Transcriptome analysis identifies signaling pathways related to meat quality in broiler chickens − the extracellular matrix (ECM) receptor interaction signaling pathway

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ABSTRACT Meat quality characteristics, including juiciness, flavor, and tenderness, can be mostly attributed to the total muscle fat content, intramuscular fat (**IMF**), and the composition of its fatty acids, which are regulated by the balance between lipid uptake, transport, synthesis, and subsequent metabolism, involving many genes and pathways. However, the detailed molecular mechanisms remain unclear. The purpose of this study was to identify the key signaling pathways related to chicken meat quality, and to provide help for improving chicken meat quality. The present study reports the RNA-sequencing analysis of pectorales and crureus of the Zhuanghe dagu chicken and the Arbor Acres Broiler chicken (**AA chicken**). We identified certain differentially expressed genes that affect IMF deposition, such as *EHHADH, TECRL, NDUFAB1, PCCB*, and *HIBCH*, which were upregulated in Zhuanghe dagu chicken , and *GCDH, TPI1, ABHD13, PSMC1, MYST2*, and *FBXO11*, which were upregulated in AA chickens. Pathway analysis using the Kyoto Encyclopedia of Genes and Genomes indicated that the extracellular matrix (**ECM**)−receptor interaction pathway is co-enriched in both tissues, and forms a sub-pathway of other enriched pathways. Intriguingly, the ECM −receptor interaction pathway genes are regulated differently in different gene pools. Collagens, which are main ECM constituents, and laminin and integrin β1 transmembrane receptors were significantly downregulated in both tissues of the AA chicken. The results showed that the ECM-receptor interaction pathway affect the quality of chicken meat by affecting the metabolism of intramuscular adipocytes. Further investigation of this signaling pathway will be helpful to the improvement of chicken meat quality.

Key words: Zhuanghe dagu chicken, Arbor Acres Broiler chicken, IMF deposition, ECM receptor interaction signaling pathway

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INTRODUCTION

Products based on chicken meat are important components of human nutrition. In recent decades, genetic selection for growth rate and yield have improved the amount of available meat in chickens. However, the higher the growth rate, the larger the fiber diameter, the higher the proportion of glycolytic fiber, and the lower the fat content in muscle, which negatively affect the quality of the meat (Dransfield and Sosnicki, 1999; Du et al., 2010; Petracci and Cavani, 2012). Improving meat quality while maintaining the growth rate remains a challenge in the poultry industry. Recently, there has been increased consumer demand for meat sourced from local or indigenous birds because of its rich flavor, unique taste, and firm texture. Furthermore, there is a perception among consumers that these birds are produced naturally using extensive farming. The growing awareness among consumers regarding poultry health and nutrition has

Abbreviations: EHHADH, Enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase; TECRL, Trans-2,3-enoyl-CoA reductase like; NDUFAB1, NADH: ubiquinone oxidoreductase subunit AB1; PCCB, Propionyl-CoA carboxylase subunit beta; HIBCH, 3-hydroxyisobutyryl-CoA hydrolase; GCDH, Glutaryl-CoA dehydrogenase; TPI1, Triosephosphate isomerase 1; ABHD13, Abhydrolase domain containing 13; PSMC1, Proteasome 26S subunit, ATPase 1; MYST2, Histone acetyltransferase MYST2; FBXO11, F-box protein 11; BAP1, BRCA1 associated protein 1; SETD2, SET domain containing 2, histone lysine methyltransferase; MCM2, Minichromosome maintenance complex component 2; ACTR3B, Actin related protein 3B; ARPC5, Actin related protein 2/3 complex subunit 5

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encouraged specialty markets for local poultry varieties produced using extensive farming systems (Devatkal et al., 2019; Al-Nasser et al., 2020). The Zhuanghe Dagu chicken (**ZD chicken**), also called the Zhuanghe chicken, originated in Zhuanghe, Liaoning province, China and is mainly bred in parts of Northeast China, Hebei, and Inner Mongolia. The ZD chicken has many advantages, such as a big body, superb meat quality, a tall trunk, large red eggs, adaptability of rough forage, cold resistance, and high disease resistance, leading it to be known as the king of northeast chickens (Gu and Li, 2020). As one of the most valuable materials for poultry breeding in China, the Chinese government listed the ZD chicken as a nationally protected domestic animal in 2000 (Wu, 2001). In the present study, the pectorales and crureus of the ZD chicken and the Arbor Acres broiler chicken (**AA chicken**) were used as test materials.

