Dissection of Myogenic Differentiation Signatures in Chickens by RNA-Seq Analysis

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Abstract: A series of elaborately regulated and orchestrated changes in gene expression profiles leads to muscle growth and development. In this study, RNA sequencing was used to profile embryonic chicken myoblasts and fused myotube transcriptomes, long non-coding RNAs (lncRNAs), and messenger RNAs (mRNAs) at four stages of myoblast differentiation. Of a total of 2484 lncRNA transcripts, 2288 were long intergenic non-coding RNAs (lincRNAs) and 198 were antisense lncRNAs. Additionally, 1530 lncRNAs were neighboring 2041 protein-coding genes (<10 kb upstream and downstream) and functionally enriched in several pathways related to skeletal muscle development that have been extensively studied, indicating that these genes may be in cis-regulatory relationships. In addition, Pearson’s correlation coefficients demonstrated that 990 lncRNAs and 7436 mRNAs were possibly in trans-regulatory relationships. These co-expressed mRNAs were enriched in various developmentally-related biological processes, such as myocyte proliferation and differentiation, myoblast differentiation, and myoblast fusion. The number of transcripts (906 lncRNAs and 4422 mRNAs) differentially expressed across various stages declined with the progression of differentiation. Then, 4422 differentially expressed genes were assigned to four clusters according to K-means analysis. Genes in the K1 cluster likely play important roles in myoblast proliferation and those in the K4 cluster were likely associated with the initiation of myoblast differentiation, while genes in the K2 and K3 clusters were likely related to myoblast fusion. This study provides a catalog of chicken lncRNAs and mRNAs for further experimental investigations and facilitates a better understanding of skeletal muscle development.

Keywords: RNA sequencing; lncRNA; mRNA; myoblasts; chicken

1. Introduction

Skeletal muscle, which accounts for approximately 40% of the body weight of mammals, is an important tissue involved in the regulation of metabolism, locomotion, and strength [1]. A loss or reduction in skeletal muscle mass results in weakness and impaired mobility and, if severe enough, in increased morbidity and mortality [2,3]. In livestock production, skeletal muscle develops into meat, which is the primary terminal product for human consumption. Therefore, the study of muscle development in agriculturally-important species is essential to achievements of increased body weight and muscle mass.

The process of muscle fiber development is nearly completed during the prenatal stage. Some reports suggest that animals with greater number of muscle fibers of moderate size produce a higher quantity of meat [4,5]. The development of skeletal muscle depends on myogenesis, which is a multistep process starting with myoblast proliferation, followed by their exit from cell cycle,
differentiation, alignment, and fusion to form multinucleated myotubes that will further differentiate into mature fibers. During myogenesis, the degree of myoblasts proliferation and differentiation largely determines how many muscle fibers are formed. Therefore, understanding the events occurring during myogenesis is essential to achieve a higher quantity of meat. So far, the genetic factors influencing this process have been extensively studied using skeletal muscle myoblasts as model system because of their capable proliferation and differentiation (for example, see [6,7] among many others). However, the mechanisms underlying myogenesis are still sophisticated, especially as noncoding RNAs are emerging as integral components of the gene regulatory network in myogenesis.

Long non-coding RNAs (lncRNA), which are transcripts normally longer than 200 bases without evident protein coding regions, have recently emerged as important novel regulators of skeletal muscle development. With the development of RNA sequencing technology, several lncRNAs in different species and tissues have been identified and characterized [8–10]. In chickens, Li and Liu have identified 1995 and 2597 lncRNA transcripts, and their work largely enriched chicken lncRNA annotation [11,12]. A series of lncRNAs, such as linc-MD1, linc-Six1, linc-H19, linc-133b, and linc-Y1Y1, have been proven to have an important impact on skeletal myogenesis [13–17]. Although many lncRNAs have been identified in chickens, a large number remains undiscovered. The profiles of lncRNAs obtained from chicken myoblasts and fused myotubes have not been reported, and lncRNAs involved in skeletal myogenesis are not yet elucidated.

