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ORIGINAL ARTICLE

Transcriptomic Insights into the Age-Dependent Regulation of Breast Muscle Growth in Broiler Chickens

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Abstract

The poultry industry is an excellent model for understanding the effects of human-driven selective pressure on animal genomes. One such system is modern poultry breeding, which uses intensive genetic selection for meat production in broilers. While the mRNA levels in broilers between days 6 and 21 post-hatch have been compared, the transcriptome of older chickens is not well defined, particularly for growth-related mRNAs. Here, RNA sequencing was used to compare the transcriptomes of 2-, 6-, 10-, and 14-week-old broilers to identify differentially enriched genes in the breast muscle. Among the genes analyzed, 445 were upregulated, and 237 were downregulated. We identified the differentially enriched genes that regulate breast muscle growth and differentiation in chickens of different ages. Our results indicated that most growth hormone and insulin-like growth factor genes were downregulated as growth proceeded. Furthermore, differences in the ratios of several positive myogenic growth regulators (*IGF2BP1*, *IGF2BP3*, *MYOD1*, *mTOR*, *SHC*, *IRS*, *SOCS*, *GK*, and *MNK*) may help to explain the different growth characteristics of different aged broilers.

Keywords: Chicken, RNA-seq, Breast muscle, Differential expression

Introduction

In the 20th century, modern agriculture promoted numerous selection traits for meat and egg production in broilers. To improve meat production, rapid growth genes such as skeletal muscle genes have been studied in broilers. In 1950, it took 16 weeks for a chicken to be market-ready; however, the timeframe was shortened to six weeks in 1990 (Warren 1958; Griffin and Goddard 1994; Konarzewski et al. 2000). This intense selective pressure indicates that broilers may be used to analyze the genes and alleles selected for human requirements. Compared with the original chickens, genetically selected modern broilers have highly improved production efficiency, rapid growth, and high feed efficiency. It is important to understand the mechanisms that regulate skeletal muscle growth because they may be crucial for chicken meat production (Niemann et al. 2011).

Comparisons of growth characteristics and gene expression patterns in modern broilers have been used to evaluate the selection of growth properties (Havenstein et al. 1994; Qureshi and Havenstein 1994; Schmidt et al. 2009). Reportedly, the transcriptional profiles of legacy and modern broilers identified 193 differentially enriched genes at day 21 post-hatching. A study indicated that different genes regulate myogenic growth in modern and legacy broilers (Davis et al. 2015). Although the mRNA levels in broilers between days 6 and 21 post-hatch have been compared, the transcriptome of older chickens is not well

defined, particularly for growth-related mRNAs. A study analyzed and compared the breast muscle transcriptomes of Recessive White Rock and Xinghua chickens (7 weeks old). They indicated that *FOXO3* is a candidate gene that may influence chicken growth (Chen et al. 2019). These findings suggest that diverse genes regulate the myogenic growth of broilers at different ages. Therefore, it is necessary to investigate the differences in the transcriptomes of chickens at different ages.

Here, we aimed to analyze the transcriptomes of four chicken groups at different ages for differentially expressed genes (DEGs). Genes associated with muscle growth and development were also investigated.

Methods and Materials

Animal Rearing and Tissue Collection

Twelve apparently healthy female, Wen Shang Luhua chickens were raised in the Gene Bank of National Chickens Genetic Resources (Jiangsu) and provided with food and water ad libitum. All birds were hatched on the same day. Samples T01, T02, and T03 were chest muscle samples from 2-week-old chickens; T04, T05, and T06 were chest muscle samples from 6-week-old chickens; T07, T08, and T09 were chest muscle samples from 10-week-old chickens; T10, T11, and T12 were chest muscle samples from 14-week-old chickens. Birds were euthanized by cervical dislocation, and tissue samples were collected from the pectoralis major muscle. Samples were flash-frozen in liquid nitrogen and stored at -80 °C before RNA isolation.

Total RNA Extraction

Total RNA was extracted from tissue samples using TRIZOL (Invitrogen, USA) following the manufacturer's protocol. SmartSpecPlus (Bio-Rad) was used to detect the absorption at 260/280 nm (A260/A280) to quantify total RNA. The integrity of the obtained total RNA was further analyzed using 1.5% agarose gel electrophoresis. Subsequently, extracted RNA was transcribed to first-strand cDNA using a First Strand cDNA Synthesis Kit (Thermo Scientific) to detect gene expression.

RNA-seq Library Construction and Sequencing

Before library construction, the collected mRNAs were purified and concentrated with oligo (dT)-conjugated magnetic beads. The obtained mRNAs were randomly cut into fragments in the fragmentation buffer. Using mRNA as a template, six-base random primers were used to synthesize the first strand of cDNA. To synthesize the second strand of cDNA, dNTPs, RNase H, and DNA polymerase I were added to the buffer. AMPure XP beads were used to purify the double-stranded cDNA by end repair and A-tailing and to select the size of the fragments. A cDNA library was obtained by polymerase chain reaction (PCR). Sequencing by Synthesis (SBS) technology using the Illumina HiSeq2500 high-throughput sequencing platform was used to predict the cDNA library and obtain many high-quality reads. The reads and bases from the platform were considered Raw Data, and a majority received a Q30 score for base quality.

Single Nucleotide Polymorphism Analysis

The TopHat2 sequence alignment program was used to align sample reads and reference genome sequences. SAM tools (Li et al. 2009) were used to identify single-base mismatches and Single Nucleotide Polymorphism (SNP) sites within the gene region between the sample and reference sequences. After transcription, the mRNA was capped, and a poly (A) tail was added. The number of SNP sites in each gene was divided by its length to obtain the SNP density.

