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Development of myofibers and muscle transcriptomic analysis in growing Yili geese

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ABSTRACT In poultries, muscle growth is a quantitative trait controlled by multiple genes. The regulatory mechanisms governing muscle tissue growth and development in poultry, particularly during the early stages of growth, are intricate. Through the examination of leg muscle transcripts from Yili geese during various stages of development, this study offers valuable insights into the molecular mechanisms underlying the growth and development of Yili geese. This study aimed to perform a comparative analysis of the histological characteristics of leg muscles and the mRNA expression profiles of leg muscles in Yili geese at different ages (2, 4, 6, 8, and 10 wk). The objective was to identify differentially expressed genes related to muscle development in Yili geese and utilize bioinformatics to predict the potential biological functions of these genes. Through histological studies on leg muscle tissues, it was discerned that male geese at 4 wk exhibit a significantly reduced muscle fiber density in comparison to females ($P < 0.01$). In contrast, by the time they reach 6, 8, and 10 wk, their muscle fiber diameter and cross-sectional dimensions significantly outpace the females ($P < 0.01$). With the advancement in age, muscle fiber density tends to decrease. It is worth noting that 4- and 6-wk-old male geese have a substantially elevated muscle fiber density when matched against females ($P < 0.01$). Conversely, at the age of 10 wk, their muscle fiber density is notably inferior to the females ($P < 0.01$). Furthermore, male geese exhibit the most rapid increase in muscle fiber diameter and cross-sectional area between 4 and 6 wk of age. The density of muscle fibers in these geese significantly decreases from 4 to 8 wk. In contrast, female geese show the most pronounced growth in muscle fiber diameter and cross-sectional area between 2 and 6 wk, with a swift decline in density following the 6-wk mark, accompanied by a gradual reduction in the rate of muscle fiber growth. A comprehensive analysis of the leg muscle mRNA expression profiles from 12 Yili geese generated a cumulative total of 502,065,268 valid sequence reads, corresponding to a data volume of 75.30 Gb. In a comparative analysis between 4-wk-old and 2-wk-old groups (**T4 vs. T2**), 8-wk-old and 2-wk-old groups (**T8 vs. T2**), and 8-wk-old and 4-wk-old groups (**T8 vs. T4**), we identified 1,700, 1,583, and 221 differentially expressed genes (**DEGs**), respectively. Differentially expressed genes were significantly enriched in Gene Ontology (**GO**) terms such as organelle organization, cytoskeletal protein binding, cation transport, myosin complex, and actin cytoskeleton. Among the significantly enriched signaling pathways, 5 pathways were found to be significantly related to growth and development: adhesion patch, extracellular matrix receptor interaction, tight junction, TGF-β signaling pathway, and MAPK signaling pathway, with a total of 38 differentially differentiated genes contained in these 5 pathways, and it was hypothesized that the above pathways as well as the DEGs in the pathways played an important role in the regulation of early growth and development of the Yili goose. This investigation serves as a foundational reference for elucidating the molecular regulatory mechanisms involved in the development of goose muscle. Furthermore, it contributes to the expansion of the theoretical framework concerning the genetic regulation of muscle growth in geese.

Key words: mRNA, goose, leg muscles, growth

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INTRODUCTION

Yili goose is an excellent and rare local poultry species in Xinjiang, which is the only kind of small and medium-sized domestic geese domesticated from gray geese in China, and has the characteristics of roughage tolerance, cold tolerance, and strong disease resistance (Zhao et al., 2022). Yili goose meat quality is better, rich in nutrition, low fat content, in the poultry products market has been in short

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supply. Poultry growth traits are quantitative traits that are regulated by multiple genes, especially during their early growth and development, the regulatory mechanisms of muscle tissue growth and development are complex, and the relevant regulatory genes are differently expressed at different growth stages. In recent years, numerous RNA-seq studies have investigated the growth performance of livestock and poultry. These studies aim to uncover the molecular mechanisms that regulate their growth traits (Qian et al., 2023; Zhang et al., 2023; Zhao et al., 2023). Hu et al. (2022) found that short photoperiod significantly promoted muscle development and significantly improved the redness of goose muscle, and identified the possible involvement of glycolysis/gluconeogenesis, calcium signaling pathway, and PI3K-Akt signaling pathway in the regulation of muscle development and meat color in geese by light through RNA-seq analysis. Tang et al. (2023) investigated transcriptional changes in the leg muscles of the Shitou goose and the Wuzong goose during a period of high growth rate and found that many DEGs have potential growth functions, such as CXCL12, SSTR4, FABP5, SLC2A1, MYLK4, and EIF4E3. The gene-gene interaction network of DEGS is mainly related to the transmission of cell signals and substances, hematological system development, and functions. Chen et al. (2015) conducted transcriptome sequencing studies on the pectoral muscles of cryptic white rock chickens and apricot chickens. They found that CCAAT enhancer binding protein beta (**CEBPB**), F-box protein 32 (**FBXO32**), forkhead box O3 (**FOXO3**), and myogenic differentiation 1 (**MYOD1**) play vital roles in muscle growth, of which FBXO32 is mainly expressed in leg muscles, heart, and pectoral muscles, and after reducing the expression of FBXO32, growth-related genes such as pyruvate dehydrogenase kinase 4 (**PDK4**), insulin like growth factor 2 receptor (**IGF2R**), and insulin like growth factor 2 mRNA binding protein 3 (**IGF2BP3**) were significantly down-regulated, suggesting that the FOXO3 gene could be a candidate gene for probing chicken growth performance. He et al. (2020) analyzed the transcriptome of leg muscles of fast- and slow-growing performance border chickens and found that there were 17 upregulated genes and 91 down-regulated genes in the slow-growing group compared to the fast-growing group and screened 4 genes related to muscle contraction, troponin C1, slow skeletal and cardiac type (**TNNC1**), troponin T2, cardiac type (**TNNT2**), myosin light chain 3 (**MYL3**) and myosin heavy chain 7 (**MYH7**), which were significantly higher in the fast-growing group than the slow-growing group in terms of the expression level of these 4 genes. At present, the research on the molecular regulatory mechanism of muscle growth and development for Yili geese is still blank. Therefore, in this study, the histological characteristics of leg muscles and mRNA expression profiles of leg muscles of 2-, 4-, 6-, 8-, and 10-wk-old Yili geese were comparatively analyzed to screen differentially expressed genes related to muscle development of Yili geese, and to predict the potential biological functions of the differentially expressed genes through bioinformatics, to provide a reference for resolving molecular regulatory mechanisms of muscle development in geese.

MATERIALS AND METHODS

Ethics Statement

Sample collection and cell treatment involved were approved by the Animal Welfare and Ethics Committee of Xinjiang Agricultural University, Urumqi, Xinjiang, China (Approval number 2020035).

Experimental Design and Sample Collection

The test was conducted with 150 healthy Yili goose chicks of the same batch and 0 d of age, 75 male and 75 female, for a period of 10 wk, during which the males and females were kept in separate groups. The experimental geese were caged online from 0 to 3 wk and raised flat on the ground in the enclosure from 4 to 10 wk. A maize-soybean meal-type basal diet was used, with ad libitum feeding and watering, and routine immunization procedures were carried out. The test geese were executed by CO_2 inhalation, and 6 male and 6 female Yili geese of 2, 4, 6, 8, and 10 wk of age were slaughtered, and the muscle tissues of the ipsilateral leg were collected for analysis of leg muscle fiber sections and transcriptome determination.

