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Identification of Differentially Expressed Genes and Lipid Metabolism Signaling Pathways between Muscle and Fat Tissues in Broiler Chickens

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In this study, signaling pathways and key differentially expressed genes (DEGs) involved in lipid metabolism in muscle and fat tissues were investigated. Muscle and abdominal fat tissues were obtained from 35-day-old female broilers for RNA sequencing. DEGs between muscle and fat tissues were identified. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses of DEGs were performed. A total of 6130 DEGs were identified to be significantly enriched in 365 GO terms, most of which were involved in biological processes, cellular components, and molecular functions in muscle and fat tissues. Three important lipid signaling pathways (pyruvate metabolism, the insulin signaling pathway, and the adipocytokine signaling pathway) were identified among the fat and muscle tissues of broilers. The key common DEGs in these pathways included phosphoenolpyruvate carboxykinase 2 (*PCK2*), acetyl-CoA carboxylase 1 alpha and beta (*ACACA* and *ACACB*), and the mitogen-activated protein kinase (*AMPK*) gene family. Hence, our findings revealed the pathways and key genes and gene families involved in the regulation of fat deposition in the muscle and fat tissues of broilers.

Key words: broiler, differentially expressed gene, fat, muscle, lipid metabolism, signaling pathway

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Introduction

The chicken, a key source of protein in the human diet, is among the most important farm animals worldwide. Over the past few decades, the growth rates of chickens have accelerated, and the yields of breast and thigh meat have improved (Cui *et al*., 2012). However, the growth of broilers is accompanied by the deterioration of meat quality and excessive deposition of abdominal and visceral fat, thereby decreasing both feed efficiency during rearing and yield of lean meat after processing (D'Andre *et al*., 2013). Intramuscular fat (IMF), located in most species in the epimysium, perimysium, and endomysium, is an important determinant of meat quality that influences tenderness, moisture content, and flavor (Cui *et al*., 2012; Sun *et al*., 2013). The regulation of IMF and abdominal fat deposition play an important role in modern broiler production (D'Andre *et al*., 2013).

Previous studies have identified differentially expressed genes (DEGs) and signaling pathways in muscle and/or fat tissues in various farm animals. For example, in the Pekin duck, DEGs between the breast muscle and skin fat samples were identified by RNA sequencing (RNA-seq), and were found to significantly enriched in several muscle development- and fat deposition-related pathways (Xu *et al*., 2014). In pigs, DEGs associated with growth and lipid deposition in the muscle and adipose tissue between all Chinese breeds and Yorkshire pigs were identified by high-throughput transcriptome sequencing, and were found to be associated with biological functions and canonical pathways involved in oxidoreductase activity, immune response, metabolic proc-