Analysis of Differential Expression

The DESeq software (Anders and Huber 2010) was used to analyze the different expression levels between sample groups to obtain DEGs between the two muscle developmental stages. Using the method described by Audic and Claverie, we defined cut-off values to assemble the DEGs. To quantify expression, the coverage of each transcript was analyzed using BEDtools v. 2.9.0 (Quinlan and Hall 2010). The coverages of all libraries were integrated into a single file. The file was predicted using R v. 3.0.1, with the Bioconductor package “DEGseq” v. 1.2 (Wang et al. 2010). The Benjamini–Hochberg correction method was used to correct the significant p-value in the differential expression analysis ($P<0.05$). The false discovery rate (FDR) was used as a key indicator for screening GEGs ($FDR<0.05$).

Annotation and Analysis of Differentially Expressed Genes

The gene ontology (GO) database was used to predict the functions of the DEGs in this study. Enrichment analysis of the differences between the samples was performed using topGO (Alexa and Rahnenfuhrer 2006). The Cluster of Orthologous Groups of proteins (COG) database was used to classify orthologs of the gene products. Further analysis was required to annotate the DEG pathways. Therefore, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to analyze the pathways of differential gene enrichment. The GO terms and KEGG pathways with p-values < 0.05 were considered significantly enriched.

Validation of Gene Expression by Quantitative Real-Time PCR (qRT-PCR)

Primers were designed using Primer-BLAST from the NCBI website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 1). The first cDNA strand was synthesized by the Prime- Script™ RT Master Mix (Perfect Real Time) kit (Takara, Dalian, China), following the manufacturer's protocol. The β-actin gene was used as the housekeeping gene. Expression was quantified using AceQ qPCR SYBR Green Master Mix (Vazyme, Nanjing, China). The 20-μL PCR reaction contained 10 μL of AceQ qPCR SYBR Green Master Mix (Vazyme, China), 0.4 μL of ROX reference dye, 0.8 μL (10 pM/μL) of forward and reverse primer, 2 μL (10 ng/μL) of prepared cDNA, and 6.8 μL of RNase-free water. Cycling parameters were 95 ºC for 5 min, followed by 40 cycles of 95 ºC for 10 s and 60 ºC for 34 s. The ABI 7500 software was used to analyze the fluorescent signals. The comparative threshold cycle ($2^{-\Delta\Delta CT}$) method was used to quantify the detected gene expression.

Table 1. qRT-PCR primers

Gene	Primer sequence (5'-3')	Product length (bp)	Annealing Temperature (ºC)
CEBPB	F:TGAGCACCCTCAGGAACTTG R:CCCCAACGAAACCGAAAACG	181	60
IGF2BP1	F:GAGGCAAAGGACACGAAGAC R:CTGCAAGGATGAGATGGTGA	150	60
IGF2BP3	F:CGGACGTTCCTCTACGGATG R:GCAGCCTTCAGGAGTTGAGT	179	60
MyoD1	F:TGGGTGAGCAGGAGG R:TTTGGTGATTCCGTGTAGTAGC	172	60
mTOR	F: TGCTGGGGTGCTGGAATATG R: ATTCGTCCCAGCATCAGCTC	156	60

Results

Sequencing Data and Quality Control

The Q-score is an integer mapping of the Base Calling misidentification. The higher the Q-score, the more reliable the recognition of nucleotide bases, which reduces the likelihood of incorrect base calling. After applying sequencing quality control, the quality of the reads was good, with most having a Q-score above Q30. All the 12 samples were of similar quality.

Single Nucleotide Polymorphism Analysis

In this study, all gene structures were optimized to analyze SNP sites (Table 2). The number of SNP sites that contained SNPs, transitions, transversions, and heterozygosity was assessed. The number of SNP sites at T01, T02, T03, T04, T05, and T06 was similar to those at T07, T08, T09, T10, T11, and T12. Samples T01, T02, T03, T04, T05, and T06 also had transversions similar to those in samples T07, T08, T09, T10, T11, and T12. To further investigate the distribution of the SNP sites, the density distributions of the SNPs were analyzed (Figure 1). Most genes had 0-1 SNP sites, and the number of genes progressively decreased with increasing number of SNP sites/kb. The results indicated that most genes showed high levels of conservation among the 12 samples.