Analysis of the Histological Structure of the Hamstrings

Goose leg muscle samples were fixed with 4% paraformaldehyde and embedded in paraffin blocks. Serial slices of 5 μm thickness were made using a slicer, the sections were stained using hematoxylin-eosin (**HE**, Servicebio, China), and after fixation, the muscle tissue sections were observed under an electron microscope and photographed. Under a 100× electron microscope, each sample section was randomly selected for 5 fields of view, and 10 muscle fibers per field of view were selected for measurement of their diameter and cross-sectional area using EZ-met software (Nikon). Muscle fiber diameter (μm), transverse area (μm^2), and density of muscle fibers (fibers/mm^2) were calculated using ImageJ software (https://imagej.net/software/imagej/), to compare the differences in histological properties of leg muscles during different growth and development periods.

RNA Extraction, RNA-seq Library Preparation, and Sequencing

Four male Yili geese each were selected at the early growth stage (2 wk of age, T2), close to the growth inflection point (4 wk of age, T4), and near the group average weight at the listed weekly age (8 wk of age, T8), and the muscle tissue of the ipsilateral leg was collected after slaughter and placed in 2 mL freezing tubes containing RNA later (Qiagen, Hilden, Germany), stood at 4°C overnight, and preserved at −80°C, for the total RNA extraction of the tissue samples. Leg muscle tissue RNA was extracted from 12 samples according to the Trizol kit instructions, and the purity, concentration,

and integrity of the RNA samples were examined by Nanodrop (NanoDrop, Wilmington, DE), Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA), and Agilent 2100 bioanalyzer (Agilent, Santa Clara, CA), respectively. After the RNA samples passed the quality test, library construction, and quality control were performed on the Illumina sequencing platform, and the Illumina HiSeq2500 (Illumina) high-throughput sequencing platform performed sequencing.

Analysis of Sequencing Data

The raw data will be filtered for connector sequences, low repetitive sequences, and low-quality sequences at both ends, and the remaining data will be valid data (clean reads), after which all analyses will be based on clean reads. Sequence comparison of the sequencing data of each sample with the Anser cygnoides genome published in the Ensembl database (https://www.ncbi.nlm.nih.gov/assembly/GCF_000971095.1) was performed using the sequence efficient comparison HISAT software (http://www.ccb.jhu.edu/software/hisat). DESeq was used to analyze the differences between 12 samples, and the calculation of hypothesis testing probability (P value) was carried out according to the model, and finally multiple hypothesis testing (FDR) corrections were carried out, and the genes with fold change >1 and corrected P value (q value) <0.05 were taken as differentially expressed genes after correction.

Gene Ontology and Kyoto Encyclopedia of Genes and Genomes Pathway Enrichment Analysis

ClusterProfler(v3.10.1) (Yu et al., 2012) and KOBAS (v2.0) (Chen et al., 2011) was used for GO and Kyoko Encyclopedia of Genes and Genomes (**KEGG**) (Kanehisa and Goto, 2000; Kanehisa et al., 2021) pathway analyses of differentially expressed mRNA. GO enrichment analysis includes biological process (**BP**), cellular component (**CC**), and molecular function (**MF**). GO terms or KEGG pathways with P value <0.05 were considered to be significantly enriched.

Protein Interaction Network Analyses

Protein-protein interactions (**PPIs**) were constructed using the STRING (Andrea et al., 2013) database by combining the results of differential expression analyses and the interaction pairs included in the database. For species not included in the database, target genes and proteins in the database were sequenced using BLAST software (v2.2.28) to find homologous proteins. Interaction networks were then constructed based on the interaction relationships of homologous proteins and visualized using Cytoscape software (v3.8.0).

Real-time Fluorescence Quantitative PCR Validation

Nine differentially expressed genes were randomly selected from the transcriptome sequencing test results for fluorescence quantification verification, GAPDH was selected as the internal reference gene, and fluorescence quantification primers were designed according to the sequences of the genes using the online tool Primer-BLAST. Primer sequences are shown in Table S1. RNA was reverse transcribed using RevertAid Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's protocol. Quantitative RT-PCR was performed using AceQ Universal SYBR qPCR Master Mix (Vazyme Biotech, Nanjing, China) and ABI StepOnePlus machine (ABI, Foster City, CA). The total reaction system was 20 μL (configured on ice), and the reaction procedure was: predenaturation at 95°C for 5 min, denaturation at 95°C for 10 s, and extension at 60°C for 30 s, with 40 cycles. As a result, the relative quantitative $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001) was used to calculate the expression of the target genes to further assess the accuracy and reproducibility of the transcriptome sequencing data.

Statistical Analysis

Statistical analysis was performed using the software IBM SPSS Statistics version 22. Te comparative analysis of 2 groups was performed using Student t test, and multiple comparative analysis was performed with 1-way ANOVA. GraphPad Prism 8 (https://www.graphpad.com) was applied for making graph. It was considered to be statistically significant when P value <0.05.

RESULTS

Observations on Myofiber Sections of the Leg Muscles of Yili Geese at Different Weekly ages

From the leg muscle tissue sections, it can be observed that the cross-section of muscle fibers is mostly approximately round or oval, some of them are polygonal, the muscle fibers are uniformly colored, pink or red, and the nuclei of the cells are all bluish-purple. As can be seen from the figure, the diameter and transverse area of the myofibrils became progressively larger with increasing age, and the number of myofibrils in each field of view progressively decreased (Figure 1A−E).

Comparative Morphological Analysis of Leg Muscle Myofibers in Male and Female Yili Geese of Different Weekly Ages

Myofiber diameter and cross-sectional area of the leg muscles of Yili geese increased gradually with the increase of age, in which the myofiber density of male geese at 4 wk of age was very significantly lower than

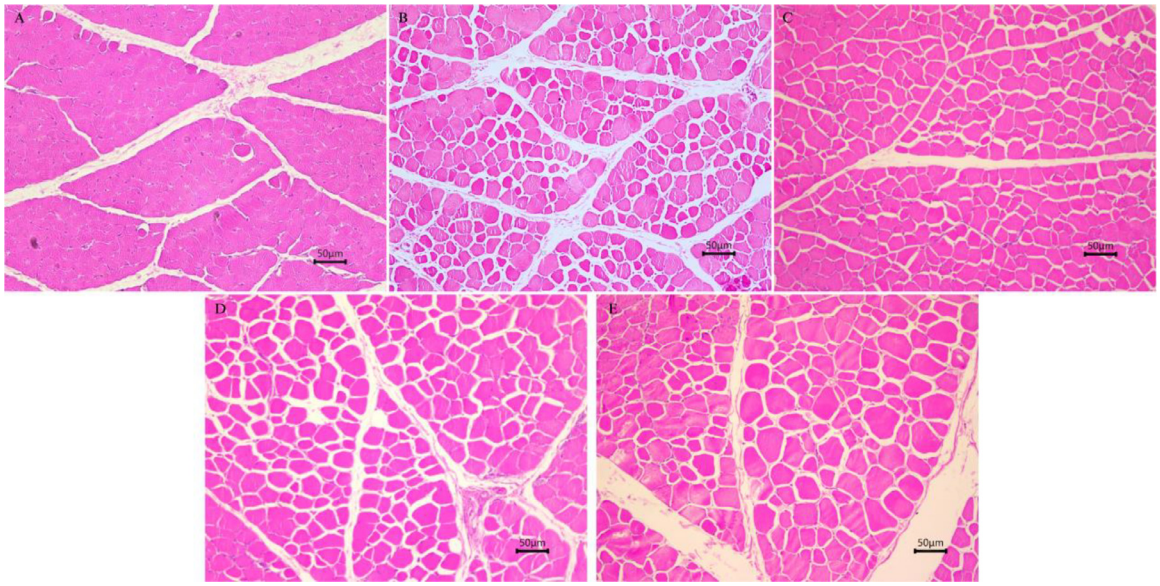

Figure 1. Representative micrographs of hematoxylin and eosin (H&E) staining of Yili geese leg muscle at different weeks of age (100×). (A) 2 wk of age. (B) 4 wk of age. (C) 6 wk of age. (D) 8 wk of age. (E) 10 wk of age. $n = 6$.