The use of chicken embryonic myoblasts is a good model system [6,18] to understand how the genetic basis of myoblasts determines the outcome of the fate of myocytes and eventual muscle formation. Here, the expression profiles of lncRNAs and messenger RNAs (mRNAs) obtained from myoblasts and fused myotubes were investigated using RNA sequencing (RNA-seq) analysis. The features of the lncRNAs and mRNAs were characterized, and sets of differentially-expressed lncRNAs and mRNAs were identified by comparing the profiles of myoblasts at different stages of differentiation. The functions of differentially-expressed genes (DEGs) were annotated and the involved pathways were enriched. This investigation provides not only precious resources for chicken lncRNA studies, but also an approach to gain further insight into the complex biological processes to achieve a better understanding of the biology of myogenesis.

2. Materials and Methods

2.1. Ethics Statement

The study protocol was approved by the Animal Care Committee of the Department of Animal Science and Technology of Yangzhou University, Yangzhou, China (permit number: SYXK (Su) 2012-0029) and conducted in accordance with the guidelines of the Animal Use Committee of the Chinese Ministry of Agriculture (Beijing, China). All efforts were made to minimize animal suffering.

2.2. Chicken Primary Myoblast Isolation and Culture

Fertilized chicken eggs were obtained from Jiangsu Jinghai Livestock Industry Group (Haimen, China) and incubated at 37.8 °C under a relative humidity of 55–60%. The breast muscles of 12-day-old chicken embryos were collected, washed three times with Hank’s balanced salt solution (Solarbio, Beijing, China) formulated without magnesium and calcium, and then minced and digested with 0.2% collagenase type I (Sangon, Shanghai, China) in a water bath at 37 °C for 15 min. The digested cells were then dispersed by pipette and filtered to remove large debris using 200-, 400-, and 600-mesh screens (Solarbio). Cell were then collected by centrifugation at 350 × g and resuspended in growth medium (GM), consisting of Dulbecco’s modified Eagle’s medium/F12 (1:1) (Sangon), supplemented with 20% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), and 1% penicillin/streptomycin (Sangon). Enrichment of myoblasts cell population was accomplished by pre-plating the cells at 37 °C for 40 min on non-coated dish (Corning, New York, NY, USA) to remove the fibroblastic cells. This process was repeated two more times. To ensure uniformity of the samples, the purified
myoblasts were mixed and seeded in twelve 60-mm dishes (Corning) at a density of $1 \times 10^5$ cells/cm$^2$. The culture dishes were incubated at 37 °C in a humidified atmosphere with 5% CO$_2$ (Incubator: Binder, Tuttlingen, Germany). Myoblast differentiation was induced by replacing the 20% fetal bovine serum with 2% horse serum (Invitrogen). Cells were collected from the GM (100% confluency) and differentiation medium at 0, 24, 72, and 120 h (referred to as A0, A1, A3, and A5, respectively). Three biological replicates were collected at each of the four time points ($n = 3$).

2.3. Immunofluorescence

Primary myoblasts were fixed with 4% formaldehyde (Solarbio) for 30 min and then washed three times for 5 min each in phosphate-buffered saline (PBS) (Solarbio). The fixed cells were then permeabilized with 0.2% Triton X-100 (Solarbio) for 15 min and then blocked with normal goat serum (Solarbio) for 30 min at 37 °C. After blocking, the cells were incubated with anti-desmin (Bioss, Beijing, China) overnight at 4 °C. Afterward, the cells were then incubated with goat anti-rabbit IgG-Cy3 (Bioss) for 1 h at 37 °C. The cell nuclei were visualized using DAPI staining solution (Beyotime Institute of Biotechnology, Shanghai, China).

2.4. RNA Isolation, Library Preparation, and Sequencing

Total RNA was isolated from each sample using TRIzol regent (Invitrogen, Carlsbad, CA, USA). The purity, concentration, and integrity of the RNA were checked using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. The RNA integrity number (RIN) of all samples was greater than 9.5.

Approximately 3 µg of RNA per sample were used to construct a complementary DNA (cDNA) library, according to the following procedures: the ribosome RNA (rRNA) was removed and strand-specific RNA-seq libraries were then generated using rRNA-depleted RNA. Briefly, after RNA fragmentation, double-stranded cDNA was synthesized by replacing dTTPs (deoxythymidine triphosphate) with dUTPs (deoxyuridine triphosphate) in reaction buffer used for second strand cDNA synthesis. The resulting double-stranded cDNA was ligated to adaptors, after being end-repaired and A-tailed. Single-strand cDNA was then obtained using USER (Uracil-Specific Excision Reagent) Enzyme (NEB, Ipswich, UK). Finally, PCR amplification was performed to enrich the cDNA libraries. Sequencing was performed on an Illumina HiSeq 2500 instrument using the TruSeq Cluster Kit v3-cBot-HS (Illumina, San Diego, CA, USA) to generate 150-bp paired-end reads.