Meat quality characteristics, such as juiciness, flavor, and tenderness, are attributed mainly to the total muscle fat content, the intramuscular fat (**IMF**), and its fatty acid (**FA**) composition (Hocquette et al., 2010). IMF comprises the amount of fat in muscles, including that in the outer layer, perimuscular region, and the endometrium (Liu et al., 2019). In contrast to adipose tissue, in which triglyceride, as the major lipid category, accounts for more than 90% of lipids, IMF includes a significant proportion of phospholipids. Muscle FAs are rich in polyunsaturated fatty acids, including arachidonic acid (20:4n-6), linoleic acid (18:2n-6), and α-linolenic acid (18:3n-3) (Wood et al., 2008). Heating of polyunsaturated fatty acids results in their oxidation to produce volatile components, for example, 2, 4-sebacal, which improve meat flavor (Calkins and Hodgen, 2007). Studies have demonstrated that juiciness, flavor, and tenderness correlate positively with the muscle total fat content (Chartrin et al., 2006; Hocquette et al., 2010; Liu et al., 2019). Previous studies used microarray technology to analyze chicken pectorales (Cui et al., 2012) and liver (Bourneuf et al., 2006), which revealed certain potential candidate genes and pathways that might influence chicken meat flavor; however, no further validation has been performed.

The commonly accepted method of comparing meat production is to use the chronological time (age). However, growth comprises both mass and time components, either of which could be used effectively to compare growth traits (Liu and Niu, 2008). It has been proposed that physiological time might be a better measure than chronological time when comparing growth and meat quality traits between fast growing broilers and slow growing native chickens, particularly when their market ages differ (Wattanachant et al., 2004; Sarsenbek et al., 2013). 6-mo-old of ZD Chickens and 6-wk-old of AA Chickens are of the same physiological age. Therefore, the present study performed RNA-sequencing (RNA-seq) analyses of pectorales and crureus samples of 6-mo-old ZD chickens and 6-wk-old AA chickens which are approximately the same body weight to determine the key signaling pathways related to muscle quality and thus contribute to the improvement of muscle quality.

MATERIALS AND METHODS

Ethics Statement

The animal welfare committee of the College of Animal Science and Veterinary Medicine of Shenyang Agricultural University approved the experimental procedures, which were performed according to the Regulations for the Administration of Affairs Concerning Experimental Animals (China, 1988) and EU Directive 2010/63/EU for animal experiments. Surgeries were performed according to recommendations proposed by the European Commission (1997). All efforts were made to minimize the animals' suffering.

Animals and Tissues

The chickens used in this study comprised ZD chickens (6 mo old) and AA chickens (6 wk old) with approximately same weight were obtained from a chicken farm in Liaoning. There were 10 chickens of each breed, of which there were five females and five males. Feeding management was carried out according to the standard routine. The daily food comprised a whole diet and feed, the experimental animals were fed diets consistent with the 1994 NRC nutrient level of chickens, and its nutritional components could satisfy the growth and development demands of chickens. All the chickens were killed by conventional neck cutting. Then, the pectorales and crureus were excised immediately, frozen in liquid nitrogen, and stored at -80 °C for the follow-up index analysis.

Total RNA Extraction and the Construction of the RNA-Seq Library

Selected six chickens (50% male: 50% female) from 10 of each breed. RNA-seq analysis was carried out on pectorales and crureus samples from 12 individuals. An Animal tissue total RNA extraction kit (Beijing Tiangen biochemical technology co. LTD, Beijing, China) was used to isolate total RNA from the muscle tissues, following the manufacturer's instructions. Agarose gel electrophoresis and a NanoDrop 8000 spectrophotometer (NanoDrop, Thermo Scientific, Waltham, MA) were used to assess the concentration and purity of the RNA, respectively. A 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) was used to determine the RNA integrity. To minimize the effect of transcriptome variation among individuals, we pooled equal amounts of the RNA samples from six individuals in each group to form one mixed sample for each group. Subsequently, a complementary DNA (cDNA) library was constructed using the mixed RNA samples. A TruSeq RNA Sample Preparation Kit (Illumina, Inc., San Diego, CA) was then used to construct mRNA libraries in accordance with the TruSeq protocol. Finally, an Illumina HiSeq 2000 instrument at the Huada gene technology co. LTD (Shenzhen, China) was used to sequence the libraries.