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esses (Tao *et al*., 2017). In Nelore cattle, analysis of genes associated with inflammation, immune response, biological processes indicated that radical S-adenosyl methionine domain containing 2 (*RSAD2*), eukaryotic translation initiation factor 2 alpha kinase 2 (*EIF2AK2*), and poly(ADP-ribose) polymerase (*PARP*) modulated backfat thickness, as determined by the construction of co-expressed gene modules. Previously, Cui *et al.* (2012) examined the gene expression profiles of the breast muscle of Beijing-You and Arbor Acres chickens sampled at different developmental stages and identified potential candidate genes associated with IMF deposition, thus indicating that IMF deposition in chicken breast muscle is regulated and mediated by genes and pathways associated with lipid metabolism and muscle development, as well as others involved in cell junctions. D'Andre *et al*. (2013) showed that the differences in fat deposition are reflected by differential gene expression in fast- and slow-growing chickens. Moreover, Ye *et al*. (2014) identified potential candidate genes associated with IMF deposition and suggested that IMF deposition in the skeletal muscle of sex-linked dwarf chickens is partly regulated by adipocytokines and insulin, as well as other downstream signaling pathways, such as the transforming growth factor-β/ SMAD family member 3 (TGF-β/SMAD3) and Wnt/β-catenin pathways. Through integrated analysis of microRNAs and mRNA expression profiles of the abdominal adipose tissues in chickens, Huang *et al*. (2015) demonstrated that the miRNA gga-miR-19b-3p accelerated pre-adipocyte proliferation as well as adipocyte differentiation through down-regulation of acyl-CoA synthetase long chain family member 1 (*ACSL1*). In addition, Zhang *et al*. (2017) reported that many highly connected genes provide a valuable resource for long non-coding RNA studies to improve our understanding of the biology of pre-adipocyte differentiation in chickens. Cui *et al*. (2018) showed that peroxisome proliferator-activated receptor (PPAR) signaling and cell junction-related pathways might contribute to IMF metabolism in chickens through DEGs, according to Kyoto Encyclopedia of Genes and Genomes analysis based on RNA sequences derived from the breast and thigh tissues of chickens. Furthermore, Liu *et al*. (2018) reported that higher triglyceride levels were observed in the abdominal tissues than in the adipose tissues of breast and thigh, which could be regulated by the expression of genes related to pathways involved in lipid metabolism (PPAR, Wnt pathway, and inositol phosphate metabolism), cell junctions (focal adhesion and regulation of actin cytoskeleton), and muscle contraction. Gunawan *et al*. (2019) performed deep RNA-seq of muscle tissues and revealed novel transcripts and pathways involved in the metabolism of unsaturated fatty acids, by using liver tissues from chickens with high and low unsaturated fatty acid content. Hence, these studies reveal the molecular mechanisms underlying fat deposition in chickens and other animals, thus providing insights into the metabolic differences between muscle and fat tissues.

The aim of the present study was to identify DEGs involved in lipid metabolism signaling pathways in the muscle and fat tissues of broilers through RNA-seq.

Materials and Methods

Birds and Management

This study was conducted in accordance with the Guidelines for Experimental Animals established by the Ministry of Science and Technology (Beijing, China). A total of 150 one-day-old healthy female Arbor Acres chicks were raised in stair-step cages under recommended environmental conditions and given *ad libitum* access to feed and fresh water, according to normal management practices at the Animal Rearing Room of Longyan University. At the age of 35 days, six broilers were randomly selected, killed after 12 h of fasting by stunning, and exsanguinated. The breast muscle and abdominal fat tissues were separated, weighed, and stored in liquid nitrogen until further analysis. The IMF content was measured following a method previously described by Cui *et al*. (2012). The abdominal fat weight and IMF content of each bird was measured in grams.

mRNA Library Construction and Sequencing

Three birds were used for RNA-seq. mRNA was isolated from the breast muscle and abdominal fat tissues using the traditional phenol-chloroform method. mRNA quality was assessed with an Agilent RNA 6000 Nano Kit on an Agilent 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA, USA) in accordance with the manufacturer's recommendations. Total RNA for the construction of an mRNA library met the following criteria: concentration $\geq$6.0 μg, RNA integrity number $\geq$8.0, and 28S/18S ratio $\geq$1.0. mRNA was then fragmented with an RNA fragmentation kit (Invitrogen Corporation, Carlsbad, CA, USA). The fragmented mRNA was used as a template with a six-base-pair primer along with buffer, deoxyribonucleotide triphosphates, ribonuclease H, and DNA polymerase I for first-strand synthesis of complementary DNA (cDNA). The cDNA products were purified with the QIAQuick PCR Purification kit (Qiagen, Beijing, China), in accordance with the manufacturer's instructions. After elution with ethidium bromide (EB) buffer, the overhang of each adaptor was annealed to the end of the cDNA molecule in a strand-specific manner, whereas the other strand of the adaptor was ligated to the terminal nucleotide of the first-strand cDNA. The target fragments were recovered by agarose gel electrophoresis and amplified by polymerase chain reaction (PCR) to construct mRNA libraries, which were individually sequenced with paired-end 150-bp reads using the Illumina HiSeq 2500 sequencing system (Illumina, Inc., San Diego, CA, USA). mRNA libraries for three muscle and abdominal fat tissue samples were constructed individually.