2.5. Quality Control

The raw data were subjected to quality control using the Quality Control tool for High Throughput Sequence Data FastQC v0.11.2 [19]. The Phred score (Q20, Q30) and G + C content of the raw data were analyzed. At the same time, clean data were obtained by discarding reads containing adapter or ploy-N and low-quality reads (>50% of base with Phred scores <5) from the raw data.

2.6. Sequencing Data Analysis and Transcriptome Assembly

All the following analysis were based on the clean data with high quality. Reference genome and gene model annotation files were downloaded from following genome website (ftp://ftp.ensembl.org/pub/release-83/fasta/gallus_gallus/dna/). An index of the reference genome was built using Bowtie v2.0.6 [20] and paired-end reads were aligned to the chicken genome (Galgal 4.0) using TopHat v2.0.9 [21]. Mapped reads belonging to each sample were assembled with the Cufflinks v2.1.1 in a reference-based approach [22]. Transcripts from all samples were then merged together with the Cuffmerge tool to construct a consensus set of transcripts across the samples.
2.7. Identification of lncRNAs

Transcripts were filtered out from 12 chicken primary myoblast samples pooled by Cuffmerge using an in-house computational pipeline, which selects transcripts as candidate lncRNAs according to size (>200 bp), number of exons ≥ 2, expression levels (fragments per kilobase of exons per million mapped fragments, FPKM ≥ 5 overlaps of coding regions with no overlap with a known gene set), overlap of noncoding regions (no overlap with known pseudogenes, pre-microRNA, transfer RNA (trNA), rRNA, or small nucleolar RNAs (snoRNAs)), and noncoding potential (CNCI [23] and CPC [24] scores > 0, Pfam-scan E-value > 0.001 [25], and PhyloCSF score > −20 [26]). Transcripts that passed through all of the filters mentioned above were collected as a candidate set of lncRNAs and were then blasted against the chicken lncRNAs retrieved from the Domestic-Animal Long Noncoding RNA Database (ALDB) v1.0 [27].

2.8. lncRNAs Targets Prediction and Annotation

The cis role of lncRNAs is to act on adjacent target genes, while the trans role is to identify every other lncRNA at the expression level. In the present study, protein-coding genes located ~10 kb upstream and downstream of the expressed lncRNAs were classified as lncRNAs of cis-target genes. Furthermore, the expression levels of the identified lncRNAs and the known coding genes from four different developmental stages were used to analyze the co-expression of lncRNAs and coding genes. The correlations between the expression levels of the lncRNAs and coding genes were evaluated using the Pearson’s correlation coefficient (r > 0.95 or < −0.95 and p < 0.05).

2.9. Screening and Clustering Analysis of Differentially Expressed lncRNAs and mRNAs

mRNA and lncRNA expression levels were calculated in fragments per kilobase of exons per million mapped fragments (FPKMs) using the Cuffdiff algorithm v2.1.1 (http://cole-trapnell-lab.github.io/cufflinks/cuffdiff/index.html). For biological replicates, a p-adjust value of >0.05 and an absolute log$_2$ value (fold change) of <1 were considered to indicate a significant difference in mRNA expression. The DEGs were subjected to K-means clustering by the Euclidean distance method associated with complete linkage using the BMKCloud platform [28].

2.10. Gene Ontology and KEGG Enrichment Analysis

Gene Ontology (GO) and Kyoto Encyclopedia of Gene and Genomes (KEGG) functional annotation enrichment analysis were conducted using the DAVID database [29]. GO terms and KEGG pathways with p-values < 0.05 were considered significantly enriched.