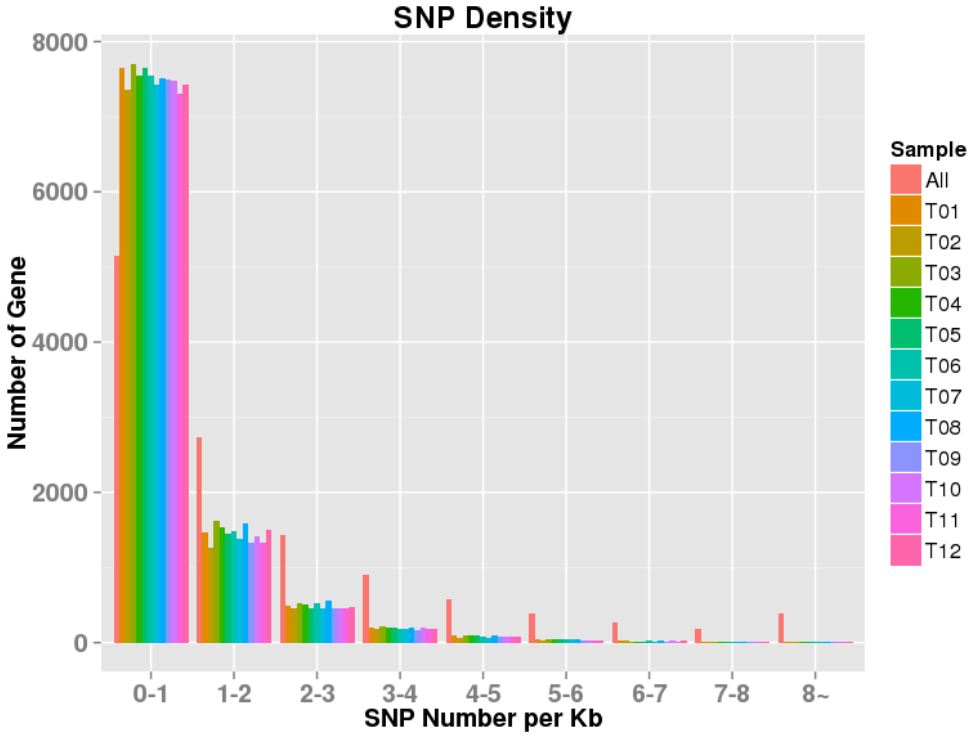

Figure 1 SNP density of the 12 samples. T01, T02 and T03 were chest muscle samples of 2-week-old chickens; T07, T08 and T09 were chest muscle samples of 6-week-old chickens; T04, T05 and T06 were chest muscle samples of 10-week-old chickens; T10, T11 and T12 were chest muscle samples of 14-week-old chickens

Table 2. Statistics for the SNP sites from 12 samples

Samples	SNP number	SNP number gene	Transition	Transversion	Heterozygosity
T01	112081	76178	77.24	22.76	59.98
T02	99954	68337	77.5	22.5	58.23
T03	120545	81652	76.92	23.08	58.92
T04	115546	76731	76.98	23.02	58.48
T05	115356	76535	77.03	22.97	57.97
T06	112988	75821	77.17	22.83	58.68
T07	105087	71451	77.3	22.7	59.44
T08	120348	80548	76.8	23.2	58.85
T09	104171	70385	77.24	22.76	59.79
T10	111115	74185	76.91	23.09	57.53
T11	101336	69176	77.1	22.9	58.65
T12	108640	73719	77.03	22.97	58.52

New Genes and Expression

Cufflinks software was used to splice new genes using mapped reads, and 1057 new genes were detected. Bioinformatic analysis was used to annotate the functions of the new genes. BLASX software was used to analyze the sequence alignment between the new genes and the NR, Swissprot, GO, COG, and KEGG databases.

Furthermore, expression analysis of the new genes was performed. In the COG functional classification of the consensus sequence, most of the new genes contributed to biogenesis and replication. The GO classification showed that numerous genes were associated with reproductive processes.

Different Gene Expression Levels

To analyze transcriptome sequencing data, it is

important to assess biological replicates and establish their relevance. The fragments per kilobase of transcript sequence per million (FPKM) base pairs-sequenced distribution of each sample is presented in Figure 2, wherein, all 12 samples were identical. A box plot of FPKM distribution was drawn to observe the gene expression distribution in the samples. The degree of dispersion from the box was similar for the biological replicates under the same conditions (Figure 3). However, the height of the box was significantly different under different conditions, indicating that the biological experiments were successfully repeated.

Numerous DEGs were detected in two groups of samples (Table 3). Cluster analysis (CA) of DEGs was performed on the samples. Genes with identical or similar expression levels were selected to analyze clustering (Figure 4). CA results indicated that the largest difference in gene expression occurred between the T01_T02_T03 and T10_T11_T12 sample sets, which is consistent with the results shown in Table 3. These results suggest that gene expression differed in different samples.

Table 3. Number of differentially expressed genes from the samples

DEG Set[a]	log2FC>1	
	upregulated	downregulated
T01_T02_T03 vs T04_T05_T06	133	203
T01_T02_T03 vs T07_T08_T09	37	65
T01_T02_T03 vs T10_T11_T12	237	445
T04_T05_T06 vs T07_T08_T09	119	236
T04_T05_T06 vs T10_T11_T12	165	143
T07_T08_T09 vs T10_T11_T12	219	290

[a]DEG Set: Names of differentially expressed genes;

