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Identification of miRNA-mRNA Networks Associated with Pigeon Skeletal Muscle Development and Growth

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Simple Summary: The growth and development of skeletal muscle determine the meat production performance of pigeons and were regulated by complex gene networks. In order to explore the function of miRNA in regulating the growth and development of pigeon skeletal muscle, we will use sequencing technology to study the transcriptome of pigeon at two embryonic stages (E8 and E13) and two growth stages (D1 and D10).

Abstract: The growth and development of skeletal muscle determine the meat production performance of meat pigeons. MiRNA plays an important role in the growth and development of skeletal muscle. However, there are few reports about miRNA regulating the growth and development of skeletal muscle in pigeons. In order to explore the function of miRNA in regulating the growth and development of pigeon skeletal muscle, we will use sequencing technology to study the transcriptome of pigeon at two embryonic stages (E8 and E13) and two growth stages (D1 and D10), A total of 32,527 mRNAs were identified in pigeon skeletal muscles, including 14,378 novel mRNAs and 18,149 known mRNAs. A total 2362 miRNAs were identified, including 1758 known miRNAs and 624 novel miRNAs. STEM clustering tool assigned each miRNA to its time expression characteristics. A total of 20 expression profiles were generated, and seven were significantly enriched (P-value < 0.05),there are two types. The first category represents DEmiRNAs that continuously down-regulated from the developmental stage to the growth stage of pigeon skeletal muscle, including cli-miR-20b-5p、miR-130-y、cli-miR-106-5p、cli-miR-181b-5p etc. The second category represents DEmiRNAs with low expression at the development and early growth stage and significantly up-regulated expression at the high growth stage, including miR-27-y、miR-1-z、cli-miR-1a-3p、miR-23-y etc. We performed GO and KEGG enrichment analysis on the miRNA target genes in the two categories, and found many biological processes and pathways related to muscle growth and development. including biological processes such as striated muscle cell differentiation、striated muscle cell development、muscle cell development、regulation of biological process、regulation of cellular process and tissue morphogenesis. Signal pathways such as ECM-receptor interaction、focal adhesion、biosynthesis of amino acids、cell growth and death、signal transduction and development. This study expands the diversity of pigeon miRNAs and helps to understand the role of miRNAs in pigeon muscle development and growth.

Keywords:pigeon; skeletal muscle; miRNA-mRNA; RNA sequencing

1. Introduction

Pigeon meat is rich in nutrition, high in protein, low in fat, and high in medicinal value. In China, pigeon meat is called "animal ginseng" and is considered as an advanced nourishment product that is increasingly favored by consumers. Meat production performance is an important index to measure the economic value of pigeons. However, the genetic improvement of meat production performance of pigeons is relatively lagging in comparison with other poultry. The growth and development of skeletal muscle determine the meat production performance of pigeons [1]. Therefore, understanding the molecular regulation mechanism of pigeon skeletal muscle growth and development is an important prerequisite for improving meat production performance by molecular breeding technology [2].

The number of muscle cells and the development of skeletal muscle fibers are closely related to animal meat production. Myogenic regulatory factor family (MRF) members play important roles in the process of skeletal muscle differentiation, including MyoD (myogenic determining factor), MyoG (myogenin), MRF4 (muscle reguLatory factor 4), and Myf5 (myogenic factor 5) [3]. However, skeletal muscle growth and development involves multi-gene expression, signal transduction, and network regulation, and there are still a large number of regulatory factors to be identified.

MicroRNAs (miRNAs) are small endogenous non-coding RNAs with a length of about 22nt. MiRNA regulates gene expression by binding 3'UTR of the target gene, and then participates in a variety of biological processes, including growth and development [4]. Increasing evidence shows that miRNA can act as a key regulator to affect the development of skeletal muscle [3, 5]. For example, miR-206 is involved in skeletal muscle development, growth/adaptation, regeneration and muscle related diseases [6]. Chen et al. found that miRNA-1 and miRNA-133 participated in regulating skeletal muscle proliferation and differentiation [7]. Kong et al. found that miR-17 and MIR-19 cooperate to promote skeletal muscle cell differentiation [8]. Cheng believes that miR -223-3p promotes skeletal muscle regeneration by regulating inflammation in mice [9].

Although studies have shown that miRNA plays an important role in the regulation of skeletal muscle growth and development, there is no report on miRNA regulation of pigeon skeletal muscle growth and development. To explore the function of miRNA in the growth and development of pigeon skeletal muscle, this study will use next-generation sequencing technology to analyze the miRNA and mRNA expression in pigeon muscle growth and development, screen differentially expressed miRNA and mRNA, and construct miRNA-mRNA regulation networks based on expression data and miRNA mRNA interaction theory. Our findings will contribute to a better understanding of the role of miRNA in pigeon muscle development and growth.

2. Materials And Methods

2.1. Animal Ethics Statement

This study was performed following the Chinese guidelines for animal welfare, and the animal protocol was approved by the Animal Welfare Committee of Yangzhou University (permit number SYXK [Su] 2012–0029).

2.2. Preparation of Experimental Animals and Tissues

The pigeons used in this study were obtained from Wuxi Sanxiangan Agricultural Technology Development Co., Ltd. (Jiangsu, China). The eggs were hatched by conventional procedures. The breast muscle tissues were collected on 8 day (E8), 13 day (E13) of the embryo; the breast muscle tissues of the 1 day (D1) and 10 day old (D10) pigeon with the same feeding and management conditions were also collected. Each period contained three biological replicates. All tissue samples were flash-frozen in liquid nitrogen and stored at −80℃.

2.3. RNA sequencing

Total RNA was extracted using a Trizol reagent kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA quality was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and checked using RNase-free agarose gel electrophoresis. After total RNA was extracted, eukaryotic mRNA was enriched by Oligo(dT) beads, while prokaryotic mRNA was enriched by removing rRNA by Ribo-Zero™ Magnetic Kit (Epicentre, Madison, WI, USA). Then the enriched mRNA was fragmented into short fragments using fragmentation buffer and reverse transcripted into cDNA with random primers. Second-strand cDNA was synthesized by DNA polymerase I, RNase H, dNTP, and buffer. Then the cDNA fragments were purified with QiaQuick PCR extraction kit (Qiagen, Venlo, The Netherlands), end repaired, poly (A) added, and ligated to Illumina sequencing adapters. The ligation products were size selected by agarose gel electrophoresis, PCR amplified and sequenced using Illumina HiSeq2500 by Gene Denovo Biotechnology Co. (Guangzhou, China).

2.4. Small RNA Library construction and sequencing

After total RNA was extracted by Trizol reagent kit (Invitrogen, Carlsbad, CA, USA), the RNA molecules in a size range of 18–30nt were enriched by polyacrylamide gel electrophoresis (PAGE). Then the 3′ adapters were added and the 36-44nt RNAs were enriched. The 5′ adapters were then ligated to the RNAs as well. The ligation products were reverse transcribed by PCR amplification and the 140-160bp size PCR products were enriched to generate a cDNA library and sequenced using Illumina HiSeqTM 2500 by Gene Denovo Biotechnology Co. (Guangzhou, China).