2.11. Validation of Gene Expression by Quantitative Real-Time Polymerase Chain Reaction Analysis

Total RNA from the 12 samples used for the RNA-seq experiment was amplified by quantitative real-time polymerase chain reaction (qPCR). Single-strand cDNA was synthesized using the PrimeScriptTM RT Master Mix kit (TaKaRa Biotechnology, Dalian, China). qPCR was performed using an Applied Biosystems 7500 Real-Time PCR System (Life Technologies, Gaithersburg, MD, USA) with specific primers. qPCR amplifications were carried out in 20 µL reaction volumes containing 1 µL of cDNA, 10 µL of SYBR Premix Ex Taq polymerase (2×) (TaKaRa, Dalian, China), 0.4 µL of ROX Reference Dye II (50×) (TaKaRa), 0.4 µL of the forward primer (10 mmol/L), 0.4 µL of the reverse primer (10 mmol/L), and 6.8 µL of dH$_2$O. The amplification started with initial denaturation step at 95 °C for 30 s followed by 40 cycles at 95 °C for 5 s and an annealing step at 60 °C for 34 s, at which point fluorescence was acquired. Finally, a dissociation curve to test PCR specificity was generated by one cycle at 95 °C for 15 s followed by 60 °C for 1 min and ramped to 95 °C with acquired fluorescence. Specific primers (Table S1) were designed based on sequences retrieved from the National Center for Biotechnology Information (NCBI) database. Gene expression levels were normalized to those of
3. Results

3.1. Proliferation and Differentiation of Embryonic Primary Myoblasts

Chicken myoblasts were derived from primary cultures of embryonic breast muscle tissues and fused myotubes were generated by culturing the myoblasts in differentiation medium for 24, 72, or 120 h (Figure 1). The morphologies of the myoblasts and fused myotubes were observed under an inverted microscope, which revealed the formation of a few, small myotubes within 24 h after induction of differentiation. Immunofluorescence staining showed that the mononucleated myoblasts were fused to one another and the formation of elongated, multinucleated myotubes had markedly increased over the 120-h period. Cells at different developmental stages were collected for use in the following procedures.

Figure 1. (A) Microscopic images of chicken primary myoblasts cultured in growth medium (GM) (100% confluence) or in differentiation medium (DM) for 24 h (DM1), 72 h (DM3) and 120 h (DM5). Black arrows represent myotubes; and (B) cells were fixed and immunostained for desmin in GM and DM. Nuclei were stained with DAPI. White arrows represent fused myotubes. Cells at different developmental stages were collected for use in the following procedures. The red and blue color represented cytoplasm and cell nucleus, respectively.

3.2. Overview of RNA-Sequencing

Observation of the proliferation and differentiation of myoblasts to mature myotubes in vitro is a valuable tool for the characterization of cellular events during myogenesis. Therefore, the differentiation of separated chicken primary myoblasts was stimulated at specific time points and observed over the course of about five days. The Illumina Hiseq 2500 platform was used to perform RNA-seq for the 12 cDNA libraries, which resulted in the generation of more than 90 million raw reads from each library (Table S2). After quality control, most of the clean reads still comprised more than 90% of the raw data, with the exception of A0-2 (89.98%). More than 74.86% of the clean reads were perfectly mapped to the reference chicken genome (release: gaGal4). The uniquely-mapped reads ranged from 64.38% to 67.29% of clean reads (Table S2).

HSP70 and β-actin to attain relative expression levels using the $2^{-\Delta\Delta Ct}$ method. Correlations between the RNA-seq and qPCR results were determined using the Spearman’s rank correlation coefficient.
3.3. Identification and Characterization of lncRNAs in Chicken Myoblasts

Of a total of 18,828 transcripts that were identified, most (86.81%) were mRNAs (Figure 2A). Among all 2484 confirmed lncRNA transcripts, 1248 were previously annotated, and 1236 were novel (Figure 2A). Other than these, only two other types of lncRNAs were identified in the chicken primary myoblasts: an overwhelming majority of long intergenic non-coding RNAs (lincRNAs) (92.0%) and a minority of antisense lncRNAs (8.0%) (Figure 2B). The 2484 lncRNAs were distributed among chicken chromosomes 1–28 and Z. Quite notably, 367 lncRNAs were located on chromosome 1 (Figure 2C). The ggplot2 package [30] in the R environment was used to compare the genomic features of lncRNA to that of mRNA transcripts, as shown in Figure 2D–F. The average length of mRNA transcripts was 2752 bp with an average of 11 exons, which was larger than size of the lncRNA transcripts. Furthermore, the identified lncRNAs tended to have shorter open reading frames (ORF) than mRNAs (555.5 on average).