Bioinformatic Analysis of the RNA-Seq Data

Using transcriptome profiling, the gene expression profiles of the pectorales and crureus of the ZD chicken and AA broiler were acquired and analyzed using GeneSpring7.0 software (http://www.silicongenetics.com). We identified the differentially expressed genes (**DEGs**) of the pectorales and crureus in both chickens as those whose expression was at least two times higher or lower in each comparison. Genes whose expression changed by less than 2-fold, or that did not change, were excluded from further analysis. Then, Gene Ontology (**GO**) and Kyoto Encyclopedia of Genes and Genomes (**KEGG**) databases were used for enrichment analysis of DEGs to ascertain the functions of DEGs involved in meat flavor formation in the pectorales and crureus of ZD and AA chickens.

Validation Using Quantitative Reverse Transcription Real-time PCR Reverse Transcription (qRT-PCR)

The reliability of the Illumina analysis was verified using qRT-PCR analysis of 17 genes believed to affect the meat quality traits of ZD Chickens or their muscle growth and development. Primer 5.0 was used to design the primers used for qRT-PCR and the 17 pairs of primer are shown in Table S1. Three samples were taken from each breed to collect tissue, and each sample was repeated three times. The total RNA of both chicken muscle tissues (pectorales and crureus) was then isolated and extracted. A PrimerScript RT Reagent Kit (Takara, Dalian, China) was used to reverse transcribe the total RNA. Subsequently, a Bio-Rad iQ5 Real-time PCR Detection System (BIO-RAD, Hercules, CA) was used to perform the qPCR reactions. Results were presented as relative fold change of the value of control group after normalizing to endogenous control β-actin. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) was used to calculate the fold changes.

Statistical Analyses

The data are reported as the mean ± the standard error of the mean based on the results of at least three replicates for each treatment. To determine statistical significance, one-way analysis of variance was used. Statistical significance was accepted at $P < 0.05$.

RESULTS

RNA-seq was employed to acquire the pectorales and crureuse transcriptome of ZD and AA chickens, with the aim of determining the potential molecular mechanism underlying chicken meat quality, flavor, and growth and development. We selected four mix sample separate cDNA libraries for RNA-seq. For each sample there were over 3 million clean reads, which corresponded to 7,953 to 9,923 expressed genes in Table 1.

Table 1. Statistics of the RNA-seq data.

Samples	Clean reads	Genome mapping rate (%)	Gene mapping rate (%)	Expressed genes
6MDXA	3388751	96.56	41.57	7953
6MDTA	3399815	97.46	43.68	8357
6WAXA	3525200	96.40	46.32	8861
6WATA	3522407	94.45	51.87	9923

6MDXA, pectorales of 6-mo-old ZD chickens; 6MDTA, crureus of 6-mo-old ZD chickens; 6WAXA, pectorales of 6-wk-old AA chickens; 6WDTA, crureus of 6-wk AA chickens.

DEGs Analysis

DEGs were identified as those genes with a false discovery rate < 0.001 and at least a 2-fold difference in expression. The expression levels were estimated using the RPKM method (Reads per Kb per Million mapped reads) to obtain the up- and downregulated genes which are shown in Figure 1.

In the comparison between the pectoral muscle data of the 6-month-old ZD chickens and the 6-week-old AA chickens (6WAXA *vs.* 6MDXA), there were 155 upregulated genes, including 42 unknown genes, and 488 downregulated genes, among which 143 were unknown. While in the comparison of crureus between the chickens (6WATA *vs.* 6MDTA), there were 399 upregulated