2.5. miRNA Identification and Quantification

Clean tags were obtained by removing low-quality tags, tags containing adapters, tags shorter than 18 nt, and tags with polyA. Clean tags were aligned with the GenBank database (Release 209.0) [10]and Rfam database [11]to identify and remove rRNA, scRNA, snoRNA, snRNA and tRNA. The clean tags were also aligned with the *Columba livia* reference genome. Tags mapped to exons or introns were removed, which might be fragments from mRNA degradation. The tags mapped to repeat sequences were also removed. Then the retained tags were aligned with the miRbase database (Release 21) to identify known miRNAs. All of the unannotated tags were aligned with the Gallus gallus reference genome. Novel miRNA candidates were identified according to their genome positions and hairpin structures predicted by software Mireap_v0.2. Tags per million (TPM) algorithm was used to quantify the expression levels of miRNAs.

2.6. mRNA Identification and Quantification

High-quality clean reads were obtained by removing reads containing adapters, reads consisting of all A bases, reads containing more than 10% of unknown nucleotides (N), and reads containing more than 50% of low quality (Q-value ≤ 20) bases. rRNAs were removed by aligning high-quality clean reads with the ribosome RNA (rRNA) database. The rRNA removed reads of each sample were then mapped to the *Columba livia* reference genome by TopHat2 (version 2.1.1), respectively. Fragments per kilobase of transcript per million mapped reads (FPKM) algorithm was used to quantify the expression levels of mRNAs.

2.7. Identification of Differentially Expressed Genes

The edgeR software was used to identify differentially expressed mRNAs (DEMs), and miRNAs (DEmiRNAs). Genes with P-value < 0.05 and fold change ≥ 2 were considered as significantly differentially expressed genes (DEGs).

2.8. miRNA expression pattern clustering

Unsupervised hierarchical clustering and PCA (principal component analysis) were performed to explore the relationships between samples using R packages. Clustering of DEmiRNAs was perform using the Short Time-series Expression Miner software (STEM) [12] on the OmicShare tools platform, a free online platform for data analysis（www.omicshare.com/tools). The parameters were set as follows: Maximum Unit Change in model profiles between time points is 1, maximum output profiles number is 20 and Minimum ratio of the fold change of DEmiRNAs is no less than 2.0. Profile with a P-value < 0.05 was considered significant.

2.9. Construction of miRNA-mRNA network

First, we used RNAhybrid 2.1.2+svm_light 6.01, Miranda3.3a and TargetScan 7.0 software to predict the DEMis targets within DEGs. miRNA sequences and family information were obtained from the TargetScan website (http://www.targetscan.org/). Then, the expression correlation between miRNA and target was evaluated using the Pearson correlation coefficient (PCC). Pairs with PCC < -0.7 and P < 0.05 were selected as co-expressed negatively miRNA-mRNA pairs. The miRNA-mRNA network was visualized using Cytoscape software v 3.6.0 (http://www.cytoscape.org/). The connectivity degree of each node of the network was calculated. Hub miRNAs were identified by slelecting the top 10 nodes with the largest connectivity degree. The hub miRNAs were visualized using Cytoscape v 3.6.0 and Origin 2021b software.

2.10. Functional enrichment analysis

To understand the underlying function of the miRNA-mRNA network, the DEGs involved in the miRNA-mRNA network were performed to Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. GO or pathway terms with FDR < 0.05 were considered significant.

2.11. qRT-PCR confirmation of differentially expressed genes

MicroRNA was reverse-transcribed respectively using miRcute Plus miRNA First-Strand cDNA Kit (TIANGEN,Beijing,China) following the manufacturer's recommendations. The miRNA forward primers were obtained commercially from Tsingke Biotechnology (Beijing,China). U6 snRNA were simultaneously used as internal control genes (Table 1). ThemiRcute Plus miRNA qPCR Kit (TIANGEN, Beijing, China) was used to perform qRT-PCR procedure following the manufacturer's recommendations. The reaction mixtures were added in a 96-well plate at 95 °C for 15 min, followed by 40 cycles of 94°C for 20 s and 65 °C for 34 s, and then followed by a melting curve using an ABI 7500 Real-Time PCR System (Applied Biosystems, foster City, CA). All experiments were preformed in triplicates for each biological repea. Quantification of selected gene expression was performed using the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method. The quantitative real-time PCR (RT-qPCR) results for all genes were statistically tested using the Student's t-test.

Table 1. Primers for RT-qPCR.

Genes	Primer sequences
miR-26-x	CAAGTAATCCAGGATAGGCT
miR-27-y	TTCACAGTGGCTAAGTTCC
cli-miR-30c-5p	GTAAACATCCTACACTCTCAG
miR-130-y	CAGTGCAATAATGAAAGGG
U6	GATGACACGCAAATTCGTGAAG

3. Results

3.1. Analysis of miRNA and mRNA expression in pigeon breast muscle

A total of 32,527 mRNAs were identified in pigeon skeletal muscles, including 14,378 novel mRNAs and 18,149 known mRNAs. A total 2362 miRNAs were identified, including 1758 known miRNAs and 624 novel miRNAs. Subsequently, we conducted hierarchical clustering and PCA analysis based on miRNA and mRNA expression across all twelve samples. The results showed that the gene expression of pigeon skeletal muscle has a stage- and time-specific pattern. Sample from embryonic stages (E8 and E13) were closely clustered into a subgroup, and samples from 1 and 10 days after hatching were clustered into a single group. The results indicated that the gene expression pattern of pigeon skeletal muscle in embryonic stages was significantly different from that in growth stages, suggesting that the genetic regulation mechanism in the two stages was different.

3.2. Identification of differentially expressed miRNA and mRNA

A total of 839 DEmiRNAs were identified among the four groups (P-value < 0.05, FC > 2) (Figure 1C). E8-D10 and E13-D10 comparisons had the largest number of DEmiRNAs (E8-D10: 484, E13-D10: 461), while the D1-D10 comparison had the smallest number of DEmiRNAs (174) (Data S1). A total of 11311 mRNA（differentially expressed genes, DEGs） were identified among the four groups (FDR < 0.05, FC > 2) (Figure 1D). E8-D10 and E8-D1 comparisons had the largest number of DEGs (E8-D10: 8242, E13-D10: 6024), while the E13-D1 comparison had the smallest number of DEGs (1959) (Data S1). These results indicate that there are significant differences in the expression of miRNA and mRNA between developmental and growth stages of pigeon skeletal muscle, suggesting that the potential mechanism of miRNA and mRNA regulating pigeon skeletal muscle development and growth is stage specific.