![Figure 2. Characterization analysis of long non-coding RNAs (lncRNAs). (A) The classification of transcripts by RNA sequencing (RNA-seq); (B) lncRNA classification; (C) chromosome distribution of lncRNA transcripts in chicken primary myoblasts; (D) the length comparative analysis of lncRNAs and messenger RNA (mRNAs); (E) the exon number distribution of lncRNAs and mRNAs; and (F) the distribution of the open reading frame (ORF) length of lncRNAs and mRNAs.](image-url)
3.4. Functional Enrichment Analysis: GO and KEGG

To investigate the possible functions of lncRNAs in chicken myoblasts, the cis-targets of all lncRNAs were predicted using a threshold of 10-kb. As shown in Table S3, 1530 lncRNAs were transcribed close to 2041 protein-coding neighbors. GO analysis was then performed to identify the functions of the cis lncRNA targets, which showed that the highest number of genes were related to cell signaling and skeletal muscle contraction processes, such as cation binding (GO:0043169), metal ion binding (GO:0046872), and ion binding (GO:0043167) in addition to the other significantly enriched GO terms ($p < 0.05$), including protein modification process (GO:0036211), cellular protein modification process (GO:0006464), and regulation of molecular function (GO:0006509), suggesting that one of the principal roles of lncRNAs may be the mediation of protein modifications to regulate the expression of adjacent genes (Table S4). Pathway analysis indicated that seven pathways were significantly enriched ($p < 0.05$), including several related to muscle development via regulation of the cytoskeleton, insulin signaling pathway, MAPK signaling pathway, FoxO signaling, and focal adhesion (Figure 3A; Table S5). Taken together, the cis results showed that the protein-coding genes were regulated by neighboring lncRNAs involved in muscle development.

Figure 3. Kyoto Encyclopedia of Gene and Genomes (KEGG) enrichment of lncRNA-targets genes using DAVID software. (A) KEGG annotation for neighbor gene function of predicted lncRNAs; and (B) statistical KEGG enrichment for co-expressed gene function of identified lncRNAs.

Then, the potential targets of lncRNAs in trans-regulatory relationship were predicted using co-expression analysis. A total of 105,926 interaction relationships (78,202 positively, and 27,724 negatively correlated) were detected between 990 lncRNAs and 7436 protein-coding genes in the reference chicken genome (Table S3). Functional analysis showed that the co-expressed genes were significantly enriched in 606 terms (451 under biological process, 83 under cellular component, and 72 under molecular function) that encompassed a variety of biological processes (Table S6). Importantly, some of the terms were related to muscle development, including muscle cell proliferation (GO: 0033002), muscle cell differentiation (GO: 0042692), myoblast fusion (GO: 0007520), myoblast differentiation (GO: 0045445), myotube differentiation (GO: 0014902), muscle cell development (GO: 0035001), striated muscle cell proliferation (GO: 0014855), and skeletal muscle cell differentiation (GO: 0035914). An additional 18 pathways were significantly enriched ($p < 0.05$), some of which were closely related to mitosis and cellular activities, such as the cell cycle, focal adhesion, the p53 signaling pathway, and extracellular matrix-receptor interactions (Figure 3B; Table S5). Overall, lncRNAs were involved in the trans-regulation of protein-coding genes associated with muscle development.
3.5. Differentially-Expressed lncRNAs and mRNAs during Primary Myoblast Differentiation

To investigate the key lncRNAs and mRNAs involved in myoblast differentiation, RNA-seq was performed to detect the differentially-expressed lncRNAs (DE-lncRNAs) and genes (DEGs) at four developmental stages of chicken primary myoblasts in vitro (A0, A1, A3, and A5). DE-lncRNAs and DEGs were identified using Cuffdiff analysis (|fold change| > 2; \( p \)-adjust < 0.05). Lastly, 906 lncRNAs and 4422 mRNAs were found to be differentially expressed during myoblast differentiation (Figure 4A,B and Table S7). The number of up-regulated mRNAs was higher than the number of down-regulated mRNAs during myoblast development, while the lncRNAs shared a common feature, with the exception of A5 vs. A3 and A5 vs. A1. To further analyze the interactions among the differentially expressed lncRNAs, 362, 353, 162, and 534 differentially-expressed lncRNAs were used in four comparisons (A1 vs. A0, A3 vs. A1, A5 vs. A3, and A5 vs. A0, respectively) to construct a Venn diagram. Meanwhile, 1812, 1601, 589, and 3497 differentially-expressed mRNAs in the aforementioned four comparisons were used to construct a Venn diagram. As shown in Figure 4C,D, 89 differentially-expressed genes (10 lncRNAs and 79 mRNAs) were common among the four comparisons.