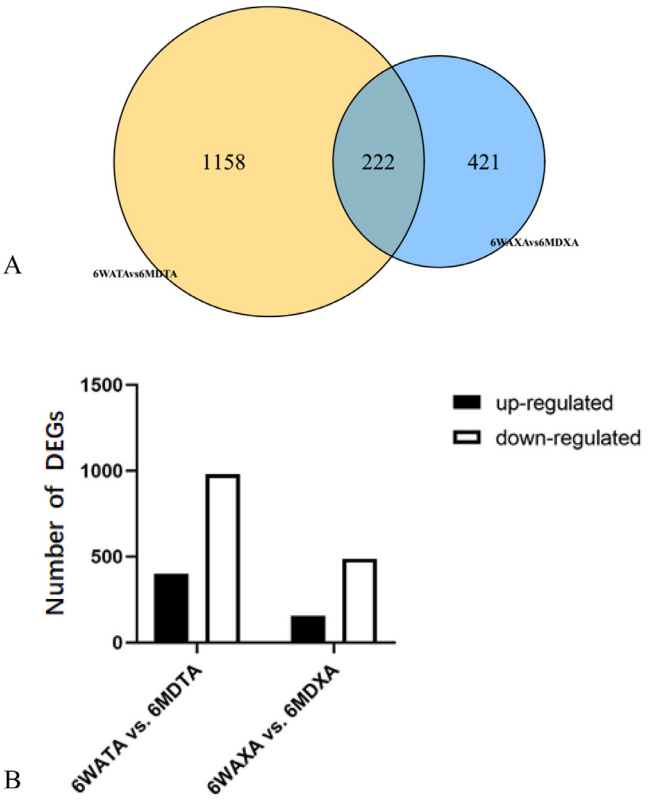


Figure 1. Number of differentially expressed genes (DEGs). (A) Numbers of unique or shared DEGs between pectorales and crureus of two breeds of chicken. (B) Numbers of DEGs showing upregulated or downregulated expression in pectorales and crureus of 2 breeds of chicken. W, week; M, month; A, AA broiler chicken; D, Zhuanghe Dagu chicken; TA, crureus; XA, pectorales.

Table 2. Expression levels of differentially expressed genes with the most significant differences.

Gene	Raw intensity -S1	Raw intensity -S2	TPM -S1	TPM -S2	*P*-value	FDR	Symbol	Transcript ID
424877	7	32	1.99	9.41	2.63E-05	0.00015	EHHADH	gi|118094871| ref|XM_422690.2|
422618	4	45	1.14	13.24	2.10E-10	2.30E-09	TECRL	gi|118090172| ref|XM_420576.2|
416571	243	967	68.99	284.43	2.13E-108	1.71E-106	NDUFAB1	gi|118097960| ref|XM_414872.2|
768706	40	83	11.36	24.41	4.22E-05	0.000232	PCCB	gi|118094795| ref|XM_001231793.1|
423979	171	426	48.55	125.3	2.99E-28	8.08E-27	HIBCH	gi|71895122| ref|NM_001031243.1|
56507	73	26	20.72	7.65	3.91E-06	2.52E-05	GCDH	gi|118111165| ref|XM_001231725.1|
396435	4065	624	1154.04	183.54	0	0	TPI1	gi|45382060| ref|NM_205451.1|

genes, of which 123 were unknown, and 981 downregulated genes, of which 281 were unknown.

Additionally, in the 6WATA *vs.* 6MDTA and 6WAXA *vs.* 6MDXA comparisons, 222 DEGs were shared. These DEGs might correlate with the origin of the meat flavor between the breeds. The genes with large differences in expression are shown in Table 2.

Validation Using qRT- PCR

Seventeen genes were selected for qRT-PCR verification of the RNA-seq data. Figure 2 shows that in general, The relative expression trends between RNA-seq and qRT-PCR of the 17 genes (6-wk-old AA chicken vs. 6-mo-old ZD chicken) were completely consistent, which supported the reliability of the Illumina sequencing data. Any discrepancies regarding ratios could be attributed to the different al**go**rithms and sensitivities of the 2 techniques.

GO Classification Analysis of the DEGs

Figure 3 shows that the DEGs in the ZD and AA chickens influenced the GO terms included in the three secondary classifications of biological process, cellular component, and molecular function.

In the category of biological process, relatively large effects were observed on cellular process, metabolic process, and single-organism, which were significantly different from the positive and negative controls. Development process, response to stimulus, and other biological regulatory functions showed large effects. This analysis showed that pathways related to energy metabolism in cells might have a marked impact on the origin of meat quality.

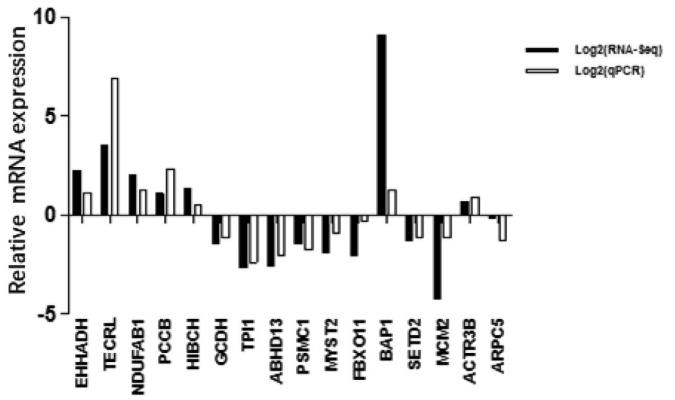


Figure 2. qRT-PCR validation of the gene expression profiles. The relative expression trends between RNA-seq and qRT-PCR of the 17 genes (6-wk-old AA chicken vs. 6-month-old ZD chicken) were completely consistent.