RNA-seq Data Processing and Analysis

Raw data reads were acquired by RNA-seq. Clean data were obtained from quality reads with SOAPnuke software (https://omictools.com/soapnuke-tool). Reads containing adapters (adapter contamination), those with more than 5% unknown bases, and those of low quality (less than 15 bases, $>$20%) were removed. Following this, all clean reads were mapped onto the reference chicken genome (*Gallus gallus*

5.0) with TopHat v2.0.12 software with default parameters (Kim and Salzberg, 2011; Ghosh and Chan, 2016). The annotation database Ensembl Genes v67 (Larsson *et al*., 2005) was used as a reference. The raw reads mapped to each gene were quantified with the easyRNASeq package (Delhomme *et al*., 2012).

Gene expression levels were calculated using RSEM v1.2.12 (Li and Dewey, 2011). A heatmap of all expressed genes was created with R software (v3.4.3) using the pheatmap package.

The gene expression values were first calculated in fragments per kilobase per million mapped fragments with the CummeRbund tool for visualizing the RNA-seq data. The DEGs between muscle and fat tissues were then detected using R software (v3.2.5) with the DESeq package (Love *et al*., 2014). Significant up-and down-regulation of DEGs was defined as $|\log2Ratio| \geq 1$ and an adjusted p-value (q) of <0.05.

Functional Enrichment Analysis of DEGs

Gene ontology (GO) analysis of DEGs was performed using the Blast2GO bioinformatics platform (Conesa *et al*., 2005). The significance level of GO terms was set as a false discovery rate-adjusted *P* value ≤ 0.01. Kyoto Encyclopedia of Genes and Genomes pathway information for these candidate target genes was determined, and an adjusted p-value ≤ 0.05 was considered significant.

Validation of RNA-seq Results using Quantitative Real-time PCR (qRT-PCR)

Total RNA was isolated from the breast muscle and abdominal fat tissues with the RNAsimple Total RNA kit (Tiangen Biotech Co., Ltd., Beijing, China). First-strand cDNA was synthesized from 2 μg of total RNA using the GoScript^{TM} Reverse Transcription System (Promega Biotech Co., Ltd., Beijing, China). Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) was used to analyze the mRNA expression of DEGs. qRT-PCR was performed on an ABI 7500 Real-time Detection System (Applied Biosystems, Carlsbad, CA, USA). The primers for qRT-PCR were designed using Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA, USA) on the basis of chicken sequences (Table S1). The total volume of the reaction mixture was 20 μL, which consisted of 10 μL of 2× PCR master mix, 100 ng of cDNA, 0.5 μL of each primer (10 μmol), and 8.0 μL of ddH_2O. To ensure similar PCR efficiencies (close to 100%) between the target genes and reference gene (β-actin), the primer and cDNA concentrations were optimized. The PCR conditions were as follows: 95℃ for 10 min, followed by 40 amplification cycles of 95℃ for 15 s, 60℃ for 20 s, and 72℃ for 32 s. The fold-change in gene expression was determined by the comparative $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Results and Discussion

Among the experimental broilers, the abdominal fat content was significantly higher than the IMF content in the breast muscle tissues (Fig. 1). Decreased abdominal fat deposition and improved IMF content are beneficial for both

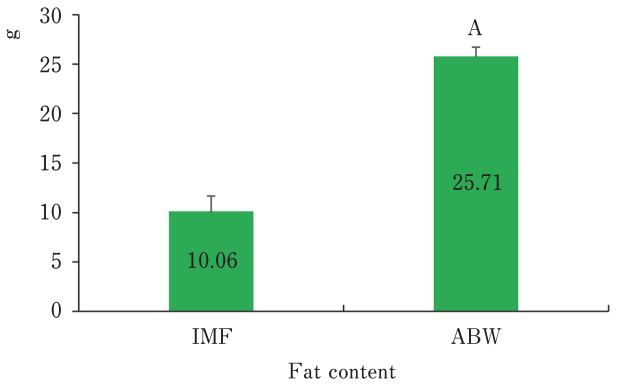

Fig. 1. **The histogram of abdominal fat content and IMF content in the breast muscle tissues.**