To extract additional biological information from the multi-dimensional transcriptome dataset, clustering analysis we performed using the \( K \)-means method to identify co-expression in DEG clusters. Four main clusters were plotted with the expression patterns of the involved genes (Figure 5, Table S8). The K1 cluster included 1436 genes with an overall decreasing trend among most of these genes. Notably, most DEGs in the K4 cluster had the highest expression levels during the initial differentiation stage. Additionally, the DEGs in the K2 and K3 clusters had some similar expression patterns, which showed an overall upregulated trend during myofibrillogenesis. Dynamic changes in gene expression reflect an intrinsic mechanism of how an organism responds to developmental and environmental signals. That is, the DEGs with different expression patterns might play different and necessary roles in myoblast proliferation and differentiation. Therefore, to better understand their roles and relevance in muscle development, GO analysis was performed to identify enriched (\( p \)< 0.05) biological process terms for each cluster (Table S9). The results showed that DEGs in the K1 cluster were mainly involved in the cell cycle, DNA replication, and mitosis, whereas the functions of DEGs in the K4 cluster were mainly involved in muscle development and muscle cell differentiation. Based on these findings, one can infer that DEGs in the K1 cluster might play...
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3.6. Verification of Gene Expression Profiles Using qPCR

To confirm the accuracy and reproducibility of the RNA-seq results, five well-known DE-lncRNA target genes that affect muscle development, including ACTC1, TNNT3, IGF2, BMP2, and IGFBP2, and their corresponding lncRNA regulators XLOC_026286, ALDBGALG0000004738, XLOC_026930, XLOC_022394, and ALDBGALG0000005188, were chosen for qPCR validation. The results showed that the expression patterns of all five lncRNAs and their cis-target genes were in excellent agreement with the RNA-seq findings (Figure 6), indicating the reliability of our RNA-seq data.
Figure 5. Hierarchical clustering of differentially-expressed genes (DEGs) (4422). (A) Heatmap plot of DEGs using the hierarchical clustering method; four clusters are shown; decreased (purple) and increased (yellow) expression of DEGs are distinguished from different stages; and (B) expression patterns of genes in the four clusters, namely K1–K4, corresponding to the hierarchical heatmap. The blue line represented the centered expression pattern of genes in each cluster.
Table 1. Overrepresented GO terms of DEGs involved in different clusters.