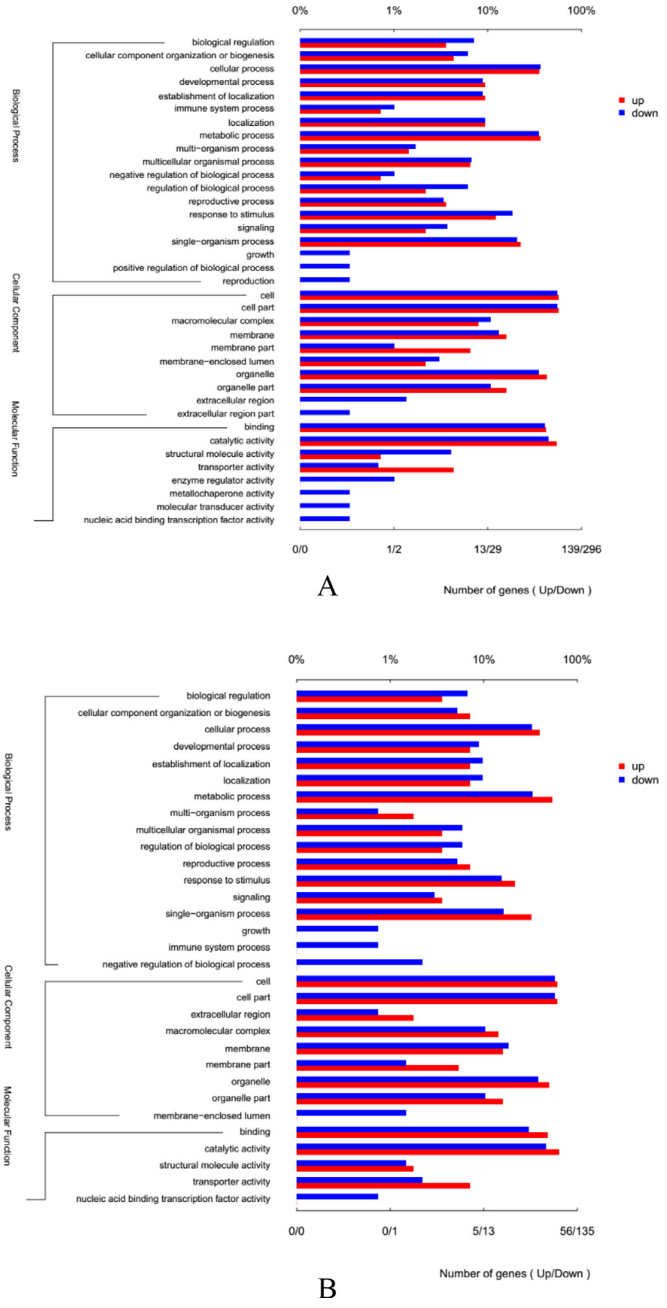


Figure 3. (A): Histogram showing the GO annotation of up- and downregulated genes between 6WATA and 6MDTA. (B): Histogram showing the GO annotation of up- and downregulated genes between 6WAXA and 6MDXA. Red indicates upregulated genes, and blue represents downregulated genes.

The results for the categories of cell components, cell and cell parts changed the most, indicating that the two different chickens had significantly different intracellular components, particularly in the ECM and extracellular structures. The GO categories of extracellular regions, macromolecular complex, membrane, organelle, and organelle part showed large differences, which suggested that the membrane, extramembranous protein, and organelle part might have an impact on the origin of meat quality.

In terms of Molecular Function, binding and catalytic activity changed the most, showing that the origin of meat quality might be related to the catalytic oxidation activity of certain enzymes. The structural molecule activity and transporter activity also changed markedly, suggesting that origin of meat quality might be determined by differences in membrane surface transport systems.

Analysis of DEGs Using KEGG Pathway Enrichment

KEGG pathway enrichment analysis suggested that certain pathways in fatty acid metabolism and synthesis might have a significant effect on meat quality. The results of KEGG pathwayenrichment analysis suggested that the ECM-receptor interaction signaling pathway is closely related to meat quality which are shown in Figure 4. The ECM-receptor interaction signaling pathway showed an enrichment rate of 0.6 to 0.8 ($P < 0.05$).