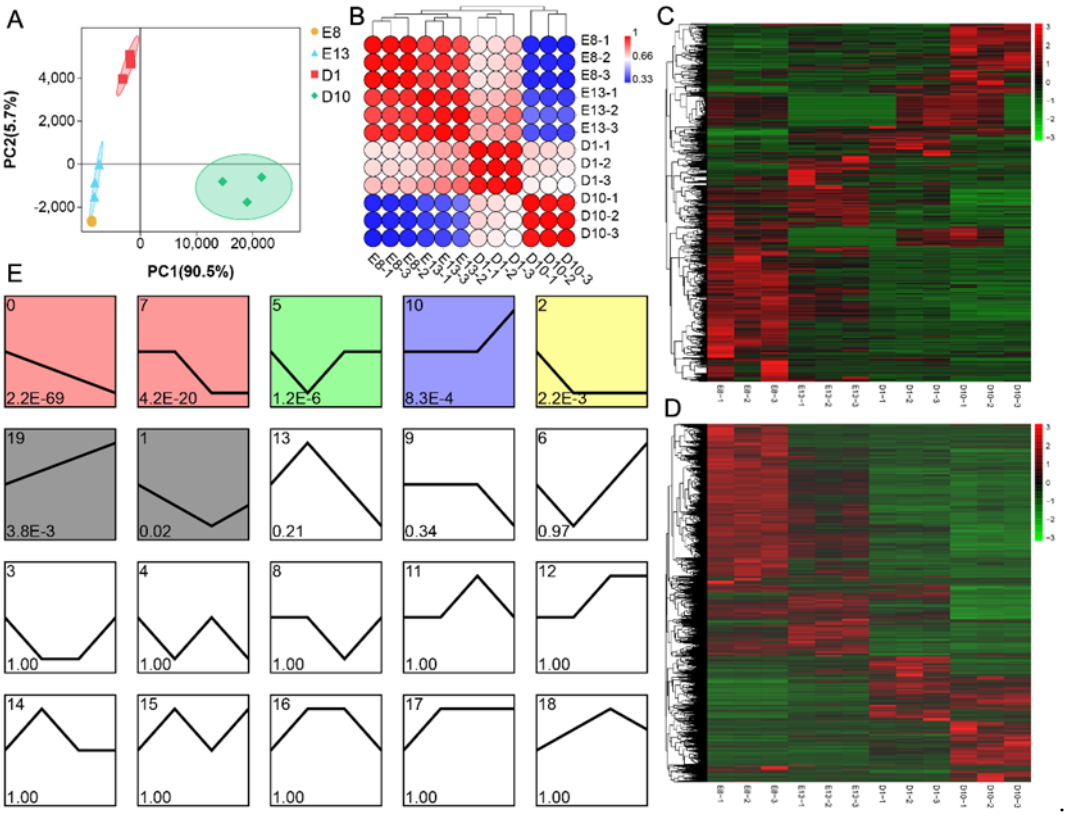

Figure 1. Expression patterns of miRNAs and mRNAs during pigeon skeletal muscle development and growth. (**A**)Principal component analysis (PCA) of the samples based on all miRNA and mRNA expression. (**B**) Correlation analysis of the samples based on all miRNA and mRNA expression. The

colors from blue to red represent a correlation from low to high. The Principal component and correlation analysis indicate distinct expression patterns of miRNA and mRNA at different stages. There were differences among the regulatory mechanisms of development and growth of skeletal muscle. (**C**) Heatmap of differentially expressed miRNA. (**D**) Heatmap of differentially expressed mRNA. Colors from green to red indicate expression level from low to high. (**E**) Expression profiles analysis of all miRNA by Short Time-series Expression Miner (STEM) program. A total of 20 profiles were clustered. Colored profiles were significant (P-value < 0.05).

3.3. miRNA expression profiles clustering

STEM algorithm was used to cluster the expression profile of DEmiRNAs in pigeon skeletal muscle growth and development[13]. STEM clustering tool assigned each miRNA to its time expression characteristics. A total of 20 expression profiles were generated, and seven were significantly enriched (P-value < 0.05) (Figure 1E). The seven significantly enriched profiles can be classified into three categories. The first category includes four profiles of profile 0, profile 1, profile 2, and profile 7. There are 143, 34, 55 and 90 DEmiRNAs in these four profiles, respectively. The first category represents DEmiRNAs that continuously down-regulated from the developmental stage to the growth stage of pigeon skeletal muscle. The second category includes profiles 10 and 19, and represents DEmiRNAs with low expression at the development and early growth stage and significantly up-regulated expression at the high growth stage. profiles 10 and 19 contain 57 and 36 DEmiRNAs, respectively. Profile 5 is the third category, which represents DEmiRNAs with low expression on the 13th day of development, including 128 DEmiRNAs.

3.4. Target gene identification of DEmiRNAs in significantly enriched profiles

In this study, We mainly focused on the dynamic expression of miRNA during the growth and development of pigeon skeletal muscle and explored miRNAs regulating the growth and development of pigeon skeletal muscle. Therefore, We mainly take DEmiRNAs in the first and second category of STEM cluster profiles as the research objects. The DEmiRNAs in the first category are highly expressed in the embryonic development stage, and the DEmiRNAs in the second category are highly expressed in the high growth period, indicating that these two categories of DEmiRNAs may play regulatory roles in the development and growth stages of pigeon skeletal muscle, respectively.

We predicted the target relationship between DEGs and DEmiRNAs in the first and second categories and analyze the expression correlation between them. Based on target relationship prediction and expression correlation analysis (PCC < -0.7 and P-value < 0.05), 1289 target genes were identified for 322 DEmiRNAs in the first category, forming13569 miRNA-mRNA pairs. A total of 3,310 target genes were identified for 93 DEmiRNAs in the second category, forming 20149 miRNA-mRNA pairs.

3.5. Construction of the miRNA-mRNA regulatory network

To further explore key miRNAs that regulate the growth and development of pigeon skeletal muscle, we constructed the miRNA-mRNA regulatory network based on the results of target relationship analysis of DEmiRNAs and DEGs. miRNA-mRNA pairs with PCC < - 0.9 were visualized by Cytoscape software. Two miRNA-mRNA regulatory networks were constructed. Network A shows the regulatory relationships between DEmiRNAs belonging to the first category and DEGs, including 1379 miRNA-mRNA pairs, composed of 128 DEmiRNAs and 294 DEGs (Figure 2A). Network B shows the regulatory relationship between DEmiRNAs belonging to the second category and DEGs, including 2666 miRNA-mRNA pairs, composed of 67 DEmiRNAs and 1108 DEGs (Figure 2B).