that of female geese ($P < 0.01$), and myofiber diameters of male geese at 6, 8, and 10 wk of age were very significantly higher than that of female geese ($P < 0.01$), the cross-sectional area of myofibers of male geese at 6, 8 and 10 wk of age was positively and significantly higher than that of female geese ($P < 0.01$), while myofiber density declined with the increase of age. Myofiber density decreased with increasing age, and was highly significant ($P < 0.01$) higher in male geese than in female geese at 4 and 6 wk of age, and highly significant ($P < 0.01$) lower in male geese than in female geese at 10 wk of age and fiber density (Table 1).

In male geese, myofiber diameter and cross-sectional area grew fastest at 4 to 6 wk of age, and myofiber density declined rapidly from 4 to 8 wk of age, while in females, myofiber diameter and cross-sectional area grew fastest at 2 to 6 wk of age, and density decreased rapidly, with myofiber growth declining after 6 wk of age (Figure 2A−C).

Table 1. Morphological comparison of leg muscle fibers of male and female Yili geese of different weeks of age.

Project	Week	Male geese	Female geese
Diameter (μm)	2	24.59 ± 1.15	24.27 ± 1.16
	4	30.27 ± 1.89^{Bb}	32.50 ± 1.77^{Aa}
	6	42.11 ± 2.59^{Aa}	39.46 ± 1.74^{Bb}
	8	46.41 ± 3.10^{Aa}	40.57 ± 2.61^{Bb}
	10	49.12 ± 2.80^{Aa}	45.72 ± 1.64^{Bb}
Cross-sectional area ($\mu m^{2)}$	2	592.93 ± 30.55	583.22 ± 33.28
	4	971.8 ± 67.06	1009.02 ± 57.74
	6	1775.13 ± 84.56^{Aa}	1447.03 ± 61.29^{Bb}
	8	1925.55 ± 71.90^{Aa}	1659.52 ± 62.15^{Bb}
	10	2554.24 ± 190.79^{Aa}	1883.96 ± 56.62^{Bb}
Density (根/$mm^{2)}$	2	1039.92 ± 162.50	1074.73 ± 177.79
	4	928.12 ± 93.39^{Aa}	833.95 ± 67.58^{Bb}
	6	649.83 ± 64.21^{Aa}	538.36 ± 47.58^{Bb}
	8	471.23 ± 72.80	473.32 ± 64.21
	10	403.23 ± 71.29^{Bb}	463.51 ± 89.92^{Aa}

Different small letters in the same group mean significant difference ($P < 0.05$), different capital letters in the same group mean highly significant difference ($P < 0.01$).

RNA-seq Data Quality Evaluation

The final amount of valid data obtained for each of the 12 leg muscle samples was above 5.93 G, with Q20 > 97.95% and Q30 > 94.26%, and the GC content of the samples ranged from 48.39 to 52.16%, making the overall data output adequate for subsequent data analysis (Table S2). The clean reads obtained after quality control of the sequencing data were compared with the goose reference genome, and 76.94 to 84.87% of the total reads were compared to the goose reference genome (Table S2).

Analysis of Differentially Expressed Genes at Different Growth Stages

There were 1,700, 1,583, and 221 differentially expressed genes in the T4 vs. T2, T8 vs. T2, and T8 vs. T4 groups, respectively, with 864 upregulated genes and 836 down-regulated genes at 4 wk of age compared to 2 wk of age, 778 upregulated genes and 805 down-regulated genes at 8 wk of age compared to 2 wk of age, and 62 upregulated genes and 158 down-regulated genes at 8 wk of age compared to 4 wk of age (Figure 3A, Table S3). Cluster analysis of the DEGs screened from the 3 comparison groups revealed that 4 samples at the same growth stage clustered in the same cluster, further illustrating the accuracy and reliability of sample collection in this study (Figure 3B). To further analyze the interactions among DEGs, Venn diagrams were constructed for the differentially expressed genes in the 3 comparison groups (T4 vs. T2, T8 vs. T2, and T8 vs. T4), and a total of 2,676 differentially expressed genes

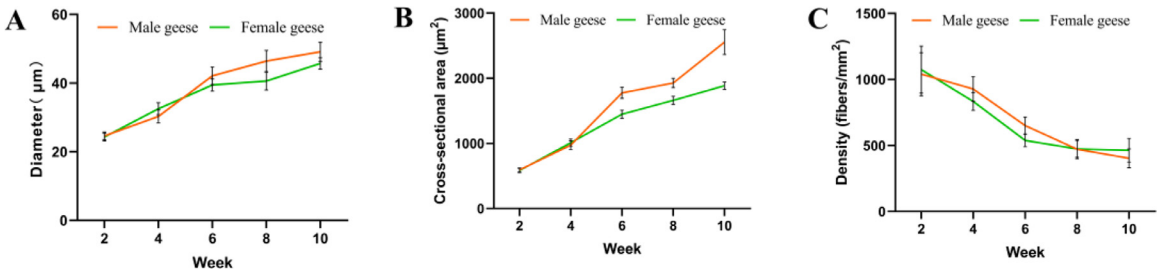

Figure 2. Morphological comparison of leg muscle fibers of Yili geese. (A) Measurement of the diameter of leg muscle fibers of geese. (B) Measurement of cross-sectional area of leg muscle fibers of geese. (C) Measurement of the number of muscle fibers per unit area of leg muscles of geese.

were screened (Figures 3C), among which 14 differentially expressed genes were expressed in all the 3 comparison groups (Table 2).