Visualization of KEGG Pathways Associated With the DEGs

Using the KEGG database, the 6MD gene was used as a reference. The KEGG pathways associated with changes in gene expression in the 6WA group were displayed in a KEGG annotation pathway map. In Figure 5, the genes and pathways associate with ECM-receptor interaction signaling pathway are indicated by a red box (representing proteins with up-regulated mRNA expression) and a green box (downregulated). The expression of reduced ECM component mRNA can be simultaneously seen in the presence of many meat-quality-related genes in the ECM-receptor interaction signaling pathway.

DISCUSSION

The Zhuanghe dagu chicken originated from Zhuanghe city, Dalian, China, and is a unique local breed that was bred during a particular historical period and under special geographical and climatic conditions. The Zhuanghe dagu chicken has strong disease resistance, a high egg yield, and delicious meat taste. It is a famous local fine breed for meat and eggs in China, and was listed as a national livestock and poultry genetic resources protected breed in 2006 (Zhou, 2010). There is growing evidence to support the fact that the meat

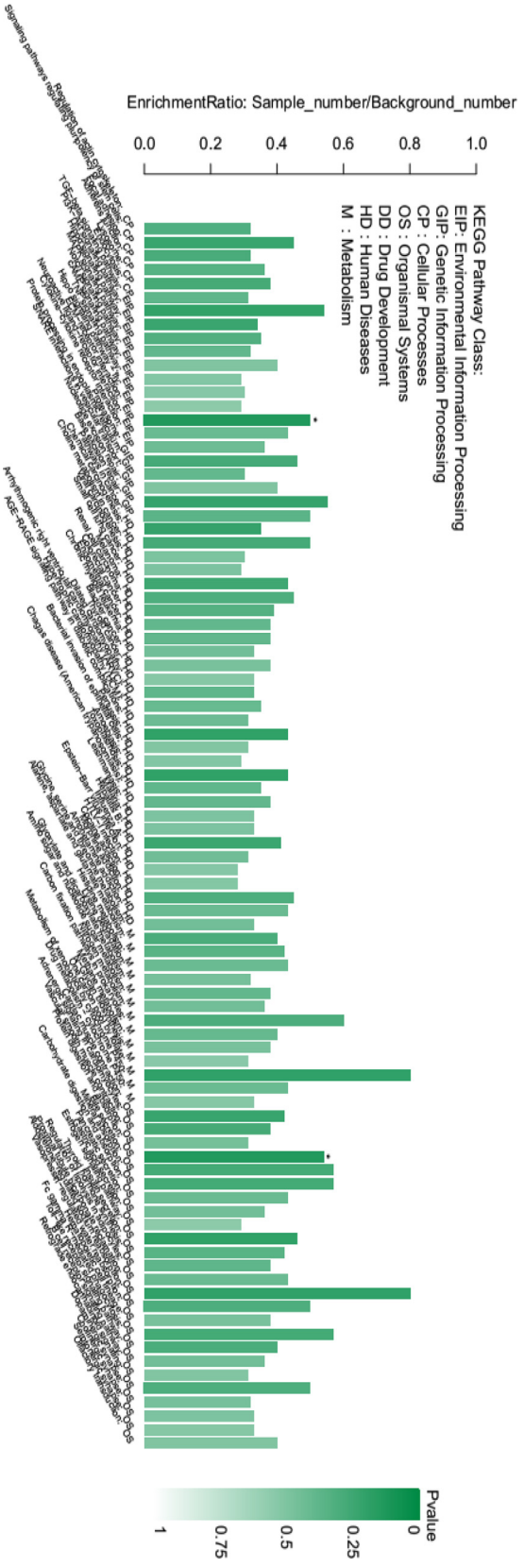
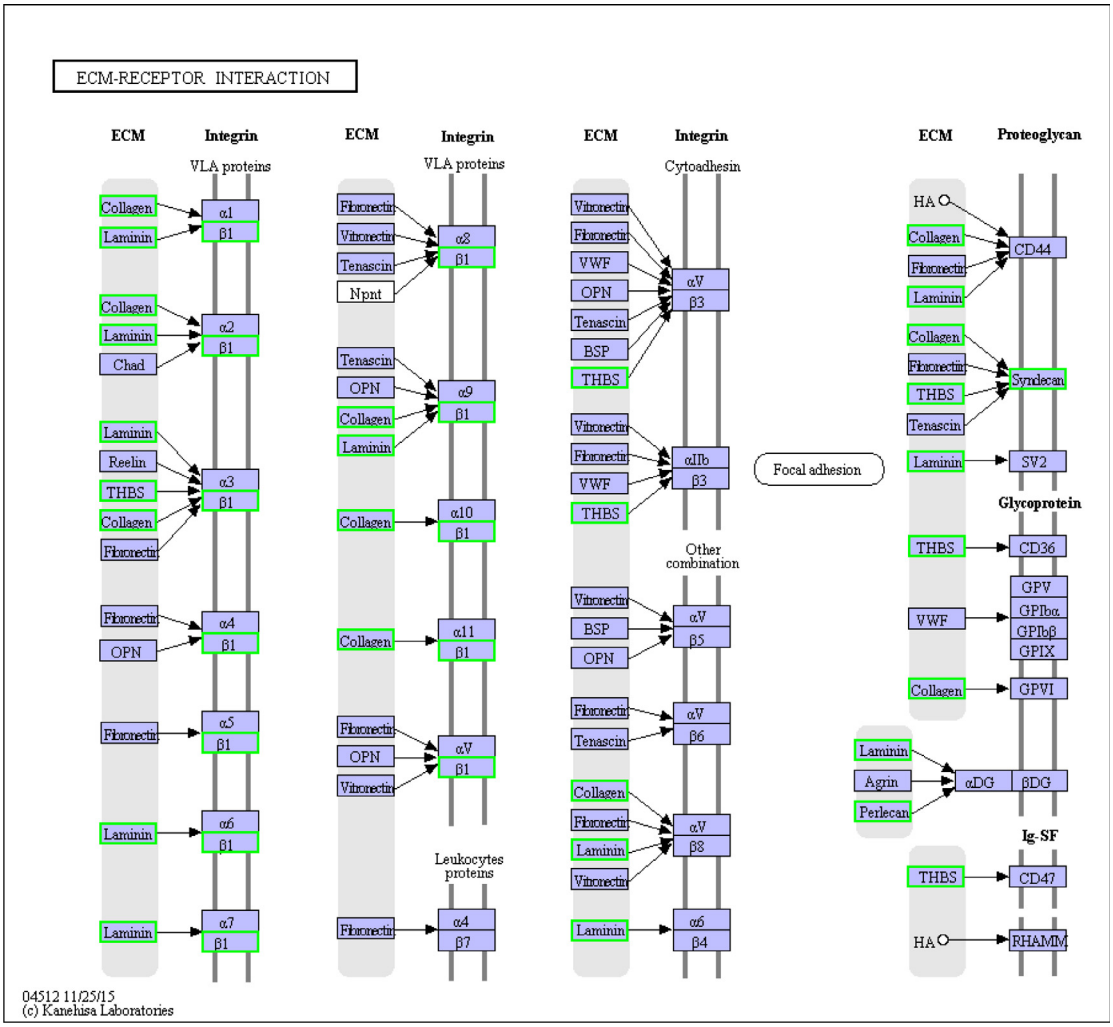


