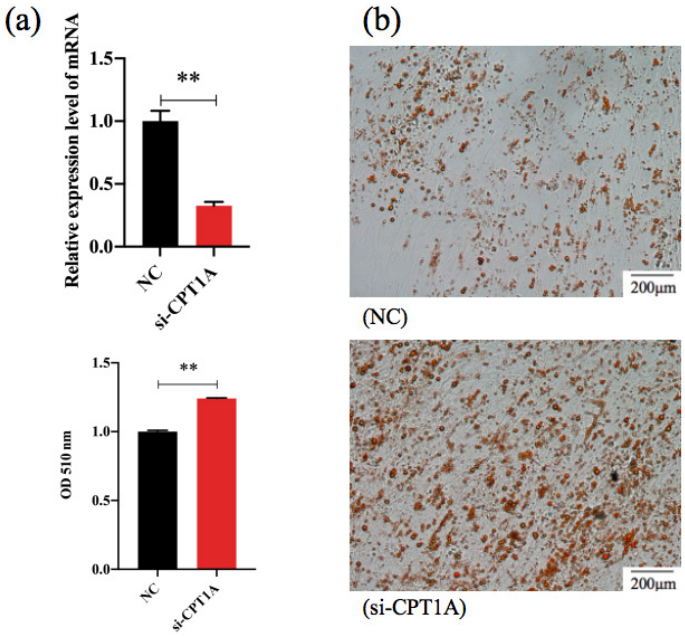

Figure 4 – The mRNA expression level of CPT1A and NC after knock down.

There was no significant difference in the expression of genes related to proliferation and apoptosis between the two groups (Figure 5). The expression levels of markers associated with adipose differentiation syntheses, such as PPARγ, FASN, and ACSL1, were relatively improved, whereas part of the expression levels of genes related to β-oxidation in process in lipid

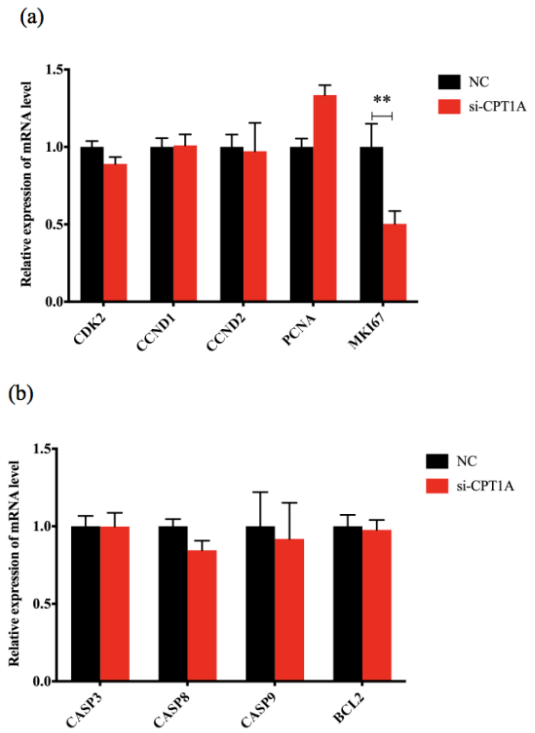

Figure 5 – (a) Proliferation-related genes expression (b) Apoptosis-related genes expression.

decomposition were significantly reduced in the si-CPT1A group. (Figure 6a).

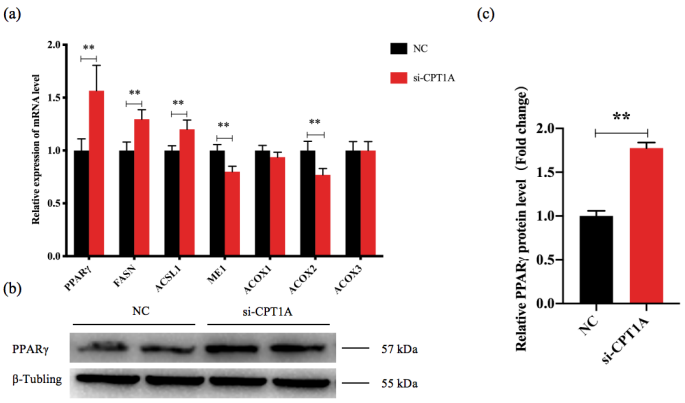

Figure 6 – CPT1A inhibits lipid differentiation through the PPAR pathway.

(a) Lipid metabolism-related genes expression (b) The protein expression of PPARγ in the intramuscular adipocytes in of two groups was analyzed by western blot (c) Relative PPARγ protein level.

DISCUSSION

Lipid metabolism is an important physiological process in the human body, including lipid synthesis, decomposition, transport, and oxidation. A variety of factors regulates the balance of lipid metabolism, and the activities of living organisms cannot be separated from the various processes in which lipids participate. Lipid metabolism supplies not only the energetic needs of the cells but also provides the raw material for cellular growth and the signaling molecules for many oncogenic pathways (Alannan *et al.*, 2020). Carnitine palmitoyltransferase-1 (CPT1) converts long-chain acyl-CoAs into long-chain acylcarnitines, which push their translocation across the mitochondrial membrane into the mitochondrial matrix, where fatty acid β-oxidation takes place (Lai *et al.*, 2020). As one of the CPT1 subtypes, based on its role in determining the total rate of fatty acid oxidation and cytosolic concentration of long-chain acyl-CoA esters, CPT1A functions in energy metabolism is involved in many physiological processes (Zammit *et al.*, 2008). The enzyme CPT1Aresides in the outer mitochondrial membrane, which catalyzes the transfer of acyl groups between coenzyme A (CoA) and L-carnitine, converting acyl-CoA esters into acyl-carnitine esters reversibly (Joshi *et al.*, 2020). These acyl-carnitines can then enter the matrix where β-oxidation takes place (Schlaepfer *et al.*, 2020). From the perspective of gene function, CPT1A is involved in regulating a variety of lipid metabolic diseases.