GO Analysis of the Differentially Expressed Genes

In this study, GO functional annotation and enrichment analyses were performed on 1,700, 1,583, and 211 DEGs obtained from the 3 comparison groups of T4 vs. T2, T8 vs. T2, and T8 vs. T4, respectively ($P < 0.05$). In the T4 vs. T2 group, DEGs involved in BP were mainly enriched in Terms such as Organelle organization and microtubule-based process; DEGs involved in CC were mainly enriched in terms such as protein-containing complex, nonmembrane—the DEGs involved in CC are mainly enriched in protein-containing complex, nonmembrane-bound organelle, Intracellular organelle part, etc.; DEGs involved in MF are mainly enriched in pyrophosphatase activity, cytoskeletal protein binding, motor activity, etc. term (Figures 4A, Table S4-1). In the T8 vs. T2 group, DEGs involved in BP were mainly enriched in terms of cation transmembrane transport, inorganic ion transmembrane transport, etc.; DEGs involved in CC were mainly enriched in terms of chromosome and myosin The DEGs involved in CC are mainly enriched in chromosome, myosin complex, and other terms; the DEGs involved in MF are mainly enriched in metallopeptidase activity, ATPase activity

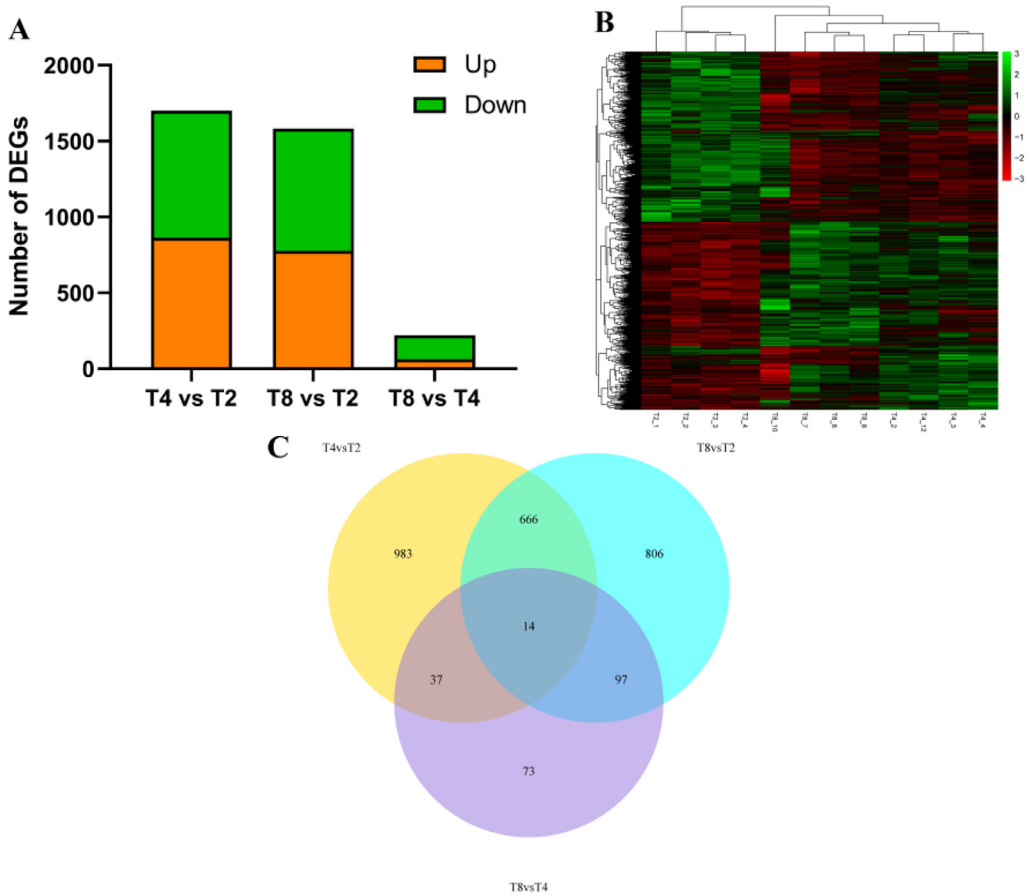

Figure 3. Analysis of differentially expressed genes at different growth stages. (A) Statistics on the number of differentially expressed genes at different growth stages. (B) Cluster analysis of DEGs. (C) Differentially expressed genes at different growth stages.

Table 2. Differentially expressed genes common to different growth stages.

Gene ID	Gene name	Gene description
106042983	*LPAR4*	Lysophosphatidic acid receptor 4
106034821	*SH3TC1*	SH3 domain and tetratricopeptide repeats 1
106048582	*LOC106048582*	Hemoglobin subunit beta-like
106030443	*ANGPT2*	Angiopoietin 2
106046557	*LOC106046557*	Kelch-like protein 4
106036765	*PLXND1*	Plexin D1
106042247	*CD93*	CD93 molecule
106038291	*LDHB*	Lactate dehydrogenase B
106042643	*NRP1*	Neuropilin 1
106032859	*NIPAL4*	NIPA like domain containing 4
106035632	*ARL15*	ADP ribosylation factor like GTPase 15
106035493	*EOGT*	EGF domain specific O-linked N-acetylglucosamine transferase
106033392	*ZFAND2A*	Zinc finger AN1-type containing 2A
106038515	*COLEC10*	Collectin subfamily member 10

and other terms (Figures 4B, Table S4-2). In the T8 vs. T4 group, DEGs involved in BP were mainly enriched in terms of ion transport and cation transport; DEGs involved in CC were mainly enriched in terms of extracellular region and transmembrane transporter complex; DEGs involved in MF were mainly enriched in terms of transmembrane transporter activity and transporter activity (Figures 4C, Table S4-3).

There were 95, 36, and 21 significantly enriched GO Terms ($P < 0.05$) in the T4 vs. T2, T8 vs. T2, and T8 vs. T4 comparison groups, respectively, which involved several pathways that have been reported to be associated with muscle growth and development, such as myosin complex, Actin cytoskeleton, Cytoskeletal part, Cytoskeleton, Cytoskeletal protein binding, Actin filament-based process, Actin cytoskeleton organization, Actin binding, etc. (Table 3). The DEGs in the above Term may be involved in the regulation of growth and development of Yili geese and can be used as candidate genes related to the regulation of goose muscle growth and development.

KEGG Analysis of the Differentially Expressed Genes

To further understand the function of DEGs in the leg muscle tissues of Yili geese at different growth stages, KEGG enrichment analysis was carried out, and there were 14, 7, and 8 significantly enriched pathways in the comparison groups of T4 vs. T2, T8 vs. T2 and T8 vs. T4, respectively ($P < 0.05$) (Figure 5, Table S5), of which 5 signaling pathways related to muscle growth and development were screened, namely, adhesion Focal adhesion, Tight junction, ECM-receptor interaction, TGF-β signaling pathway, and MAPK signaling pathway, with a total of 38 signaling pathways. A total of 38 differentially expressed genes were included in these 5 pathways (Table 4), and it was hypothesized that these pathways and the DEGs in the pathways played important regulatory roles in the growth and development of Yili geese.

Interaction Network Analysis of Genes Related to Muscle Growth and Development

To further investigate the interactions of the differentially expressed genes involved in the above muscle growth and development pathway, protein interactions were predicted using the STRING database, and hub gene screening was performed. In the network diagram (Figure 6), integrin subunit beta 3 (**ITGB3**) had the highest degree value, and the remaining hub genes encoded proteins of integrin subunit beta 6 (**ITGB6**), actin beta (**ACTB**), and laminin subunit alpha 4 (**LAMA4**) (degree value ≥8).

Real-Time Fluorescence Quantitative PCR Validation

In this experiment, 9 known DEGs were randomly selected from the transcriptome sequencing results, which were myosin heavy chain 10 (**MYH10**), myosin light chain kinase 3 (**MYLK3**), myosin light chain kinase family member 4 (**MYLK4**), integrin subunit beta 6 (**ITGB6**), insulin like growth factor 1 (**IGF1**), collagen type II alpha 1 chain (**COL2A1**), collagen type VI alpha 6 chain (**COL6A6**), forkhead box M1 (**FOXM1**), protein kinase AMP-activated noncatalytic subunit gamma 3 (**PRKAG3**), and the trend of the expression levels of the above genes in the leg muscles of the Yili geese at different growth stages was consistent with the results of the RNA-seq analyses (Figure 7), which indicated that the results of the transcriptome sequencing data analyses were reliable in the present study and that they could be carried out for the next step of the research and analysis.