Figure 4. Histogram of the KEGG pathway enrichment analysis of differentially expressedgenes between 6WATA and 6MDTA. The x-axis shows the name and classification of the pathway; the y-axis represents the enrichment rate; * indicates $P < 0.05$. The side-color gradient represents the increasing P value (faint to dark).

quality of big-bone chickens is superior to that of commercial broilers (Fanatico et al., 2007; Pietrzak et al., 2013). Consequently, consumers and researchers are



The ECM-receptor interaction signaling pathway

Figure 5. KEGG annotation pathway map of the downregulated ECM−receptor interaction signaling pathway. Blue background boxes indicate proteins encoded by background genes in the chicken transcriptome data. White background boxes indicate proteins encode by genes from non-chicken species. Boxes with red borders represent the protein products of upregulated differentially expressed genes. Boxes with green borders represent the protein products of downregulated differentially expressed genes.

paying attention to factors that affect chicken meat quality.

Genetic and environmental factors combine to make meat quality a complex trait, and there can be large variations in meat quality within and between animals (Rehfeldt et al., 2004). One key meat quality trait is IMF, which directly affects meat sensory properties (Ruiz et al., 2001; Chen et al., 2005), as well as juiciness, flavor, tenderness, and overall acceptability. Consumers consider meat sensory traits as extremely important for meat acceptability. IMF seems to play important roles in eating quality (Dodson et al., 2010; Brooks et al., 2011). Therefore, increasing the IMF level to produce poultry meat with high sensory quality without affecting total carcass yields, is desired by consumers and the poultry industry. Although the IMF is useful in improving meat quality, the mechanism underlying in increased IMF content is unknown (Hocquette et al., 2010).