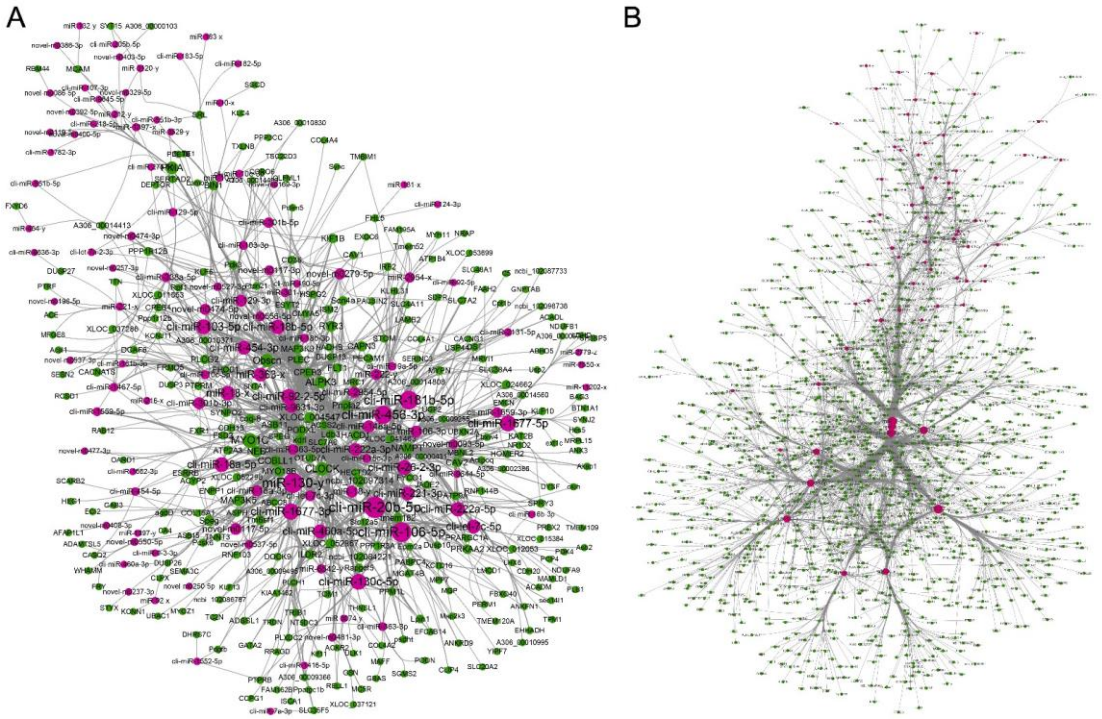

Figure 2. Visualization of the miRNA-mRNA regulatory networks. (**A**) Visualization of the regulatory network of DE miRNA and DEGs in profiles 0, 1, 2 and 7. (**B**) Visualization of the regulatory network of DE miRNA and DEGs in profiles 10 and 19. The red node represents miRNA, the green node represents mRNA, and the size of the node represents the degree of degree.

3.6. Hub miRNA identification

We used Cytoscape software to calculate the connectivity degree of nodes in the miRNA-mRNA network, and miRNAs with high connectivity degrees are considered to play important roles in the network. In this study, miRNAs with degree value ranking top 10 were considered as the hub genes. Hub miRNAs of network A are cli-miR-20b-5p、miR-130-y、cli-miR-106-5p、cli-miR-181b-5p、cli-miR-456-3p、cli-miR-1677-3p、cli-miR-1677-5p、cli-miR-130c-5p、cli-miR-103-5p and cli-miR-18a-5p. Hub miRNAs of network B are miR-27-y、miR-1-z、cli-miR-1a-3p、miR-23-y、cli-miR-30d-5p、miR-1-y、miR-133-y、miR-26-x、cli-miR-30c-5p and miR-101-y（Table 2）.Then the subnetwork containing hub miRNAs and their target mRNAs were visualized using Cytoscape software. The expression patterns of these hub miRNAs during the development and growth of pigeon skeletal muscle were also analyzed. The results showed that the average degree of hub miRNAs in network A was 44, and cli-miR-20b-5p had the highest connectivity degree (60). All the ten hub miRNAs were highly expressed during embryonic development and significantly downregulated during the growth stage of pigeon skeletal muscle (Figure 3). The average degree of hub miRNAs in network B was 192, and miR-27-y had the highest degree (259). All the ten hub miRNAs were significantly up-regulated during the skeletal muscle growth stage (Figure 4).

Table 2. Hub miRNAs related to the growth and development of pigeon skeletal muscle.

Network	Hub miRNA	Degree
Network A	cli-miR-20b-5p	60
	miR-130-y	53
	cli-miR-106-5p	53
	cli-miR-181b-5p	48
	cli-miR-456-3p	46

	cli-miR-1677-3p	39
	cli-miR-1677-5p	37
	cli-miR-130c-5p	37
	cli-miR-103-5p	34
	cli-miR-18a-5p	33
Network B	miR-27-y	259
	miR-1-z	254
	cli-miR-1a-3p	217
	miR-23-y	210
	cli-miR-30d-5p	192
	miR-1-y	191
	miR-133-y	184
	miR-26-x	154
	cli-miR-30c-5p	150
	miR-101-y	110