DISCUSSION

Myofibrils are the basic units that make up the muscle, and by measuring the diameter, transverse area, and density of muscle fibers, the differences in the histological characteristics of leg muscles at different stages of growth and development were studied, which helped to understand the growth and developmental patterns of muscle fibers in the Yili goose. Muscle growth and development can be divided into 2 stages, proliferation, and hypertrophy, where proliferation refers to the increase in the number of embryonic adult myoblasts (Li et al., 2016), and hypertrophy refers to the increase in the length and thickening of the muscle fibers after fledgling, which is mainly manifested in the changes in the diameter and density of the muscle fibers (Huo et al., 2021). In this study, we found that the diameter and density of muscle fibers of leg muscles of Yili geese increased with age, the diameter and cross-sectional area of muscle fibers of male geese grew the fastest at 4 to 6 wk of age, and the density of muscle fibers decreased rapidly at 4 to 8 wk of age, while

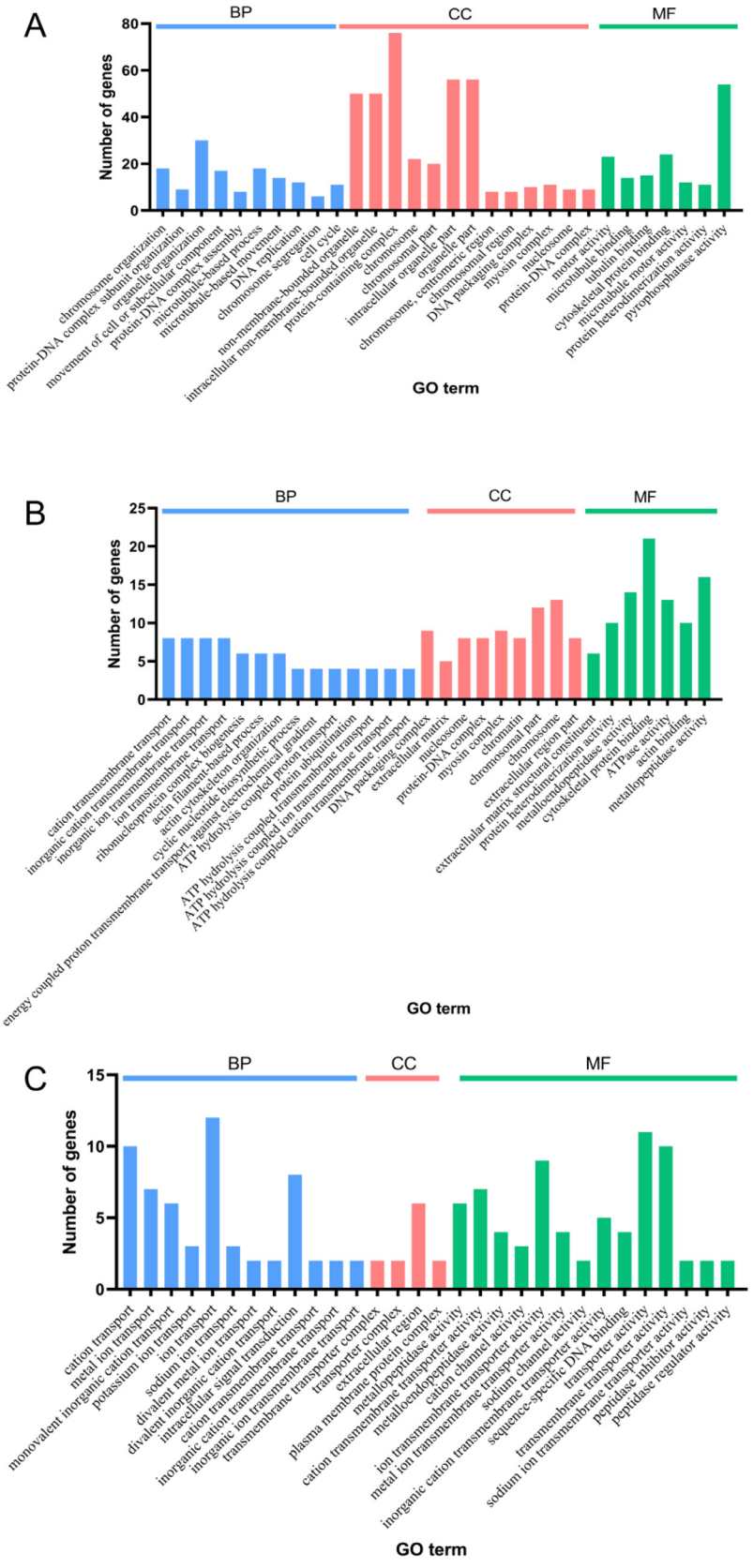

Figure 4. GO enrichment analysis of differential genes. (A) T4 vs. T2. (B) T8 vs. T2. (C) T8 vs. T4.

that of female geese grew the fastest in diameter and cross-sectional area of muscle fibers at 2 to 6 wk of age, and the density decreased rapidly, and the growth of muscle fibers decreased gradually after 6 wk of age, which was consistent with the trend of body weight growth of both males and females of Yili geese (Cao et al., 2021).

As age and body weight increase, the physiological needs, endocrine profiles, and exercise profiles of

Table 3. GO term related to muscle growth and development in Yili geese.

Group	GO term	Function description	*P* value	Genes
T4 vs. T2	0016459	Myosin complex	0.0020	*MYO1B, MYO15A, MYO16, MYO1C, MYO5A, MYH10, MYO1A, MYH9*
	0044430	Cytoskeletal part	0.0151	*MYO1B, MYO15A, MYO16, MYO1C, MYO5A, MYH10, MYO1A, MYH9*
	0015629	Actin cytoskeleton	0.0174	*MYO1B, MYO15A, MYO16, MYO1C, MYO5A, MYH10, MYO1A, MYH9*
	0005856	Cytoskeleton	0.0360	*MYO1B, MYO15A, MYO16, MYO1C, MYO5A, MYH10, MYO1A, MYH9*
	0008092	Cytoskeletal protein binding	0.0023	*KIF20B, DIAPH3, KIF20A, TWF1, KIF18A, DSTN, FSCN1, CTNNA1, MSN, ABLIM1*
T8 vs. T2	0030029	Actin filament-based process	0.0388	*TMOD1, XIRP1, LMOD1, DIAPH3*
	0030036	Actin cytoskeleton organization	0.0388	*TMOD1, XIRP1, LMOD1, DIAPH3*
	0016459	Myosin complex	0.0228	*MYO1A, MYH15, MYO19, MYO1B, MYO15A, MYO1C*
	0008092	Cytoskeletal protein binding	0.0177	*TMOD1, ABLIM1, TWF1, TWF2, XIRP1, LMOD1, DIAPH3*
	0003779	Actin binding	0.0348	*ABLIM1, TWF1, TWF2, XIRP1, DIAPH3*
T8 vs. T4	—			

different sexes of livestock vary, resulting in different rates of muscle fiber growth. In the present study, it was found that the muscle fibers of male and female Yili geese in the early stages of growth and development, the diameter and cross-sectional area of the leg muscles of Yili geese increased gradually with the increase of weekly age, and the diameter and cross-sectional area of the muscle fibers of male geese were highly significantly higher than those of female geese at the ages of 6, 8, and 10 wk, and the results of the fiber density showed the opposite trend of the fiber diameter and cross-sectional area, and there was an interaction between the diameter and the density of the fibers of the muscle fibers of the different days of age.