producers and consumers. In this study, lipid metabolism pathways and DEGs among the muscle and fat tissues of broilers were identified. Importantly, the DEGs and gene families were used to identify the pathways involved in the regulation of fat deposition in the muscle and fat tissues. Following RNA-seq, the proportions of clean reads obtained from the abdominal fat and muscle tissues of the broilers were similar, ranging from 44.08 to 44.92 Mb with mapping rates of 80.57 and 66.81%, respectively, thus meeting the requirements of sequencing. Of the 6130 DEGs between the fat and muscle tissues, 2969 (48.4%) were up-regulated and 3161 (51.6%) were down-regulated (Table S2). GO analysis was performed for these DEGs, and the enriched GO terms ($Q<0.05$) included "biological process," "cellular component," and "molecular function" (Table S3). Most of the DEGs associated with lipid metabolism were enriched in the terms "lipid metabolic process," "cellular lipid metabolic process," "cellular lipid catabolic process," and "fatty acid metabolic process." Among the DEGs, a total of 19 signaling pathways were identified through pathway functional enrichment analysis ($q<0.05$; Table S4). Hierarchical clustering showed clear discrimination into correct groups of fat and muscle tissues (Fig. 2). To further validate the results of RNA-seq, qRT-PCR was performed to examine the relative expression of the top ten DEGs. The qRT-PCR results were consistent with the fold-changes in DEGs (Table 2).

A total of 14 lipid metabolism pathways were identified, as shown in Table 3. Detailed information is provided in Table S4. Three (21.4%; including pyruvate metabolism, insulin signaling pathway, and adipocytokine signaling pathway) of 14 pathways were significantly enriched, and 31, 64, and 30 DEGs were found to be involved in pyruvate metabolism, the insulin signaling pathway, and the adipocytokine signaling pathway, respectively. The pyruvate metabolism signaling pathway plays important roles in lipid metabolism and/or muscle development, as reported previously for fat and/or muscle tissues (Ji *et al*., 2012; D'Andre *et al*., 2013; Zheng *et al*., 2014; Tao *et al*., 2017; Xue *et al*., 2017). The insulin signaling pathway plays an important role in the regulation of lipid metabolism (Saltiel and Kahn, 2001), and can influence

the synthesis of fats and fatty acids (Wong and Sul, 2010). The metabolic differences between genetically lean and fat chickens have been partly attributed to alterations in insulin signaling in the liver (Dupont *et al*., 1999), which affects lipid metabolism in abdominal fat (Dupont *et al*., 2012; Resnyk *et al*., 2013), liver, and muscle tissues (Dupont *et al*., 2009). Adipocytokines are involved not only in the deregulation of glucose and lipid homeostasis, but also in inflammation due to pro-inflammatory factors, which are involved in the negative crosstalk between adipose tissue and skeletal muscle (Sell *et al*., 2006). The adipocytokine signaling pathway has been shown to regulate IMF deposition in chickens (Ye *et al*., 2014).