In this study, pectorales and crureus transcriptomes of ZD and AA chickens with different genetic backgrounds were compared and analyzed. Many DEGs that might affect IMF deposition were identified between the breeds. The DEGs *EHHADH, TECRL, NDUFAB1, PCCB*, and *HIBCH,* were upregulated in ZD chickens, and *GCDH, TPI1, ABHD13, PSMC1, MYST2*, and *FBXO11* were upregulated in AA chickens (Table 2). Then, we verified the accuracy of the transcriptome sequencing by qRT-PCR technology. Using KEGG enrichment analysis, we found that the ECM receptor interaction pathway was a significantly enriched IMF-related pathway. The expression of reduced ECM component mRNA can be simultaneously seen in the presence of many meat-quality-related genes in the ECM-receptor interaction signaling pathway. We believe that ECM mediates a mechanism involved in the increasing of the IMF.

Reports suggest that IMF differs from other fats in three ways: Metabolic activities, adipocyte size, and developmental timing (Hocquette et al., 2010). In cattle and pigs, non-muscular adipocytes are larger than intramuscular adipocytes (Miller et al., 1991; Gardan et al., 2006). Based on the DEGs' functional annotation

analysis in the two tissues, we hypothesized that the ECM-receptor interaction pathway is involved in tissue-specific variations in IMF levels. The ECM is a crucial component of tissue architecture and has an important function in adipogenesis(Mariman and Wang, 2010). However, the ECM in IMF tissue has received little research the analysis of ECM components is difficult in this tissue.

Fat cells are surrounded by the basement membrane (a thick ECM), of which type IV collagen is the main component (Pierleoni et al., 1998). The basement membrane of adipose tissue is necessary for the survival of adipocytes. The lipid and cytoplasm within adipocytes are separated by a single molecular layer of lipid, which can be easily damaged by mechanical stress.

In 1974, Green and Meuth proposed that for differentiation and fat storage, collagen synthesis is a prerequisite (Green and Meuth, 1974). In 1990, Nandan et al. found that fat cell differentiation was required for collagen synthesis (Nandan et al., 1990). In 2004, Bouwman et al. confirmed used stable isotope labeling to study the protein dynamics of mature non-dividing 3T3-L1 fat cells, which demonstrated that the ECM is important for the function and survival of fat cells (Bouwman et al., 2004).

The ECM of fat cells has the same components as other cell types; however, the number of components determines the cellular specificity of the ECM (Mariman and Wang, 2010). Among them, type VI collagen is the most specific and is widely enriched in fat cells (Iyengar et al., 2005; Khan et al., 2009). Type VI collagen consists of three subunits: alpha 1 (VI), alpha 2 (VI), and alpha 3 (VI) (Chu et al., 1988). Aratani and Kitagawa (Aratani and Kitagawa, 1988) observed marked upregulation of collagen IV, nidogen-1 (entactin), and various laminin complexes during adipocyte differentiation. Wang et al. (Wang et al., 2007) found that when the net cell triglyceride content was reduced, cell shrinkage, and decreased expression of genes encoding matrix proteins and various processing enzymes occurred, causing a slowdown in the accumulation of the ECM.

Transmembrane molecules such as β1 mediate specific interactions between the ECM and cells. Our results showed that different members of the ECM collagen proteins, such as laminin and transmembrane molecule β1 were downregulated in both tissues of the AA chicken. Liu J et al. have reported that changes in the expression of transmembrane molecules are associated with adipocyte differentiation (Liu et al., 2005). The regulation of other genes involved in the ECM −receptor interaction might be chicken breed dependent. According to the results of the comparative transcriptome analysis of the two tissues, the interaction between transmembrane receptors and ECM components of the cell might affect tissue specific adipogenesis (Lee et al., 2013), which would affect the quality and flavor of chicken meat.

In summary, the ECM-receptor interaction might affecting the differentiation of intramuscular adipocytes, and lipid synthesis and metabolism, thereby changing the IMF content, which would affect the meat flavor of broilers. These results will contribute to improving the quality and flavor of broiler chickens.

Declaration

CONFLICT OF INTERESTS

No potential conflict of interest relevant to this article was reported.

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AVAILABILITY OF DATA AND MATERIAL

Upon reasonable request, the datasets of this study can be available from the corresponding author.

AUTHORS CONTRIBUTIONS

Conceptualization: Jiancheng Yang, Jianmin Hu.
Data curation: Rifeng Xu.
Formal analysis: Rifeng Xu.
Methodology: Gaofeng Wu.
Validation: Yanting Du.
Writing - original draft: Jishuang San.
Writing - review & editing: Yanting Du, Jishuang San

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Not applicable.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2021.101135.

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