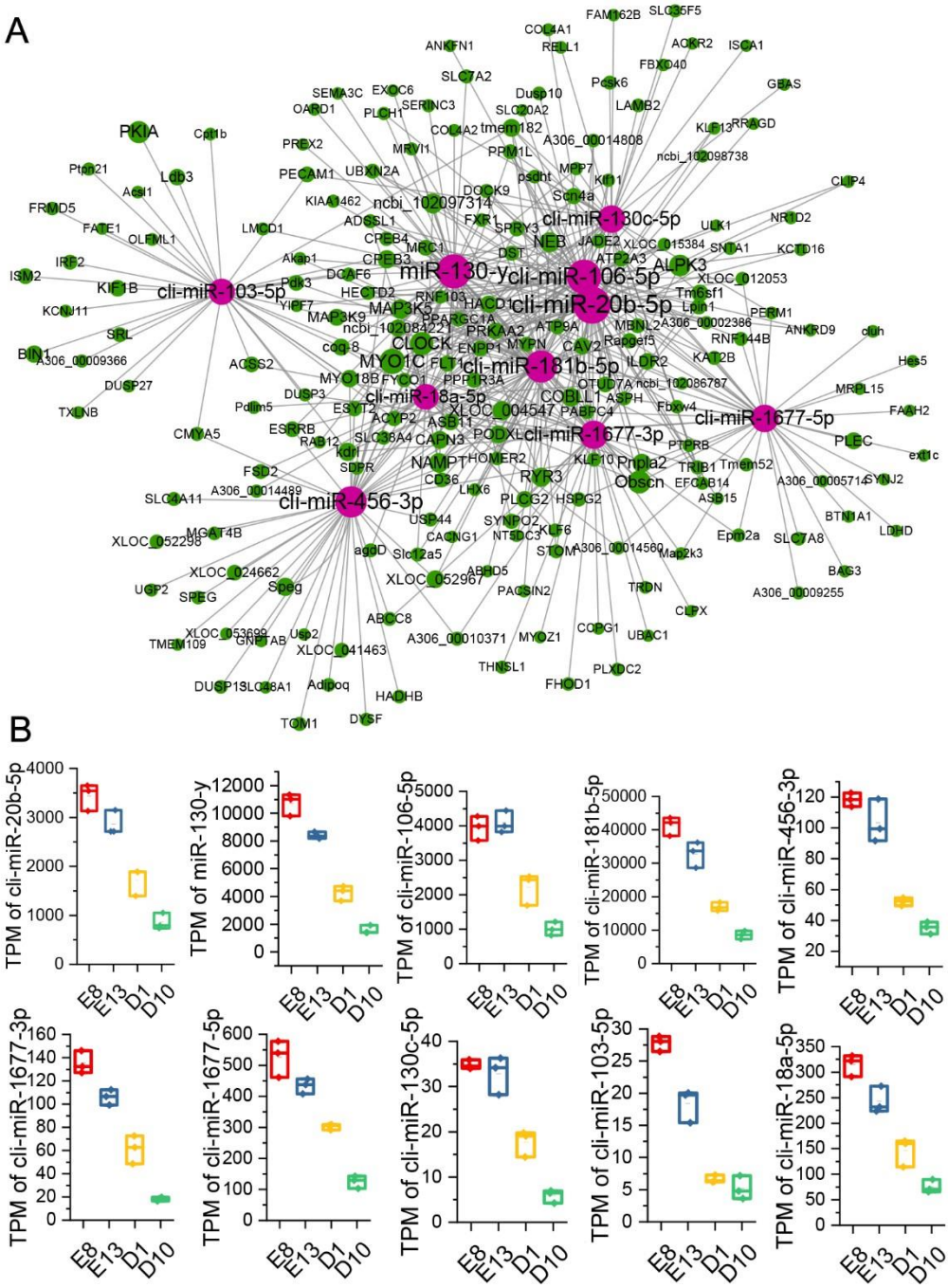

Figure 3. Identification and visualization of hub miRNAs in Network A. (**A**) The subnetwork containing hub miRNAs. The red node represents miRNA, the green node represents mRNA, and the size of the node represents connectivity degree, respectively. (**B**) The expression patterns of the ten hub miRNAs during the growth and development of pigeon skeletal muscle. All the ten hub miRNAs are highly expressed during the embryonic development stage, and their expression is significantly down-regulated in the growth stage.

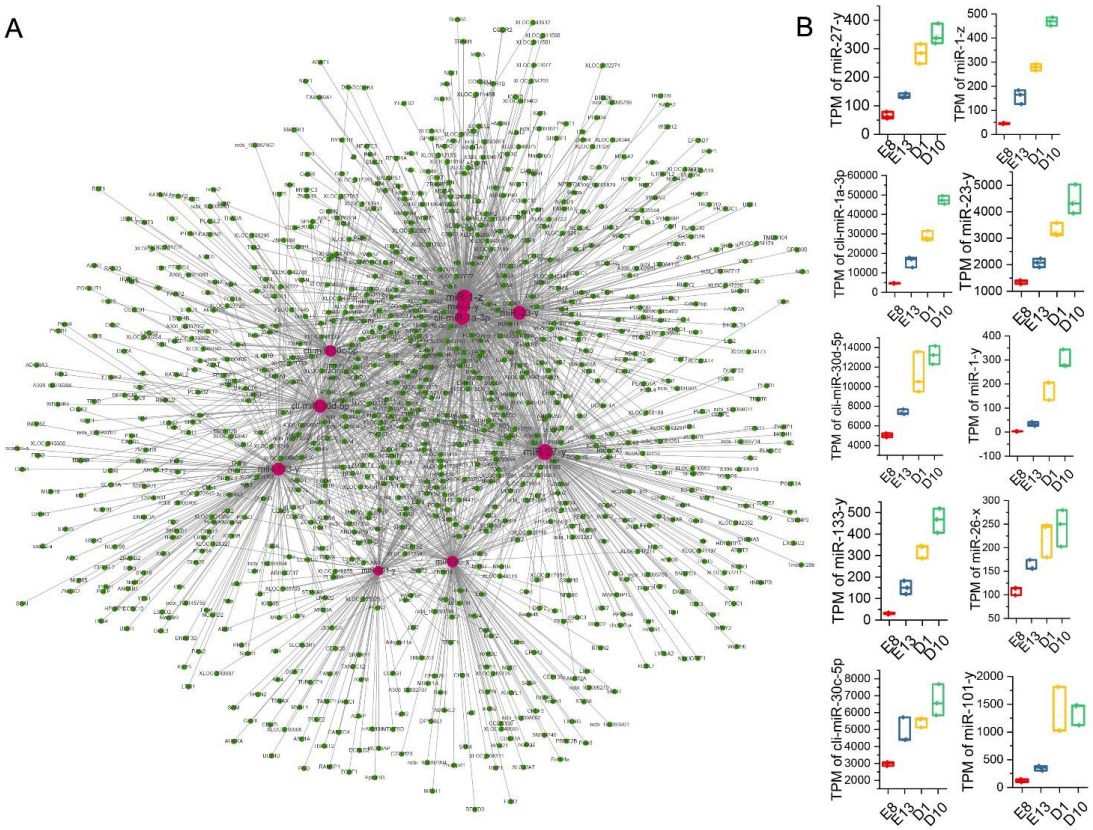

Figure 4. Identification and visualization of hub miRNAs in Network B. (**A**) The subnetwork containing hub miRNA. The red node represents miRNA, the green node represents mRNA, and the size of the node represents degree, respectively. (**B**) The expression patterns of the ten hub miRNAs during the growth and development of pigeon skeletal muscle. The expression of all the ten hub miRNAs was significantlyup-regulated during the growth stage.

3.7. Functional enrichment analysis of target genes

To further explore the possible molecular mechanism by which miRNA regulating the growth and development of pigeon skeletal muscle, we performed GO and KEGG enrichment analysis on target mRNAs of miRNAs in the first and second categories of profiles (Figure 5). GO enrichment analysis showed that target genes of miRNAs in the first category of profiles are significantly enriched in 47 biological processes (FDR < 0.05), including biological processes such as striated muscle cell differentiation, striated muscle cell development and muscle cell development. Target genes of miRNAs in the second category of profiles were significantly enriched in 242 biological processes (FDR < 0.05), including regulation of biological process, regulation of cellular process and tissue morphogenesis biological processes. KEGG pathway enrichment showed that target genes of miRNAs in the first category of profiles are significantly enriched in 29 signal pathways (FDR < 0.05), including signal pathways such as ECM-receptor interaction, focal adhesion and biosynthesis of amino acids. Target genes of miRNAs in the second category of profiles were significantly enriched in four signal pathways (FDR < 0.05), including signal pathways such ascell growth and death, signal transduction and development.