| Cluster (# of genes) | Example genes | Enriched GO terms | # of gens | p-value |
|----------------------|---------------|-------------------|-----------|---------|
| **K1 (1,436)** | YY1, TGFβ1, SOX8, | Cell cycle process | 138 | 3.81 × 10^{-22} |
| | SDX1, SIX2, PAX3, | DNA metabolic process | 123 | 5.17 × 10^{-22} |
| | PX7, MYF5, MYF6, ID1, ID2, | Cell cycle | 156 | 9.67 × 10^{-22} |
| | E2F7, E2F3, E2F1, | Chromosome organization | 132 | 4.51 × 10^{-21} |
| | E2F2, CDC7, CDC6, | DNA replication | 59 | 2.94 × 10^{-20} |
| | CDC45, CDC20, | Mitotic cell cycle process | 92 | 1.86 × 10^{-17} |
| | CDK1, CDK2, CDK6, CDK7 | Nuclear division | 73 | 5.56 × 10^{-17} |
| | | DNA repair | 76 | 8.60 × 10^{-17} |
| | | Mitotic cell cycle | 98 | 3.19 × 10^{-16} |
| | | Chromosome segregation | 54 | 2.18 × 10^{-15} |
| **K2 (440)** | MYOC, IGF2, MYO1D, FGF7, FGF18, FABP4, ECM2, DCX, FAP, FAT4, KLF15, LBP, SHOX, TF | Extracellular structure organization | 18 | 5.03 × 10^{-7} |
| | | Extracellular matrix organization | 18 | 5.03 × 10^{-7} |
| | | Chemotaxis | 24 | 1.06 × 10^{-5} |
| | | Taxis | 24 | 1.13 × 10^{-5} |
| | | Cellular response to chemical stimulus | 59 | 2.49 × 10^{-5} |
| | | Response to external stimulus | 52 | 3.50 × 10^{-5} |
| | | Regulation of cell migration | 27 | 6.87 × 10^{-5} |
| | | Cellular response to organic substance | 49 | 7.19 × 10^{-5} |
| | | Cell adhesion | 41 | 9.90 × 10^{-5} |
| | | Biological adhesion | 41 | 1.09 × 10^{-4} |
| **K3 (1,762)** | FLI1, KLF4, WNT9A, CTR, WNT5A, EGFR, BMP5, FAK, FGF14, FGF9, RAC2, MYH6, MYH10, MYCN, MXD4, IGFBP2, IGFBP4, IGFBP5 | Locomotion | 139 | 6.05 × 10^{-8} |
| | | Movement of cell or subcellular component | 148 | 1.79 × 10^{-6} |
| | | Enzyme linked receptor protein signaling pathway | 86 | 3.16 × 10^{-6} |
| | | Transmembrane receptor protein tyrosine kinase signal | 61 | 7.29 × 10^{-6} |
| | | Organ morphogenesis | 93 | 7.34 × 10^{-6} |
| | | Biological adhesion | 118 | 9.30 × 10^{-6} |
| | | Skeletal system development | 59 | 1.04 × 10^{-5} |
| | | Cell adhesion | 117 | 1.25 × 10^{-5} |
| | | Skeletal system morphogenesis | 36 | 1.42 × 10^{-5} |
| | | Neurogenesis | 127 | 1.59 × 10^{-5} |
| **K4 (784)** | TNNT3, SRF, RGS2, Myosin, MYOG, TMEM68C, DLL1, NOTCH1, MYOD, MEF2C, GDF8, MEF2D, MAPK13, DII2, BMP4, β-FGF, ACTC1, MTO1, SMYD1 | Muscle structure development | 61 | 3.24 × 10^{-20} |
| | | Muscle system process | 41 | 6.97 × 10^{-18} |
| | | Muscle contraction | 37 | 3.07 × 10^{-17} |
| | | Striated muscle cell differentiation | 35 | 2.35 × 10^{-15} |
| | | System process | 87 | 5.03 × 10^{-14} |
| | | Muscle cell differentiation | 41 | 1.18 × 10^{-13} |
| | | Muscle tissue development | 41 | 2.72 × 10^{-13} |
| | | Muscle organ development | 36 | 1.40 × 10^{-12} |
| | | Striated muscle tissue development | 38 | 1.51 × 10^{-12} |
| | | Muscle cell development | 26 | 3.83 × 10^{-12} |
Figure 6. qPCR validation of differentially-expressed lncRNAs and their cis-target genes. FPKM: fragments per kilobase of exons per million mapped fragments.
4. Discussion

Skeletal muscle growth and development consists of a series of exquisitely regulated changes in gene expression from the embryo to adult stages. Deciphering these developmental changes in agriculturally-important species is essential to the production of high-quality meat products. One of the greatest surprises was that most transcription originated from the noncoding regions of the genome [31]. The existence of non-coding RNAs (ncRNAs), including small ncRNAs (such as piwi-interacting RNA (piRNA), small interfering RNA (siRNA), and microRNA (miRNA)) and lncRNAs revealed the complexity of genome expression. However, the roles of small ncRNAs have been relatively well characterized in skeletal muscle biology [32,33]. In contrast, lncRNAs were identified only recently and we are still in the infancy of studying this novel class of ncRNAs in skeletal muscle. Despite the fact that transcriptomic research on chicken lncRNA have been carried out, transcriptome analysis of chicken muscle is performed only at the embryonic or adult stage [11,34]. In comparison to the complexity of muscle tissue at different developmental stages in vivo, embryonic myoblasts in vitro provide an easier method to understand the cellular events occurring during skeletal myogenesis. In the present study, the Illumina Hiseq 2500 platform was used to determine the identity and expression level of lncRNAs and mRNAs involved in muscle formation in an in vitro cell culture system, where myoblasts were allowed to fuse to multinucleated myotubes. To the best of our knowledge, this study is the first to screen and sequence lncRNAs and mRNAs involved in the regulation of the proliferation and differentiation of chicken myoblasts. As a result, an average of 14.43 Gb of clean bases were obtained from myoblasts and fused myotubes. Most of the 1154 million clean reads obtained (77.8%) were perfectly mapped to the current Galga 4.0 genome assembly, which was higher than the percentage obtained for the transcriptomes of sperm (73.1%) [12] and leg muscle (73.4%) [35]. The extensive sequencing depth contributed to the very high sequence coverage of the reference chicken genome. With the number sequencing depth and higher mapping ratio, more transcripts were obtained.