Skeletal muscle development is a very complex process, regulated by multiple genes at different levels, and

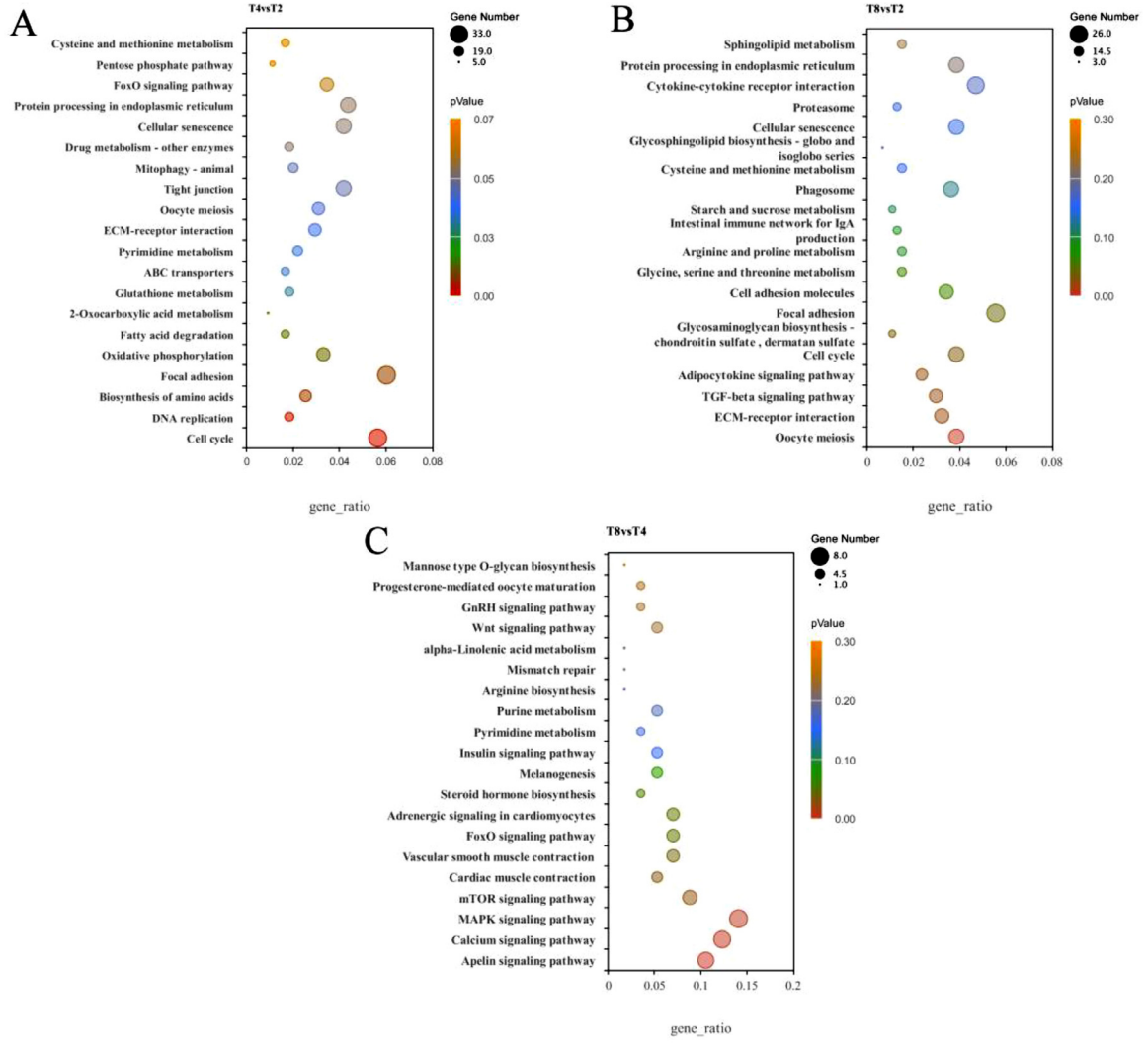

Figure 5. KEGG enrichment analysis of differential genes. (A) T4 vs. T2. (B) T8 vs. T2. (C) T8 vs. T4.

Table 4. Signaling pathway related to muscle growth and development in Yili geese.

Group	Pathway	*P* value	Genes
T4 vs. T2	Focal adhesion	0.0071	*MYLK4, COL4A5, PARVA, BCAR1, LAMB2, LAMA4, COL4A6, PAK3, LAMB1, RHOA, ACTN1, COL6A6, COL6A3, MAPK1, COL2A1, ITGB6, ACTB, PIK3R2, ITGB3, FLNB*
	ECM-receptor interaction	0.0389	*LAMA4, COL4A6, LAMB1, AGRN, COL6A6, COL6A3, COL2A1, ITGB6, ITGB3*
	Tight junction	0.0455	*SYNPO, RHOA, ACTN1, PRKAG3, MYH10, ACTB, MSN, MYH9*
T8 vs. T2	ECM-receptor interaction	0.0216	*COL4A6, COL2A1, LAMB1, LAMA4, AGRN, COL1A2*
	TGF-beta signaling pathway	0.0218	*LTBP1, TGFB2, TGIF1, GREM1, BMP6, SMAD1, ACVR2A*
	Focal adhesion	0.0411	*COL4A6, MYLK4, IGF1, COL2A1, LAMB1, LAMA4, FLNC, FLNB, MYLK3, MYL10*
T8 vs. T4	MAPK signaling pathway	0.0081	*IGF1*

transcriptional regulation plays an important role in muscle formation. In this study, we analyzed the leg muscle tissues of male Yili geese at different growth stages by RNA-Seq and screened a total of 2,676 DEGs, and identified several genes that play important regulatory roles in the muscle development process of Yili geese, such as tropomodulin 1 (**TMOD1**), myosin heavy chain 9 (**MYH9**), MYH10, MYLK3, MYLK4, myosin light chain 10 (**MYL10**), ITGB6, transforming growth factor beta 2 (**TGFB2**), IGF1, and synaptopodin (**SYNPO**), etc. GO annotation and KEGG enrichment analyses of these DEGs revealed that some DEGs were significantly enriched in pathways such as myosin complex, cytoskeletal fraction, actin cytoskeleton, adhesion plaques, extracellular matrix receptor interactions, tight junctions, TGF-β signaling pathway, and MAPK signaling pathway.