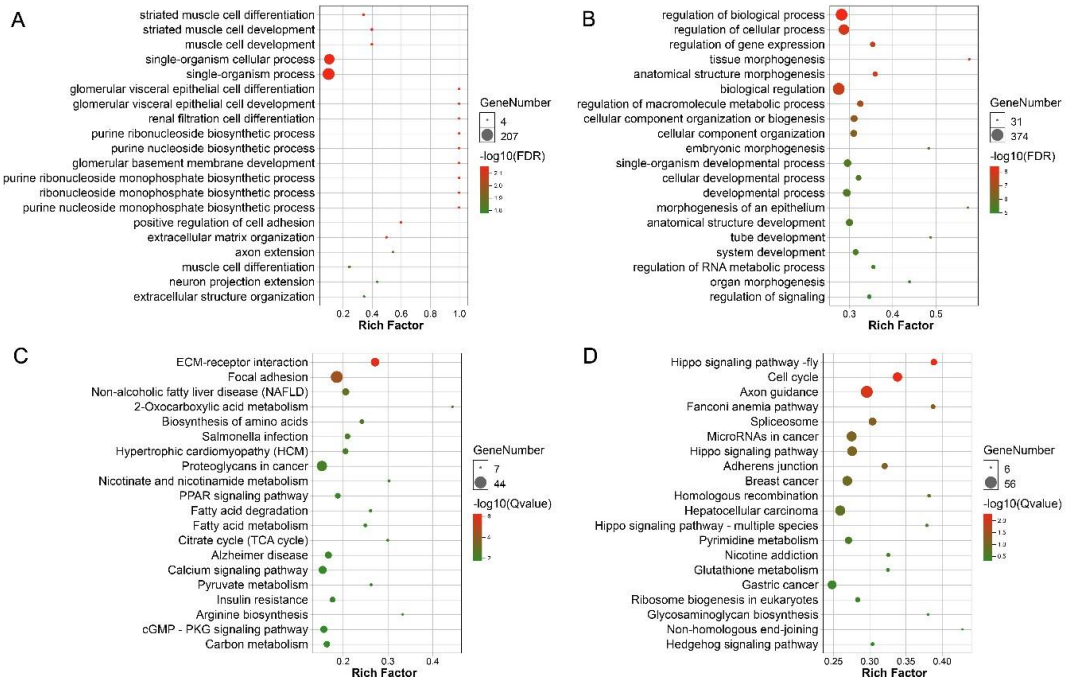

Figure 5. GO and KEGG enrichment of target genes. (A) The GO enrichment analysis of target genes of miRNAs in the first category of profiles. (B) The GO enrichment analysis of target genes of miRNAs in the second category of profiles. (C) The KEGG pathway enrichment analysis of target genes of miRNAs in the first category of profiles. (D) The KEGG pathway enrichment analysis of target genes of miRNAs in the second category of profiles.

3.8. qRT-PCR

To confirm the differentially expressed genes between embryonic and growth groups obtained by RNA-Seq, four differential expressed genes (miR-130-y, miR-27-y,miR-26-x ,and cli-miR-30c-5p) in the network were selected and their expression patterns were quantified by qRTPCR. The results were in line with our sequencing result, which highlighted the reliability of our sequencing data (Figure 6).

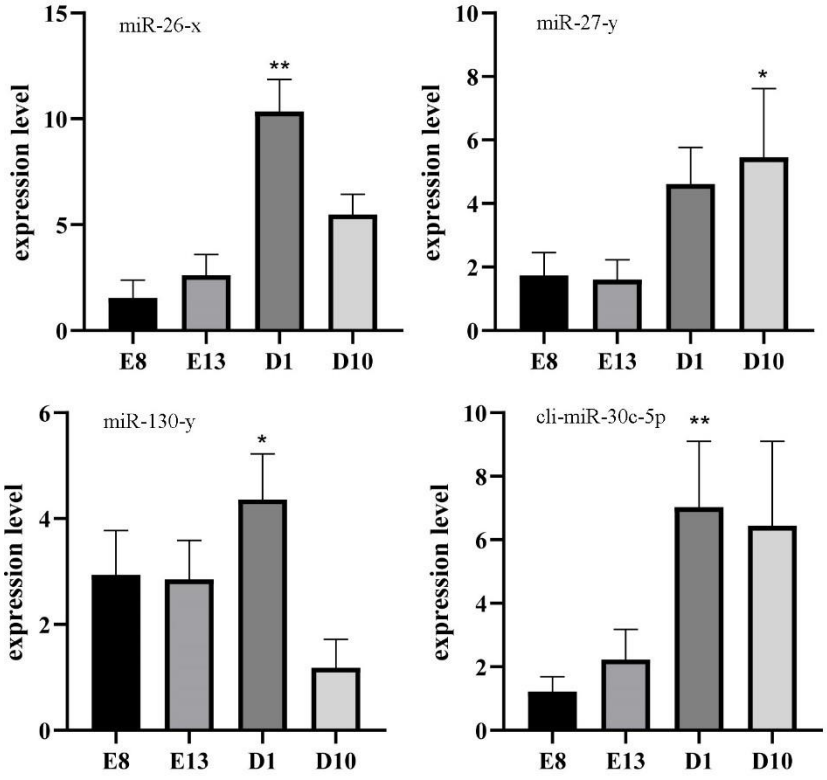

Figure 6. Validation of hub genes using RT-qPCR.

4. Discussion

Meat performance is one of the important indicators to measure the economic value of pigeons. The growth and development of skeletal muscle determine the meat performance of pigeons [14]. To elucidate the molecular regulation mechanism of pigeon skeletal muscle growth and development is an important prerequisite for using molecular breeding technology to improve pigeon meat production performance. However, compared with other poultry, the molecular mechanism of genetic regulation of pigeon skeletal muscle growth and development remains unclear, especially in miRNA regulation. In the present study, to screen miRNAs that regulate pigeon skeletal muscle growth and development and explore its possible molecular mechanism, next-generation sequencing technology was used to characterize the dynamic expression of miRNA and mRNA during the development and growth of pigeon skeletal muscle. To the best of our knowledge, this study presents the first integrated analysis of miRNA and mRNA expression in pigeon skeletal muscle. Based on the expression of miRNA and mRNA, hierarchical cluster analysis was performed on samples from different periods. The results showed that the samples from the embryonic stage (E8 and E13) were clustered into a subgroup, and there were significant differences in gene expression between the samples from the embryonic and growth stages. PCA analysis further showed a close distance between samples from the embryonic stage and a far distance between samples from the embryonic stage and the growth stage. These results suggest different regulation mechanisms of pigeon skeletal muscle between growth and embryonic development stages.

STEM algorithm is widely used to cluster the expression data sampled in time sequence and analyze its expression pattern[12, 13].We performed cluster analysis on DEmiRNAs using the STEM algorithm. Two categories of significantly enriched profiles were identified. The first category included a total of 322 DEmiRNAs, which were highly expressed during embryonic development and significantly down-regulated during the growth stage. It suggested that these DEmiRNAs may play important regulatory roles in pigeon skeletal muscle embryonic development. The second category included a total of

93 DEmiRNAs, which were lowly expression during the embryonic stage and significantly up-regulated during the growth stage, suggesting that these DEmiRNAs may play important regulatory roles in pigeon skeletal muscle growth.