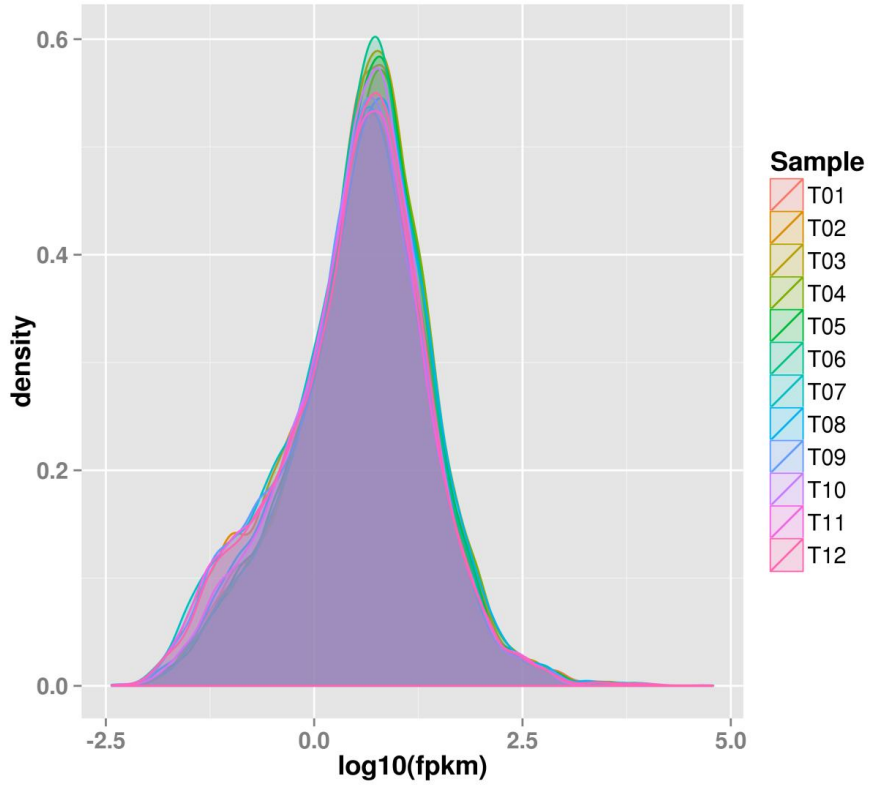

Figure 2 FPKM distribution of the 12 samples. T01, T02 and T03 were chest muscle samples of 2-week-old chickens; T07, T08 and T09 were chest muscle samples of 6-week-old chickens; T04, T05 and T06 were chest muscle samples of 10-week-old chickens; T10, T11 and T12 were chest muscle samples of 14-week-old chickens

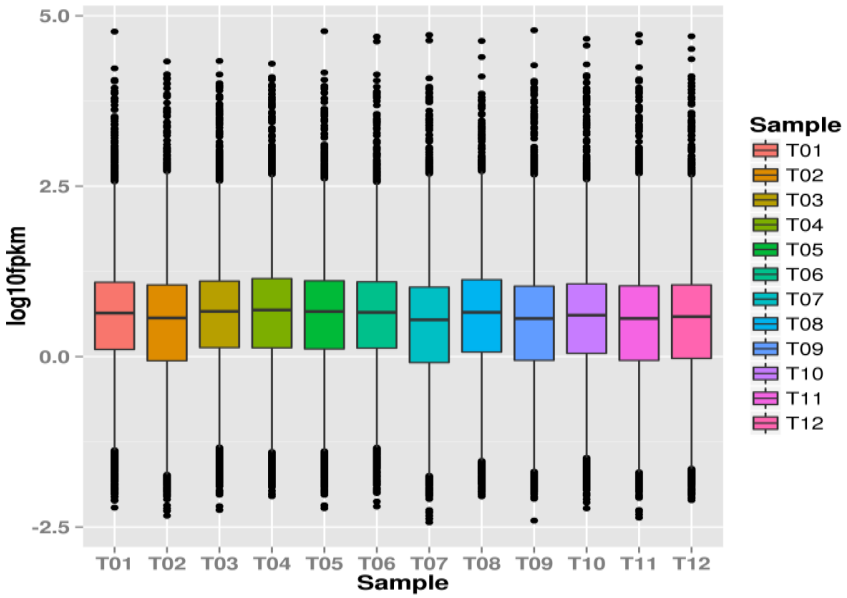

Figure 3 Degree of dispersion from the box for different biological replicates. T01, T02 and T03 were chest muscle samples of 2-week-old chickens; T07, T08 and T09 were chest muscle samples of 6-week-old chickens; T04, T05 and T06 were chest muscle samples of 10-week-old chickens; T10, T11 and T12 were chest muscle samples of 14-week-old chickens

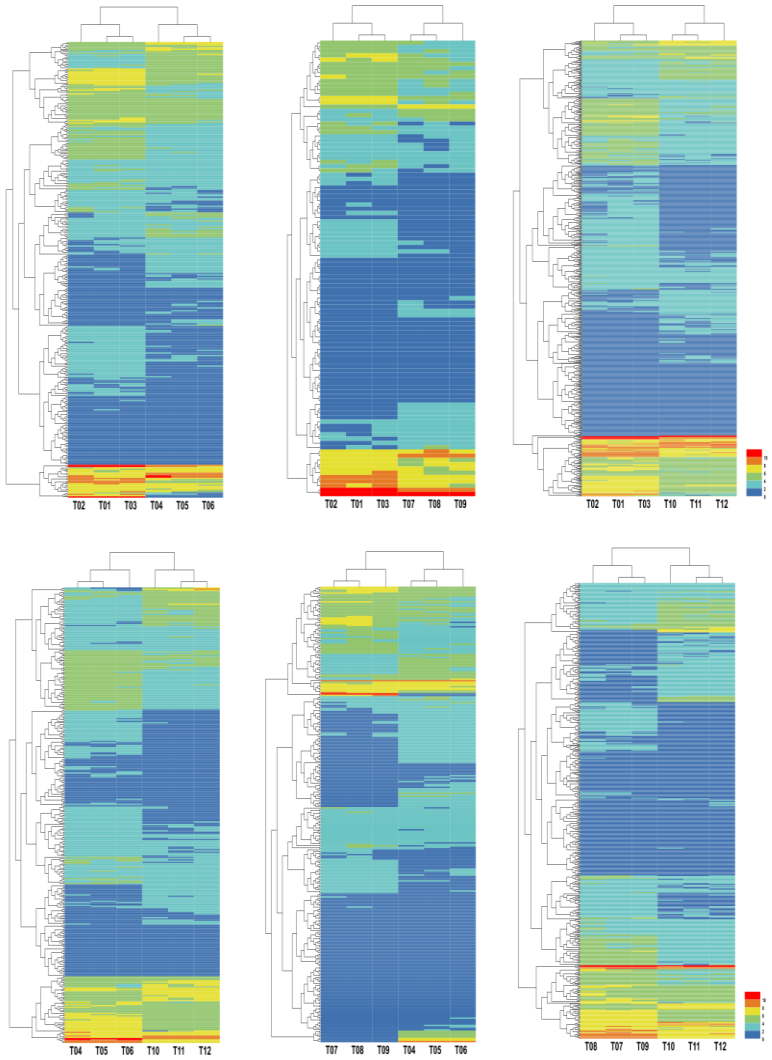

Figure 4 Clustering analysis of differentially expressed genes

Annotation and Analysis of the Genes

The numbers of DEGs are shown in Table 3. Although the numbers differed among the functional databases, the trend of the DEG set was consistent when annotated using different databases. The GO database was used to annotate and classify the functions of DEGs. The annotations of DEGs from sample sets T01_T02_T03 and T10_T11_T12 were similar (Figure 5). Compared with those in T01_T02_T03 and T07_T08_T09, fewer genes were produced in the molecular function and biological process categories. The significant differences in the proportion of genes in the two functional categories indicated that the enrichment trend differed between the DEGs and all genes. This suggests that it is necessary to analyze the relationship between function and differences in gene expression.