In this experiment, the gene expression level of CPT1A in the liver, sebaceous fat, abdominal fat, and other lipogenic parts, which are closely associated to lipid metabolism of Daheng broiler chickens, was

higher than that in other regions, indicating that CPT1A is vigorously involved in the process of lipid generation or lipid metabolism in poultry. Previous studies on CPT1A have covered multiple populations, including lipopolysaccharides such as the liver, focusing on the relationship between its regulation and energy stress (Schlaepfer *et al.*, 2020). We focused on meat production to explore the common function of CPT1A in intramuscular fat, other fats, and the specific regulatory role of CPT1A. Lipid metabolism and deposition are closely associated with the health of broilers and the quality of meat, attracting people's attentionto the quality of chicken. Hence, the study of intramuscular fat is of great importance for human production activities.

In inducing differentiation after the separation of intramuscular adipocytes, the formation process of lipid droplets can also be seen. We can see the process of lipid droplets from sparse to dense, from single lipid droplets to aggregation into clusters, and finally into sheets to form larger lipid droplets. During the whole process of fat proliferation and induced differentiation, the expression level of CPT1A was significantly higher than that of 0D, which further showed that CPT1A was actively involved in the process of lipid generation or lipid metabolism. This was consistent with previous studies (Dai *et al.*, 2018; Wei *et al.*, 2021). We transfected adipocytes with an interference vector and knocked down the expression of CPT1A. The degree of lipid production in the CPT1A knockdown group was significantly higher than that in the negative control group. The same indicated that CPT1A had an inhibitory effect on lipid production, and knocking down CPT1A could directly increase lipid synthesis. However, the downstream gene expression levels revealed that the expression levels of genes related to proliferation and apoptosis did not drastically change after the knockdown of CPT1A, indicating that CPT1A did not regulate the process of lipogenesis by affecting the proliferation and apoptosis of adipocytes.

However, during lipid differentiation, the PPARγ pathway was significantly activated. The PPARγ is a major regulator of adipocyte gene expression and insulin signaling between adipocytes and is involved in adipocyte differentiation, transport, and storage. When activated, PPARγ can control lipid mobilization in adipocytes by promoting adipogenesis and regulating the expression of proteins and cytokines secreted by adipocytes, such as leptin and adiponectin (Zhong *et al.*, 2020), suggesting that CPT1A may activate PPARγ pathway-related genes outside the mitochondrial membrane to promote lipid differentiation. The Western Blot experiment on PPARγ further confirmed this conclusion. FASN is also a marker gene for adipose differentiation and synthesis, as a basic metabolic enzyme, whose main function is to catalyze the synthesis of acetyl CoA (CoA) and malonic monoylcoA into A long-chain soft fatty acid (Chiralass *et al.*, 2004). After the knockdown of CPT1A, the expression of this gene was also significantly increased, indicating an increased effect of lipid synthesis. Simultaneously, the expression of ASCL1 gene, which is closely associated with TG synthesis and storage, also increased significantly. ACSL1 can activate the membrane transporter to deliver FFA into cells (Liu *et al.*, 2013) effectively.

Then again, within the cell mitochondrial membrane, the expression level of β-oxidation related gene expression was partly changed, including ME1 and ACOX2, indicating that the catabolism of lipids in the membrane involved in acetyl CoA's β-oxidation process is also affected. Still, there are also related oxidation genes that are not affected, CPT1A prevents substances such as acylcarnitine from entering the mitochondrial lining. This is consistent with previous studies showing that CPT1A does not act as a rate-limiting enzyme for β-oxidation alone, possibly because it functions together with other genes such as CROT to transport substances such as acylcarnitine into mitochondria. As demonstrated in Previous studies, CROT is localized in the peroxisomes. It transfers remnants from peroxisomal β-oxidation to the mitochondria and fuels them with fatty acids independently of CPT1A (Van der Leij *et al.*, 2000). Thus, the knockdown of CPT1A can promote lipid differentiation and inhibit the formation of lipid droplets by the oxidative pathway, but the promotion of lipid differentiation is the main pathway.

CPT1A plays a vital role in regulating lipid metabolism in lipids in different tissues. Lin's research proved that the regulation of CPT1A by LRRK2 may be via the activation of AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor α (PPARα) in HepG2 Cells (Lin *et al.*, 2020). Zhang *et al.* (2014) research presented muscle CPT1 mRNA was expressed at higher levels in the longissimus dorsi and subcutaneous fat. At the same time, the author indicates that CB1 can affect intramuscular fat deposition by regulating CPT1A and CPT1B mRNA expression. The PPARA signal pathway likely plays a major role in this process in ovarian adipocytes. CPT1A plays a different role in different species, and adipose tissue is taken together, but it also has certain

functional commonalities. The specific mechanism of regulating intramuscular fat deposition by CPT1A can be better utilized in production practice in future poultry production.

CONCLUSION

This paper concluded that CPT1A is actively involved in the lipid production of intramuscular fat. Knockdown of CPT1A can activate the PPAR pathway and induce adipocyte differentiation to form lipid droplets. However, CPT1A does not participate in fat proliferation and apoptosis but affects the lipid metabolism process in mitochondria.

CONFLICTS OF INTEREST STATEMENT

All authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This research was funded by Sichuan Science and Technology Program (Grant NO. 2021YFYZ0031) and the Demonstration and Promotion of Ecological and Healthy Breeding Technology of High Quality Broilers in Tibetan Plateau (Grant NO. 2021ZHFP0163).

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