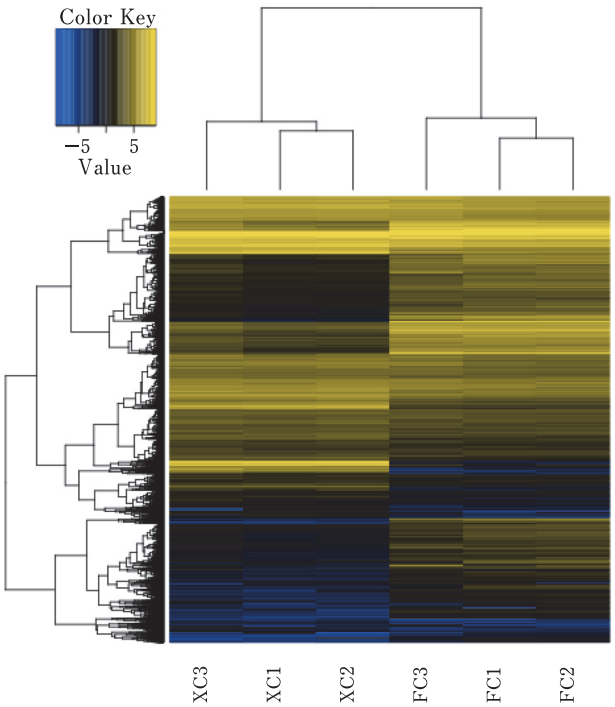

Fig. 2. **Heatmap of DEGs in each sample.** Heatmap showing DEGs between muscle and fat tissue samples. Samples FC1, FC2, and FC3 represent abdominal fat tissues. Samples XC1, XC2, and CC3 represent breast muscle tissues.

Among the three pathways, the common DEGs may be candidate genes involved in the regulation of differences in lipid metabolism between abdominal fat and muscle tissues. We found one common DEG (phosphoenolpyruvate carboxykinase 2; *PCK2*) between the three signaling pathways, two common DEGs [acetyl-CoA carboxylase 1 (*ACACA*) and 2 (*ACACB*)] between pyruvate metabolism and insulin signaling pathway, and 11 common DEGs (*AKT1*, *AKT3*, *G6PC3*, *IRS1*, *LOC100857298*, *MAPK8*, *PPARGC1A*, *PRKAA2*, *PRKAB2*, *PRKAG1*, and *PRKAG3*) between the insulin signaling pathway and adipocytokine signaling pathway (Fig. 3; Table S4). *PCK2* encodes the gluconeogenic mitochondrial isozyme phosphoenolpyruvate carboxykinase (PEPCK-M). In a previous study, *PCK2* was identified as a candidate gene involved in diabetes and obesity (Beale *et al*., 2007). *ACACA* and *ACACB*, which were identified in pyruvate metabolism and the insulin signaling pathway, are essential for lipogenesis and have been shown to influence IMF deposition (Liu *et al*., 2017) and abdominal fat traits (Hu *et al*., 2010) in chickens.

Of the 11 common DEGs among the insulin signaling pathway and adipocytokine signaling pathway, four protein kinase AMP-activated catalytic subunits alpha 2, beta 2, gamma 1 and gamma 3 (*PRKAA2*, *PRKAB2*, *PRKAG1*, and *PRKAG3*) belonged to the 5′ AMP-activated protein kinase (AMPK) gene family. AMPK is a heterotrimeric protein

Table 1. **Mapping reads of each sample after RNA-seq**

Items[1]	FC1	FC2	FC3	XC1	XC2	XC3
Total clean reads (Mb)	44.08	44.64	44.92	45.14	45.13	44.27
Mapped reads (Mb)	34.90	36.58	36.20	29.71	30.45	29.72
Mapping rate (%)	79.17	81.94	80.59	65.83	67.47	67.13

Samples FC1, FC2, and FC3 represent abdominal fat tissues. Samples XC1, XC2, and XC3 represent breast muscle tissues.

Table 2. **Comparison of RNA-seq and qRT-PCR data (log2 fold-changes) between the fat and muscle tissues of broilers**

DEGs	RNA-seq	qRT-PCR	DEGs	RNA-seq	qRT-PCR
GJB1	9.19	15.89	*KLHL31*	−14.17	−3588.00
ACVR1C	9.47	4.43	*NRAP*	−13.11	−3558.48
LOC428702	10.03	47.53	*MYH1G*	−14.02	−9271.79
RET	6.51	37.55	*TRIM55*	—	−423.78
NFASC	6.15	13.22	*CAV3*	−13.20	−3257.15

qRT-PCR, real-time quantitative PCR; DEGs, differentially expressed genes.