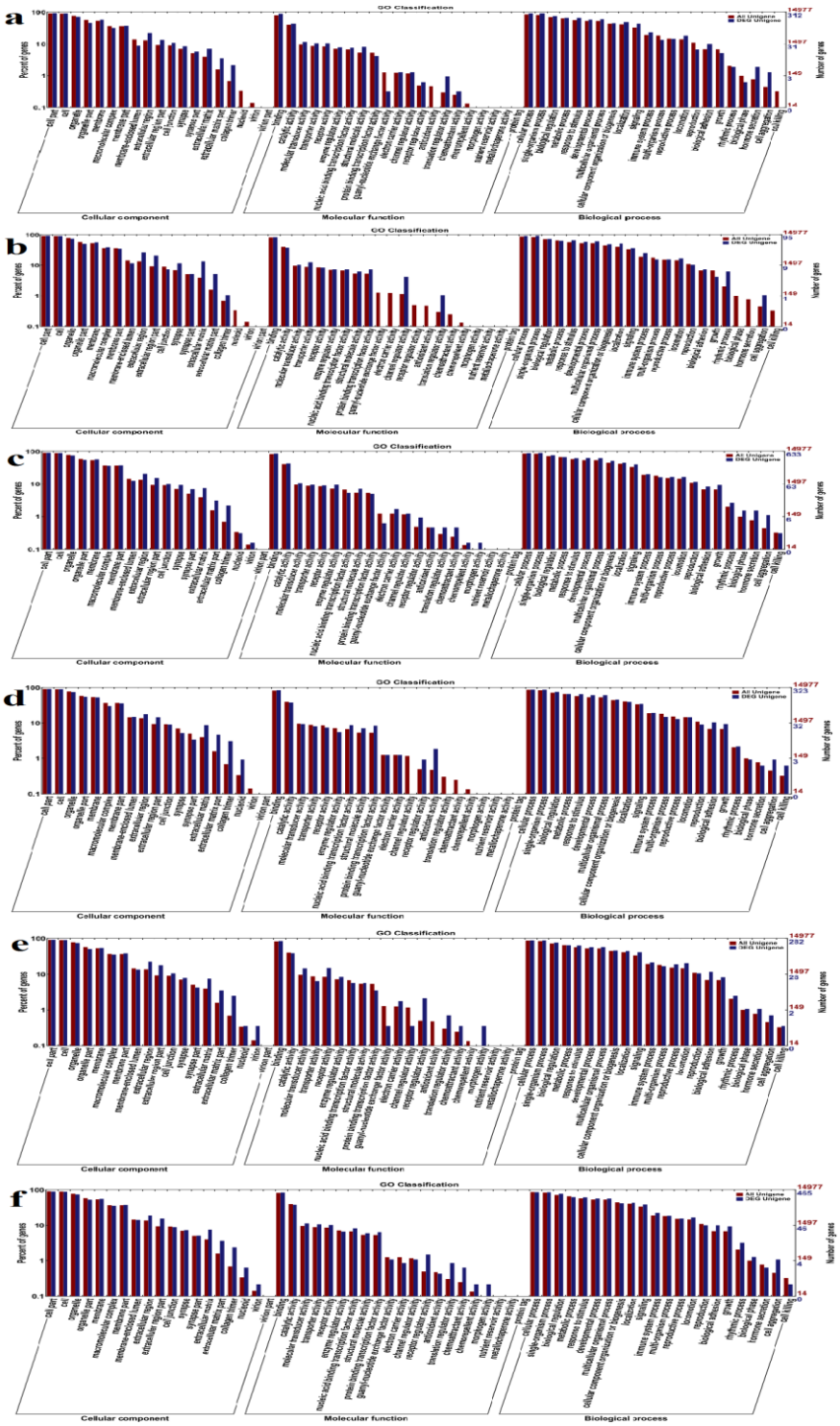

Figure 5:GO analysis of differentially expressed genes from the four sample groups
a: T01_T02_T03_vs_T04_T05_T06; b: T01_T02_T03_vs_T07_T08_T09;

c: T01_T02_T03_vs_T10_T11_T12; d: T04_T05_T06_vs_T10_T11_T12; e: T07_T08_T09_vs_T04_T05_T06; f: T07_T08_T09_vs_T10_T11_T12.

Pathways Analysis

The KEGG database contains the main public data on metabolic pathways and was used to analyze the metabolic pathways of the DEGs in this study. The annotation results were classified according to path type (Figure 6). Genes expression involved in metabolism and cellular processes showed significant differences between samples. The number and percentage of genes from different pathways showed differences between T01_T02_T03 vs. T04–T05–T06, T01_T02_T03 vs. T07_T08_T09, and T01_T02_T03 vs. T10–T11–T12. These data suggest that the number of genes was higher with a longer time interval. KEGG pathway analysis revealed a downregulation of the *SHC*, *IRS*, *SOCS*, *GK*, and *MNK* genes in the T10_T11_T12 sample set compared with the T01_T02_T03 sample set. These genes are involved in protein synthesis, proliferation, differentiation, lipid homeostasis, and glucose homeostasis, which correlates with chicken growth. The *SHC*, *SOCS*, *GK*, and *MNK* genes were downregulated in the T10_T11_T12 sample set compared with the T07_T08_T09 sample set, whereas only the *GK* gene was upregulated in the T10_T11_T12 sample set compared with the T04_T05_T06 sample set. This suggests that more growth-related genes were downregulated as chicken growth progressed.