Myosin, the basic building block of myogenic fibers, is the most abundant protein in muscle and plays an important role in cell movement and intracellular substance transport. The myosin family is a highly conserved family of proteins that are widely present in eukaryotic cells, and MYHC, encoded by the MYH gene family, is a key subunit in the myosin class II molecules (**MHCII**), of which MHCII is widely present in rhabdomyolysis, smooth muscle, and nonmyocytes (He et al., 2020). MYHC is the basic building block of myosin, a skeletal muscle-specific contractile protein expressed during muscle development, and plays an important role in ensuring the proper functioning of muscle cells (Wimmers et al., 2007). A variety of myosin heavy chain genes and their isoforms exist in animals and are encoded by different MYH gene family members, and the type and expression of these genes determine the type of muscle fibers, whereas the growth and development of skeletal muscle, physiological and biochemical characteristics, and muscle quality are all influenced by the influence of muscle fiber type and composition (Lefaucheur, 2010). Most myoblasts simultaneously express 3 nonmyosin MyHC isoforms, myosin heavy chain IIA, IIB, and IIC, with MYH9 encoding the protein MYHIIA and MYH10 encoding MYHIIB, which are involved in important cellular functions such as cytoplasmic division, cellular motility, and maintenance of cellular shape (Hasan et al., 2021). In this study, MYH9 and MYH10 genes were significantly enriched in signaling pathways such as tight junctions, regulation of actin cytoskeleton and cellular adhesion molecules, and the genes were significantly down-regulated in the T4 vs. T2 group, and it was hypothesized that these genes were reduced in expression at 4 wk of age, which might be related to the process of myofiber development and differentiation.

Myosin light chain kinase (**MLCK**) is a class of serine/threonine-specific protein kinase that regulates the light chain of type II myosin by phosphorylation, and is widely found in skeletal, smooth, and cardiac muscle as well as in nonmuscle cells of animals, and is encoded by 4 different genes, MYLK1, MYLK2, MYLK3 an MYLK4, encoded by 4 different genes, MYLK1, MYLK2, MYLK3, and MYLK4, which can enhance myosin ATPase activity

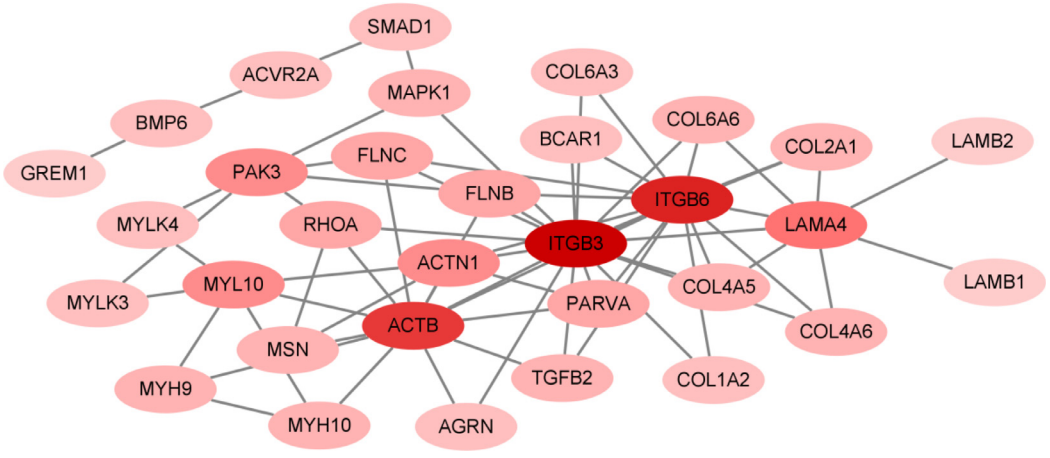

Figure 6. The protein interaction network diagram of differentially expressed genes related to muscle growth and development in Yili geese.

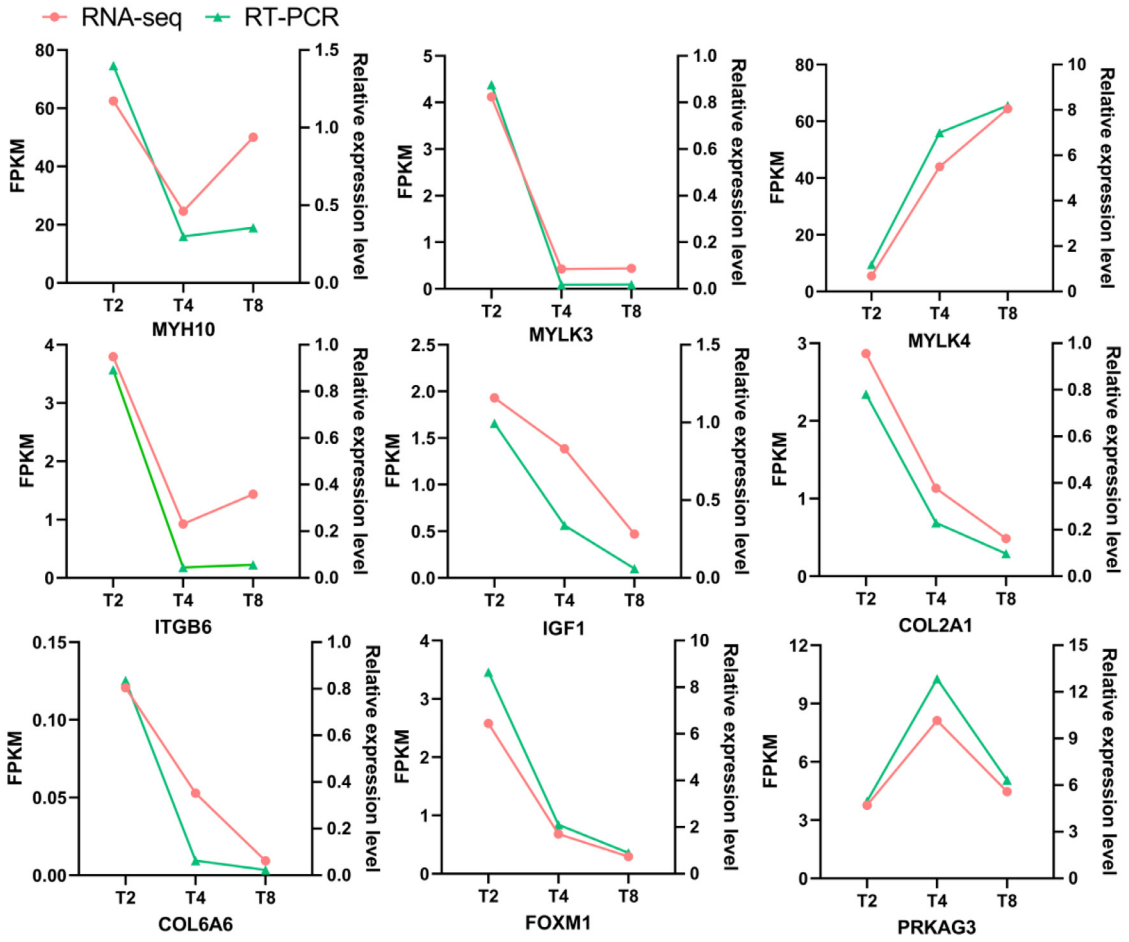

Figure 7. Comparison of RNA-seq and RT-PCR expression analysis.

by catalyzing the phosphorylation of serine residues at the end of myosin-regulated light chains, thus facilitating the interaction between myosin and actin (Yu et al., 2016). In this study, the expression of the MYLK2 gene was significantly higher at 4 and 8 wk of age than at 2 wk of age, the expression of the MYLK3 gene decreased gradually with the increase of weekly age, and the opposite was true for the MYLK4 gene, which is involved in the signaling pathways such as adhesion plaque, calcium signaling pathway, and the regulation of actin cytoskeleton, which may be related to the muscle growth and intercellular signaling, and play a key role. In a comparative transcriptomic study of the leg muscles of Shitou goose and Wuzong goose, it was similarly found that MYLK4 may be associated with growth and development (Tang et al., 2023).