To further screen candidate miRNAs that regulate pigeon skeletal muscle growth and development, we constructed two miRNA-mRNA regulatory networks based on the expression correlation and target relationship prediction analysis of DEmiRNAs and DEGs. Network A included DEmiRNAs in the first category of profiles and network B included DEmiRNAs in the second category of profiles. According to the connectivity degree, 20 candidate miRNAs were identified, including miR-130-y, cli-miR-106-5p, cli-miR-181b-5p, cli-miR-456-3p, miR-1-z, cli-miR-1a-3p, miR-23-y, cli-miR-30d-5p etc. A number of miRNAs are known to be involved in the regulation of muscle growth and development. Luo found that miR-20b-5p promotes muscle cell differentiation and inhibits muscle cell proliferation by directly binding to the 3'UTR of E2F transcription factor 1 (E2F1) mRNA [15].Zhao et al. found that miR-181b-5p can promote skeletal muscle growth by reducing the MSTNb protein level of tilapia [16]. Guanidinoacetic acid (GAA) supplements can promote muscle cell differentiation and skeletal muscle growth through the activation of the AKT/mTOR/S6K signaling pathway induced by miR-133a-3p and miR-1a-3p [17]. And miR-30c-5p is related to the treatment of skeletal fibroids with myocardial injury [18], Postmenopausal osteoporosis (PMOP) is a metabolic bone disease caused by unbalance between osteoblast bone formation and osteoclast bone resorption, and DGCR5 up-regulates Runx2 through miR-30d-5p to induce osteogenic differentiation, which contributes to ameliorate PMOP, further affect the growth of skeletal muscle [19]. miR-18a-5p has also been confirmed to promote smooth muscle cell proliferation and migration by activating the AKT/Extracellular Regulated Protein Kinases (ERK) signaling pathway [20]. The above miRNA were identified as hub miRNAs in our study, suggesting their potential role in the growth and development of pigeon skeletal muscle. In addition, the functions of cli-miR-130-y、cli-miR-1677-3p、cli-miR-1677-5p、cli-miR-130c-5p、cli-miR-103-5p、miR-27-y、miR-1-z、miR-23-y、cli- miR-1-y、miR-133-y、miR-26-x and miR-101-y in muscle growth and development have not been reported.

To explore the potential mechanism of miRNA regulating the growth and development of pigeon skeletal muscle, we performed go and KEGG enrichment analysis of mRNA in network A and network B, respectively. GO enrichment analysis showed that target genes in network A were significantly enriched in biological processes related to skeletal muscle cell differentiation and development, such as striated muscle cell differentiation, striated muscle cell development, and muscle cell development. It indicates that miRNAs in network A may play important regulatory roles in the differentiation and development of pigeon skeletal muscle cells. Target genes in network B are significantly enriched in biological processes such as regulation of biological process, regulation of cellular process and tissue morphogenesis, indicating that the above biological processes may be related to the growth of pigeon skeletal muscle. We also found that target genes in Network A and Network B were enriched in different biological processes, which indicating different mechanisms by which miRNAs regulate embryonic development and growth of pigeon skeletal muscle.

KEGG is a database for systematic analysis of gene function and genome information, which can be used as a whole network to study gene and expression information [21]. Pigeon muscle growth is a complex process influenced by multiple genes and controlled by multiple pathways. Many of the pathways enriched by mRNA in network A are related to muscle growth and development. For example, mRNAs in network A were enriched in ECM receptor interaction, focal adhesion and biosynthesis of amino acids pathways. ECM-receptor interaction and Focal adhesion pathways are frequently found in different developmental stages of chicken breast muscle [22, 23],the content and synthesis of amino acid also affect the development of skeletal muscle [24]. Focal adhesions are large-scale protein complexes on the surface of cell substrates. It physically connects the extracellular matrix with the cytoskeleton. It has long been speculated to mediate cell migration [25].

Proximal multiplier activated receptors (PPARs) regulates genes involved in development, metabolism, inflammation and many cellular processes in different organs. PPAR is also present in muscles and exerts a pectoral muscle specific response when activated by its ligand [26]. Ca2+ itself or Ca2+-dependent signaling pathways play fundamental roles in various cellular processes from cell growth to death, The most representative example can be found in skeletal muscle cells [27].

Similarly, mRNAs in network B is also enriched in pathways related to muscle growth and development. For example, muscle growth is regulated by signal transduction pathways that sense and compute local and systemic signals and regulate various cellular functions [28]. Hippo signaling pathway is an important mediator for tissue growth of some epithelial cell types. Study has showed that hippo signaling pathway serves as a key element in muscle fibers and muscle satellite cells [29] and is required for early muscle development [30]. Ribosome biogenesis in eukaryotes has become an important regulator of skeletal muscle growth and maintenance [31]. Our findings suggested the potential roles of the above pathways in regulating pigeon skeletal muscle development and growth, however, their are still need to be further studied. Besides, target mRNAs in network A and B are significantly enriched in different pathways, which again indicates that there are differences in the potential mechanisms of miRNA to regulate skeletal muscle growth and embryonic development.

5. Conclusions

Our research results identified a total of 32,527 mRNAs were identified in pigeon skeletal muscles. A total 2362 miRNAs were identified. STEM clustering tool assigned each miRNA to its time expression characteristics. A total of 20 expression profiles were generated, and seven were significantly enriched (P-value < 0.05), there are two types. The first category represents DEmiRNAs that continuously down-regulated from the developmental stage to the growth stage of pigeon skeletal muscle, including cli-miR-20b-5p、miR-130-y 、cli-miR-106-5p 、cli-miR-181b-5p etc. The second category represents DEmiRNAs with low expression at the development and early growth stage and significantly up-regulated expression at the high growth stage,including miR-27-y、miR-1-z、cli-miR-1a-3p、miR-23-y etc. This study expands the diversity of pigeon miRNAs and helps to understand the role of miRNAs in pigeon muscle development and growth.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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References