The growth genes from the four sample sets provided positive information for the analysis. The expression of some growth genes showed large differences between the sample sets (Table 4). The expression of the *CEBPB* gene was upregulated in the T01_T02_T03 sample set compared with the T04_T05_T06, T07_T08_T09, and T10_T11_T12 sample sets. The expression levels of *IGF2BP1*, *IGF2BP3*, and *MYOD1* in T10, T11, and T12 were lower than those in T01, T02, and T03 cells. The results also showed that the *mTOR* gene was upregulated in the T10_T11_T12 sample set compared with the other sample sets. Furthermore, the mRNA levels of the growth hormone and insulin-like growth factor genes were analyzed. This analysis indicated that most growth hormones and insulin-like growth factor genes were downregulated with increasing growth time.

Table 4. Expression of growth genes from different samples

Group	Gene	Regulated
T01_T02_T03 vs T04_T05_T06	*CEBPB*	down
T01_T02_T03 vs T07_T08_T09	*CEBPB*	down
T01_T02_T03 vs T10_T11_T12	*CEBPB*	down
	IGF2BP1	down
	IGF2BP3	down
	MYOD1	down
	mTOR	up
T04_T05_T06 vs T07_T08_T09	*mTOR*	----
T04_T05_T06 vs T10_T11_T12	*mTOR*	up
T07_T08_T09 vs T10_T11_T12	*mTOR*	up

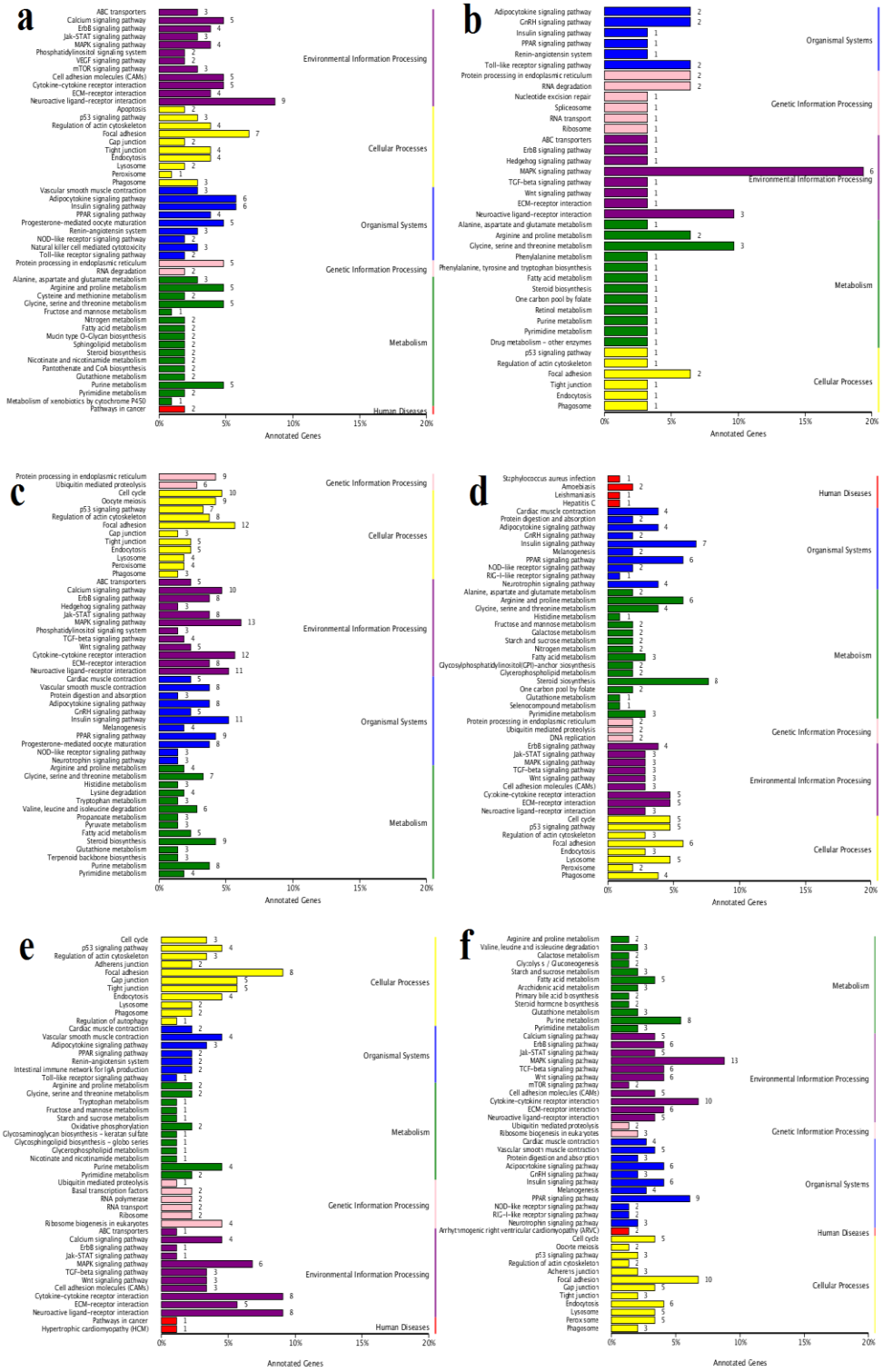

Figure 6 Annotation results of the 12 samples, in accordance with path type. a: T01_T02_T03_vs_T04_T05_T06; b: T01_T02_T03_vs_T07_T08_T09; c: T01_T02_T03_vs_T10_T11_T12; d: T04_T05_T06_vs_T10_T11_T12; e: T07_T08_T09_vs_T04_T05_T06; f: T07_T08_T09_vs_T10_T11_T12