Laminin (**LN**) is an extracellular matrix protein present in the basement membrane that, in addition to promoting cell adhesion and migration, provides structural support to stimulate intracellular signaling cascades through interactions with cell-surface receptors, thereby inducing cell-specific differentiation (Goddi et al., 2021). LN receptors have a high affinity for intracellular actin, and LN surface receptors connect extracellular LN to intracellular backbone-actin (Gao et al., 2017). Lama2 encodes a protein known to interact directly with the dystrophin complex, and mutations in Lama2 lead to muscular dystrophy(Swaggart et al., 2011). The results of this study revealed that LAMB1, LAMB2, and LAMA4 were differentially expressed in the 3 comparison groups and were involved in signaling pathways related to cell growth such as regulation of developmental processes, regulation of multicellular biogenesis, cell adhesion, adhesion patches, ECM receptor interactions, etc.

The insulin-like growth factor is a multifunctional cellular regulator and the IGFs gene family stimulates the proliferation, differentiation, and metabolism of myogenic cells, as well as the anabolism of myotubes and myofibroblasts (Duclos, 2005). It has been found that IGF-1 is essential for the mitogenic activity of adult myoblasts and is mainly mediated by 2 signaling pathways, namely the mitogen-activated protein kinase (MAPK/ERK1/2) pathway and the PI3K/Akt pathway, both of which are associated with cell cycle progression and cellular activity (Fu et al., 2018). IGF-1 plays a key role in myogenesis during embryonic development, but the mechanism of IGF-1-mediated proliferation of adult myoblasts is unclear (Yu et al., 2015). IGF1 can directly affect the expression of duck MYOD and myogenic factor 5 (**MYF5**) genes and can upregulate MRFs (Papasani et al., 2009). IGFs have important roles in the growth and development of chicken muscle cells and, the transformation of myofiber types, and IGF1 stimulates growth hormone production (Tang et al., 2010).

IGF-1 stimulates the proliferation and differentiation of satellite cells and myofibroblasts, promotes regenerative myogenesis, and maintains muscle fiber robustness (Forcina et al., 2019). The results of the present study showed that IGF1 gene expression was down-regulated in the leg muscles of 8-wk-old Yili geese, compared to 2- and 4-wk-old geese, which may be related to the slowing down of muscle cell differentiation and growth.

Functional enrichment analysis of differentially expressed genes was carried out to reveal the molecular mechanisms regulating the differences in growth phenotypes of Yili geese during different growth periods. GO enrichment analysis in this study showed that the differentially expressed genes were mainly involved in processes such as myosin complex, cytoskeletal fraction, actin cytoskeleton, cytoskeleton, cytoskeletal protein binding, actin filament-based processes, actin cytoskeletal organization, actin binding, and so on. Many differentially expressed genes affecting poultry growth and development are associated with these GO terms, suggesting that poultry growth is a complex process that is coregulated by multiple genes as well as multiple pathways. In this study, KEGG enrichment analysis revealed that the pathways significantly enriched at different periods were mostly related to energy and amino acid metabolism, such as cysteine and methionine metabolism, lysine degradation, and arginine biosynthesis. In a transcriptomic study of muscle development in Zhedong white geese, the differential genes were similarly found to be mostly associated with metabolic pathways such as glycolysis/gluconeogenesis (Hu et al., 2022). Through the comparative analysis of differential pathways at different stages, we screened 5 pathways related to muscle growth and development in Erythrina geese, namely: adhesion plaques, extracellular matrix receptor interactions, tight junctions, TGF-β signaling pathway, and MAPK signaling pathway.

Adhesion plaques are macromolecular protein complexes that connect the extracellular matrix at the ends of specialized actin fibers and play a key role in the stability of cell membrane function and cellular signal transduction (Romer et al., 2006). Extracellular matrix (**ECM**) is a complex mixture of functional macromolecules that interact with cells through the occurrence of interactions. The ECM can directly or indirectly control cellular activities such as cell adhesion, migration, differentiation, proliferation, and apoptosis, mainly caused by interactions with integrins and other cell surface-associated components, and ECM proteins play a key role in skeletal muscle development and maintenance of the stability of the internal environment (Thomas et al., 2015). Tight junctions form the structural basis of the paracellular pathway and are also a necessary barrier for the growth and development of multicellular organisms, restricting the free entry and exit of substances such as free ions and proteins into and out of the paracellular pathway (Citi, 2019). The TGF-β pathway is composed of the TGF-β receptor and receptor substrate Smad protein family signaling molecules, and transforming growth factor β (**TGF-β**) is a multifunctional protein (Sandra, 2019). TGF-β superfamily ligands participate in cell identify, growth, and development (Wang et al., 2020). MAPK is a stress-activated protein kinase implicated in skeletal muscle catabolism (Clary et al., 2011). The MAPK signaling pathway is a positive regulator in muscle development (Huang et al., 2018). In this study, differentially expressed genes were found to be significantly enriched in signaling pathways such as cytoskeletal protein binding, adhesion patches, and tight junctions when comparing 4-wk-old with 2-wk-old, and differentially expressed genes were found to be significantly enriched in signaling pathways such as cytoskeletal protein binding and adhesion patches when comparing 8-wk-old with 2-wk-old, which were hypothesized to be the main reasons for the differences in muscle growth and development of the Yili geese in different periods. The Focal adhesion pathway is significantly enriched in genes such as MYLK4, COL4A5, PARVA, etc., of which COL4A5 is thought to be possibly related to the transmission of cellular signals and substances, and the development and function of the blood system. A sound blood system can provide many nutrients for the growth of body tissues and promote the organism's growth (Tang et al., 2023).

In this study, protein interactions were analyzed for differentially expressed genes, and protein nodes such as ITGB3, ITGB6, ACTB, and LAMA4 were identified and among them, ACTB and LAMA4 were found to be shared genes of multiple functional pathways in the KEGG pathway analysis. ITGB6 is an integrin located in the cellular membrane that is associated with focal adhesions (Klohonatz et al., 2019). ACTB protein is 1 of 6 different human actin isoforms and is 1 of the 2 nonmuscle cytoskeletal actins (Rydbirk et al., 2016). ACTB is involved in cell motility, cell structure and cell integrity (Tao et al., 2016). In this study, the expression of ITGB3, ITGB6, ACTB, and LAMA4 was significantly upregulated at the age of 2 wk and then at the age of 4 wk, after which the expression was not significant, suggesting that the above genes may have a regulatory role in the early growth and development of the muscle fibers of the Yili geese and that the specific regulatory mechanisms need to be further investigated.

CONCLUSIONS

In this study, we constructed 12 male Yili geese leg muscle cDNA libraries by transcriptome technology and identified 5 pathways related to muscle growth and development: adhesion plaques, extracellular matrix receptor interactions, tight junctions, TGF-β signaling pathway, and MAPK signaling pathway. A total of 38 differential genes were included in these 5 pathways in this study, of which the hub genes *ITGB3, ITGB6, ACTB*, and *LAMA4* are important regulatory genes associated with the regulation of the growth and development of goose muscle fibers. However, the biological function and molecular mechanism of these key genes need to be further verified. The functional genetic variants can be applied to the

molecular breeding of geese growth performance. In brief, these results will help elucidate the molecular mechanism of geese muscle fiber development, and provide important candidate genes for the molecular breeding of geese growth performance.

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DISCLOSURES

All authors declare that they have no competing interests.

SUPPLEMENTARY MATERIALS

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