1. Guo, L.; Huang, W.; Chen, B.; Jebessa Bekele, E.; Chen, X.; Cai, B.; Nie, Q. gga-mir-133a-3p Regulates Myoblasts Proliferation and Differentiation by Targeting PRRX1. **Front Genet 2018**, 9**:** 577.
2. Xu, K.; Han, C.X.; Zhou, H.; Ding, J.M.; Xu, Z.; Yang, L.Y.; He, C.; Akinyemi, F.; Zheng, Y.M.; Qin, C., *et al.* Effective MSTN Gene Knockout by AdV-Delivered CRISPR/Cas9 in Postnatal Chick Leg Muscle. **Int J Mol Sci 2020**, 21(7).
3. Bartel, D.P. MicroRNAs: target recognition and regulatory functions. **Cell 2009**, 136(2)**:** 215-233.
4. Xia, W.; Zou, C.; Chen, H.; Xie, C.; Hou, M. Immune checkpoint inhibitor induces cardiac injury through polarizing macrophages via modulating microRNA-34a/Kruppel-like factor 4 signaling. **Cell Death Dis 2020**, 11(7)**:** 575.
5. Bushati, N.; Cohen, S.M. microRNA functions. **Annu Rev Cell Dev Biol 2007**, 23**:** 175-205.
6. Ma, G.; Wang, Y.; Li, Y.; Cui, L.; Zhao, Y.; Zhao, B.; Li, K. MiR-206, a key modulator of skeletal muscle development and disease. **Int J Biol Sci 2015**, 11(3)**:** 345-352.
7. Chen, J.F.; Mandel, E.M.; Thomson, J.M.; Wu, Q.; Callis, T.E.; Hammond, S.M.; Conlon, F.L.; Wang, D.Z. The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. **Nat Genet 2006**, 38(2)**:** 228-233.
8. Kong, D.; He, M.; Yang, L.; Zhou, R.; Yan, Y.Q.; Liang, Y.; Teng, C.B. MiR-17 and miR-19 cooperatively promote skeletal muscle cell differentiation. **Cell Mol Life Sci 2019**, 76(24)**:** 5041-5054.
9. Cheng, N.; Liu, C.; Li, Y.; Gao, S.; Han, Y.C.; Wang, X.; Du, J.; Zhang, C. MicroRNA-223-3p promotes skeletal muscle regeneration by regulating inflammation in mice. **J Biol Chem 2020**, 295(30)**:** 10212-10223.
10. Clark, K.; Karsch-Mizrachi, I.; Lipman, D.J.; Ostell, J.; Sayers, E.W. GenBank. **Nucleic Acids Res 2016**, 44(D1)**:** D67-72.
11. Griffiths-Jones, S.; Bateman, A.; Marshall, M.; Khanna, A.; Eddy, S.R. Rfam: an RNA family database. **Nucleic Acids Res 2003**, 31(1)**:** 439-441.
12. Ernst, J.; Bar-Joseph, Z. STEM: a tool for the analysis of short time series gene expression data. **BMC Bioinformatics 2006**, 7**:** 191.
13. Wang, X.; Yan, P.; Liu, L.; Luo, Y.; Zhao, L.; Liu, H.; Tang, Q.; Long, K.; Jin, L.; Ma, J., *et al.* MicroRNA expression profiling reveals potential roles for microRNA in the liver during pigeon (Columba livia) development. **Poult Sci 2020**, 99(12)**:** 6378-6389.
14. Wang, Y.; Cai, H.; Yan, H.; Chen, X.; Shu, Z.; Zhang, A.; Yang, B.; Guo, J. Research on skeletal muscle growth and development rules in poultry and its manipulation. **Feed Industry 2014**.
15. Luo, W.; Li, G.; Yi, Z.; Nie, Q.; Zhang, X. E2F1-miR-20a-5p/20b-5p auto-regulatory feedback loop involved in myoblast proliferation and differentiation. **Sci Rep 2016**, 6**:** 27904.
16. Zhao, Z.; Yu, X.; Jia, J.; Yang, G.; Sun, C.; Li, W. miR-181b-5p May Regulate Muscle Growth in Tilapia by Targeting Myostatin b. **Front Endocrinol (Lausanne) 2019**, 10**:** 812.
17. Wang, Y.; Ma, J.; Qiu, W.; Zhang, J.; Feng, S.; Zhou, X.; Wang, X.; Jin, L.; Long, K.; Liu, L., *et al.* Guanidinoacetic Acid Regulates Myogenic Differentiation and Muscle Growth Through miR-133a-3p and miR-1a-3p Co-mediated Akt/mTOR/S6K Signaling Pathway. **Int J Mol Sci 2018**, 19(9).
18. Liu, Q.; Du, G.Q.; Zhu, Z.T.; Zhang, C.; Sun, X.W.; Liu, J.J.; Li, X.; Wang, Y.S.; Du, W.J. Identification of apoptosis-related microRNAs and their target genes in myocardial infarction post-transplantation with skeletal myoblasts. **J Transl Med 2015**, 13**:** 270.
19. Wu, Z.H.; Huang, K.H.; Liu, K.; Wang, G.T.; Sun, Q. DGCR5 induces osteogenic differentiation by up-regulating Runx2 through miR-30d-5p. **Biochem Biophys Res Commun 2018**, 505(2)**:** 426-431.
20. Zhang, Y.; Chen, X. miR-18a-5p Promotes Proliferation and Migration of Vascular Smooth Muscle Cells by Activating the AKT/Extracellular Regulated Protein Kinases (ERK) Signaling Pathway. **Med Sci Monit 2020**, 26**:** e924625.
21. Kanehisa, M.; Goto, S. KEGG: kyoto encyclopedia of genes and genomes. **Nucleic Acids Res 2000**, 28(1)**:** 27-30.
22. Li, Y.; Chen, Y.; Jin, W.; Fu, S.; Li, D.; Zhang, Y.; Sun, G.; Jiang, R.; Han, R.; Li, Z., *et al.* Analyses of MicroRNA and mRNA Expression Profiles Reveal the Crucial Interaction Networks and Pathways for Regulation of Chicken Breast Muscle Development. **Front Genet 2019**, 10**:** 197.
23. Xue, Q.; Zhang, G.; Li, T.; Ling, J.; Zhang, X.; Wang, J. Transcriptomic profile of leg muscle during early growth in chicken. **PLoS One 2017**, 12(3)**:** e0173824.
24. Wang, Y.; Zhao, H.; Fei, D.; Shao, Y.; Liu, J.; Jiang, G.; Xing, M. Discrepant effects of copper (II) stress on different types of skeletal muscles in chicken: Elements and amino acids. **Ecotoxicol Environ Saf 2019**, 167**:** 227-235.
25. Kim, D.H.; Wirtz, D. Focal adhesion size uniquely predicts cell migration. **Faseb j 2013**, 27(4)**:** 1351-1361.
26. Manickam, R.; Duszka, K.; Wahli, W. PPARs and Microbiota in Skeletal Muscle Health and Wasting. **Int J Mol Sci 2020**, 21(21).
27. Choi, J.H.; Jeong, S.Y.; Oh, M.R.; Allen, P.D.; Lee, E.H. TRPCs: Influential Mediators in Skeletal Muscle. **Cells 2020**, 9(4).
28. Wackerhage, H.; Ratkevicius, A. Signal transduction pathways that regulate muscle growth. **Essays Biochem 2008**, 44**:** 99-108.
29. Watt, K.I.; Goodman, C.A.; Hornberger, T.A.; Gregorevic, P. The Hippo Signaling Pathway in the Regulation of Skeletal Muscle Mass and Function. **Exerc Sport Sci Rev 2018**, 46(2)**:** 92-96.
30. Peterson, M.T.; Henry, C.A. Hedgehog signaling and laminin play unique and synergistic roles in muscle development. **Dev Dyn 2010**, 239(3)**:** 905-913.
31. Figueiredo, V.C.; McCarthy, J.J. Regulation of Ribosome Biogenesis in Skeletal Muscle Hypertrophy. **Physiology (Bethesda) 2019**, 34(1)**:** 30-42.