Validation of DEGs using qRT-PCR

qRT-PCR was performed to detect the central and highly connected genes *CEBPB*, *IGF2BP1*, *IGF2BP3*, *MYOD1*, and *mTOR*. The 12 samples described above were used for qRT-PCR analysis. The results for all genes were statistically analyzed using the t-test method. The expression patterns of the five genes were in excellent agreement with the RNA-seq results (Figure 7).

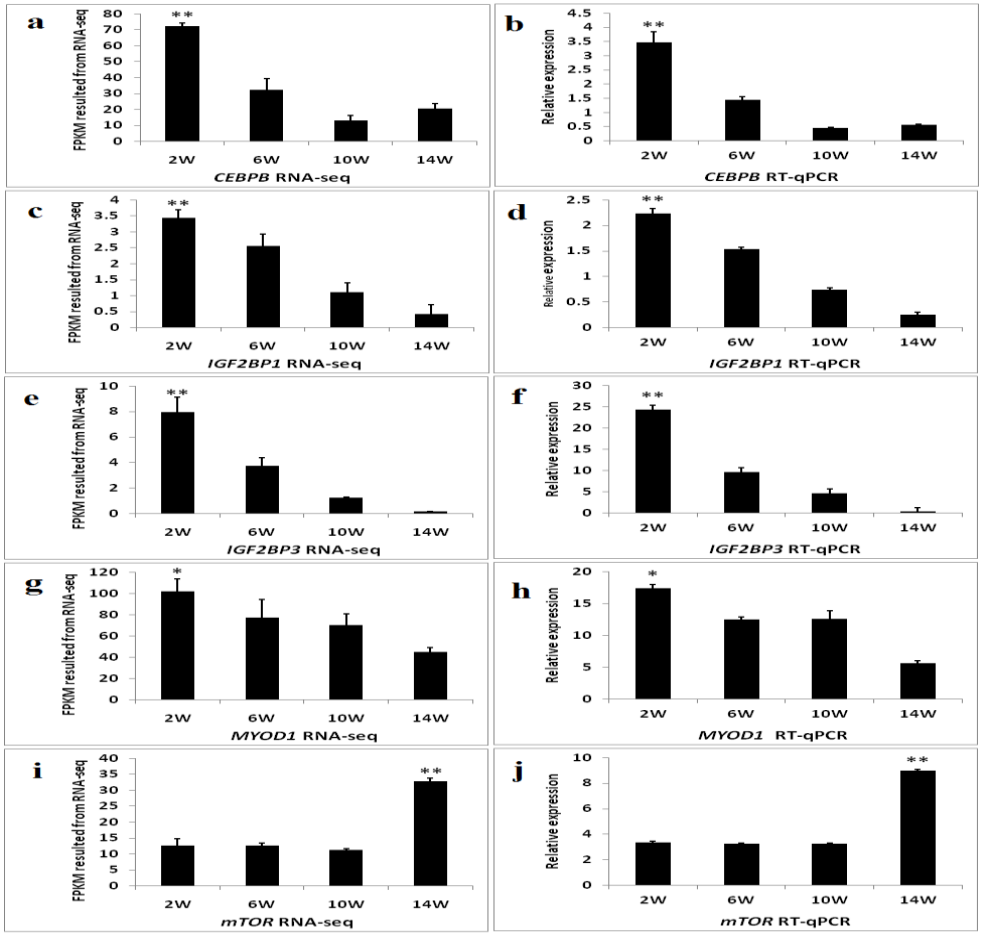

Figure 7 Expression of *CEBPB*, *IGF2BP1*, *IGF2BP3*, *MYOD1*, and *mTOR* genes.
a: RNA-seq results for the *CEBPB* gene. b: RT-qPCR results for *CEBPB* gene.
c RNA-seq results for the *IGF2BP1* gene. d RT-qPCR results for the *IGF2BP1* gene.
e RNA-seq results for the *IGF2BP3* gene. f RT-qPCR results for the *IGF2BP3* gene.
g: RNA-seq results for the *MYOD1* gene. h: RT-qPCR results for the *MYOD1* gene.
i: RNA-seq results for the *mTOR* gene. j RT-qPCR results for the *mTOR* gene.
*** P<0.05, ** P<0.01**

Discussion

Reportedly, gene expression shows biological variability (Schulze et al. 2012) between individuals. This variability has been observed through transcriptome sequencing technology, qPCR biochip analysis, and other technologies. The most effective method to study biologically significant differences in gene expression is the establishment of biological replicates in an experimental design. Therefore, multiple biological samples must be prepared under identical conditions. Testing must be repeated under stringent conditions, using many replicates to increase the reliability of the analysis.

In this study, SNP sites in 12 samples were analyzed to investigate the growth of genes expressed in breast muscle at the molecular level. Several factors can complicate SNP site identification. A large gap exists between SNP sites; however, homologous comparisons have revealed single-base mismatches with minimal spacing. SNP sites are typically used to mark genetic, evolutionary, and breeding programs (Blanca 2012). Some SNP sites may be connected to mutant phenotypes, providing a valuable resource for genetic studies and the various biochemical study processes (Fambrini et al. 2004; Fujino et al. 2008). This study used a different mutant analytic method, compared to other studies (Xu et al. 2013). These SNPs provide the basis for studying muscle development-related genes.