Table 3. **Lipid metabolism pathways of the DEGs**

N	Name	Map	P_value	Q_value
1	Pyruvate metabolism	map00620	0.00	0.00
2	Insulin signaling pathway	map04910	0.00	0.02
3	Adipocytokine signaling pathway	map04920	0.05	0.32
4	PPAR signaling pathway	map03320	0.22	0.74
5	Lipoic acid metabolism	map00785	0.30	0.90
6	Biosynthesis of unsaturated fatty acids	map01040	0.30	0.90
7	Fatty acid degradation	map00071	0.32	0.90
8	mitogen activated kinase-like protein (MAPK) signaling pathway	map04010	0.32	0.90
9	Fatty acid elongation	map00062	0.36	0.97
10	Fat digestion and absorption	map04975	0.65	1.00
11	Fatty acid biosynthesis	map00061	0.50	1.00
12	Wnt signaling pathway	map04310	0.52	1.00
13	Linoleic acid metabolism	map00591	0.78	1.00
14	α-linolenic acid metabolism	map00592	0.87	1.00

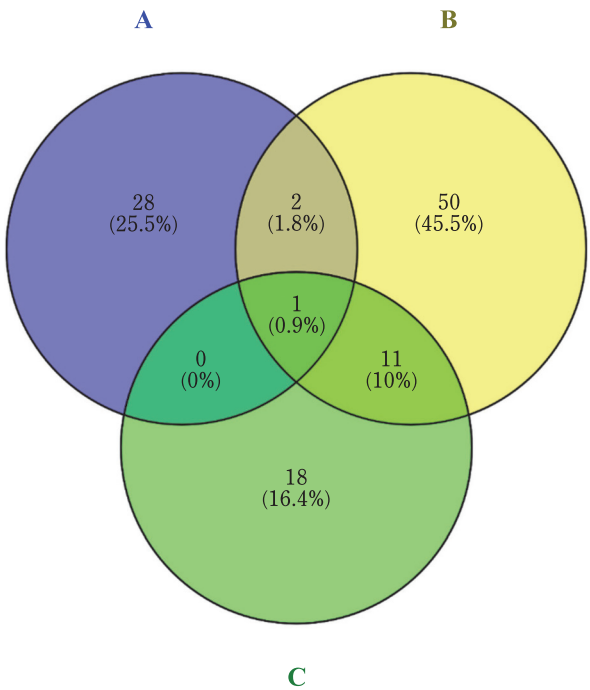

Fig. 3. **Venn diagram of DEGs associated with lipid metabolism.** DEGs implicated in (A) pyruvate metabolism, (B) the insulin signaling pathway, and (C) the adipocytokine signaling pathway.

complex that inhibits cholesterol synthesis, lipogenesis, triglyceride synthesis, and adipocyte lipogenesis, activates adipocyte lipolysis, and modulates insulin secretion by pancreatic β-cells (Winder and Hardie, 1999). *PRKAA2* and *PRKAB2* encode the AMPK catalytic subunits α2 and β2, respectively, whereas *PRKAG1* and *PRKAG3* encode the AMPK non-catalytic subunits γ1 and γ3. Variants and expression levels of these genes have been shown to influence the fat and muscle content in animals and humans (Spencer-Jones *et al*., 2006; Lin *et al*., 2010; Du *et al*., 2017). Two genes (*AKT1* and *AKT3*) among the 11 common DEGs belonged to the AKT gene family. They encode proteins that are members of the serine/threonine kinase family and are involved in signaling during cell growth, proliferation, apoptosis, transcription, angiogenesis, migration, and glucose metabolism. AKT2 is indispensable for human pre-adipocyte proliferation, apoptosis sensitivity, and adipogenesis (Cheng *et al*., 2015). Both genes play important roles in mediating the effects of insulin on cellular metabolism (Fischer-Posovszky *et al*., 2012). However, further studies are needed to elucidate the roles of the other common DEGs in lipid metabolism in broilers.

In conclusion, the findings of our study revealed that the following three lipid signaling pathways are common between the fat and muscle tissues of broilers: pyruvate metabolism, the insulin signaling pathway, and the adipocytokine signaling pathway. The key common DEGs enriched in these pathways (including *PCK2*, *ACACA, ACACB*, and the *AMPK* gene family) were also identified. These findings provide evidence of the roles of specific pathways and key genes and gene families involved in the regulation of fat deposition in the muscle and fat tissues of broilers.

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Conflicts of Interest

The authors declare no conflict of interest.

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