Differential expression in different sample sets was detected by comparative analysis of transcriptomes. Compared with those in the T01_T02_T03 sample set, more growth genes

were downregulated in the T10_T11_T12 sample set, suggesting that breast muscle growth may rely on the highly expressed genes. Furthermore, the expression of more genes was altered between the T01_T02_T03 and T10_T11_T12 sample sets when evaluated with other pairwise comparisons. This indicated that the difference in gene expression increased with increasing age. Davis et al. identified 189 differentially enriched genes between the 2 lines at 6 days post-hatch, including several regulators of myogenic growth and development. At 21 days post-hatching, the transcriptional profiles between lines identified 193 differentially enriched genes associated with myogenic growth (Davis et al. 2015). These findings are consistent with those of our study. They indicated that differences in the ratios of positive (*IGF1*, *IGF1R*, *WFIKKN2*) and negative (*MSTN* and *ACE*) myogenic growth regulators might explain the differences in enhanced growth characteristics of modern broilers. However, our study did not detect changes in *IGF1*, *IGF1R*, or *WFIKKN2* expression levels, suggesting that these genes are crucial in breast muscle development from the beginning of the post-hatching period. These genes were downregulated with an extended growth time, indicating that breast muscle growth follows a regular pattern as age progresses.

Table 4 shows several genes that may be crucial for breast muscle development. The *CEBPB* gene was downregulated in the 14-week-old broilers compared with the 2-, 6-, or 10-week old broilers, indicating that *CEBPB* is significant for chicken breast muscle growth; these findings are consistent with those of Chen's study (Chen et al. 2015). The downregulated genes, *IGF2BP1*, *IGF2BP3*, and *MYOD1*, in the T10_T11_T12 sample set suggested extensive breast muscle growth in 14-week-old broilers and is consistent with Lei's report (Lei et al. 2008). The *IGF2* gene may encode key growth factors with autocrine and paracrine functions for chicken skeletal muscle regeneration and development (Gal-Levi et al. 1998; Chargé and Rudnicki 2004). Furthermore, *IGF2* is a crucial regulator of muscle regeneration because it stimulates muscle cell differentiation and regulates muscle cell hypertrophy (Kaliman et al. 1999; Zanou and Gailly 2013). Using a genome-wide mRNA screen and functional analysis (Chen et al. 2015), found that FOXO3 is a candidate gene for chicken growth. However, the role of *FOXO3* in breast muscle growth in different-aged chickens was not elucidated. The use of different tissues may have caused this difference in results. Furthermore, we found that a negative regulation factor (*mTOR*) was upregulated in the T10_T11_T12 sample set compared with other samples. The mTOR signaling is a key target for myostatin function in skeletal muscle (Amirouche et al. 2009; Loyau et al. 2014; Wen et al. 2014; Kong et al. 2016). As *mTOR* gene expression can block breast muscle growth, it may play a role in the muscle growth of different-aged chickens.

The RT-PCR results were consistent with the RNA-seq results and confirmed the changes in *CEBPB*, *IGF2BP1*, *IGF2BP3*, *MYOD1*, and *mTOR* gene expression. Previous studies evidenced that *CEBPB* may regulate multiple genes in response to *GH* (Cui et al. 2011). Furthermore, *CEBPB* activates adipogenesis and inhibits myogenesis. The *MYOD1* gene can differentiate many types of cells into muscle cells and is crucial in regulating myogenesis (Choi et al. 1990). A recent study indicated that *MYOD1* facilitates and modulates the assembly of muscle enhancers (Blum 2014). The *IGF2* gene may encode important growth factors with paracrine and autocrine functions in chicken skeletal muscle regeneration and production (Chargé and Rudnicki 2004). *IGF2BP1* and *IGF2BP3* genes, which are key members of the *IGF* family, play important roles in the activation of the *IGF* gene (Lederer et al. 2014). The mTOR signaling pathway regulates myostatin function in the skeletal muscles (Kong et al. 2016). These genes change with age in the breast muscle of chickens and may be crucial for chicken skeletal muscle regeneration and development.

In the insulin signaling pathway, *SHC*, *IRS*, *SOCS*, *GK*, and *MNK* genes were downregulated in the T10_T11_T12 sample set compared with the T01_T02_T03 sample set. The insulin pathway also plays an important role in muscle growth (Jiang et al. 2017; Stanley et al. 2017). Therefore, *SHC*, *IRS*, *SOCS*, *GK*, and *MNK* may play important roles in breast muscle growth in broilers. The results showed that several genes were downregulated as growth progressed, suggesting that the expression of growth genes is characteristic of different stages.

This study used RNA sequencing to compare the

transcriptomes of 2-, 6-, 10-, and 14-week-old broilers to identify differentially enriched genes in breast muscle. We identified genes that regulate breast muscle growth and differentiation in chickens of various ages. Our results may explain the different growth characteristics observed in broilers of different ages.

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Author contributions

Guo-Hui Li, Jing-sheng Wu, and Wei Han conceived and designed the experiments. Cheng-Hao Zhou participated in the bioinformatics studies. Hui-yong Zhang and Qian Xue conducted the bioinformatics studies and performed the qRT-PCR validation. Jian-mei Yin, Jian-bo Yang, and Yi-Xiu Jiang participated in the animal material collection. Guo-Hui Li and Cheng-Hao Zhou analyzed the data and wrote the manuscript. All the authors have read and approved the manuscript for publication.

Conflict of interest

The authors report there are no competing interests to declare.

Ethical approval

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the Yangzhou University. This study was approved by the Animal Ethics Committee of Yangzhou University.

Data Availability Statement

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