Due to the rigorous screening criteria, a total of 2484 high-confidence lncRNAs were identified, of which 2288 were lncRNAs and 198 were antisense lncRNAs. Furthermore, only 14% of all lncRNAs were mapped with reported chicken lncRNAs from the ALDB database (8923), which was consistent with the results of previous reports [36,37]. A possible reason for this difference is that chicken lncRNAs tend to be highly tissue-specific. Moreover, the lncRNAs transcripts were widespread in chromosomes, including 28 autosomes and the Z-chromosome, which at least reflected the complexity and functional diversity of these lncRNAs. Notably, the proportion of lncRNAs located in chromosome 1 was the highest compared with other chromosomes, which was similar to a previous study of chicken leg muscle [11]. Many reports have revealed that lncRNAs share many common characteristics among species, including shorter transcript and open reading frame lengths, and fewer exons, as compared to mRNAs [38,39]. These features were also observed in this study, indicating the reliability of lncRNA identification.

Recent studies have demonstrated that the functions of lncRNAs can be inferred from adjoining or co-expressed protein-coding genes [40–42]. In the cis prediction, the protein products of the neighboring genes of all identified lncRNAs were used to predict their possible roles during myogenesis. Consequently, functional enrichment of the neighboring coding genes revealed that major enriched pathways were associated with muscle development. Combined with differential expression analysis, many of the lncRNAs and their target genes were differentially expressed, including some potentially involved in myogenesis regulation. Of these, lncRNA XLOC_026286 was predicted to act on the target gene ACTC1, a predominant striated α-actin isoform that is expressed in the fetal skeletal muscle and adult heart [43,44]. Some reports have demonstrated that ACTC1 over-expression in postnatal skeletal muscle could effectively rescue ACTA1-related diseases [45,46], congenital myopathies caused by mutations in ACTA1 [47]. lncRNA ALDBGALA0000004738 was predicted to act on the potential target gene TNNT3, a skeletal muscle contractile gene that has been reported to be associated with distal arthrogryposis syndromes [48]. lncRNA XLOC_026930 was predicted to act on the neighboring gene IGF2, an embryonic regulator of myogenesis and an autocrine
factor that initiates myoblast differentiation in vitro [49,50]. IncRNA XLOC_022394 was predicted to act on the target gene BMP2, a member of the transforming growth factor β super family. In this study, the BMP2 gene showed a notable overall trend of downregulation, suggesting its negative roles in myoblast differentiation. This is in accordance with a previous notion that BMP2 functions as a negative regulator in the differentiation of human myoblasts [51]. These findings suggest that IncRNAs might regulate myogenesis and muscle regeneration through actions on neighboring genes. However, these predicted IncRNA-mRNA pairs require further experimental verification.

Still, many IncRNAs were found to also function in trans mode to target gene loci distant from the transcription sites of the IncRNAs [33]. Co-expression analysis identified 990 IncRNAs that were related to 7436 coding genes based on the expression correlation coefficient (r) of >0.95 or <0.95. Functional enrichment analysis of the co-expressed genes was mainly involved in the cell cycle, mitosis, and cellular activities that are closely related to myofibrillogenesis. Furthermore, co-expression analysis showed that a cluster of IncRNAs often targeted coding genes that were specifically expressed in muscle and were involved in muscle development (e.g., MyoD, MyoG, Myf5, MEF2C, TNNT3, GDF8, and TMEM8C). Combined with differential expression analysis, DEGs of interest were selected to perform a reverse lookup of co-expressed IncRNAs to help refine the most likely IncRNA-mRNAs pairs for subsequent experimental validation. For example, 16 DE-lncRNAs were co-expressed with TMEM8C, the muscle-specific protein known to be absolutely essential for fusion, development, and regeneration of myoblasts in mammals [52,53]. We can forecast boldly that the 16 DE-lncRNAs might play the same important roles as TMEM8C in skeletal muscle development. In addition, 26 DE-lncRNAs were reverse-expressed with FAK, a key mediator of integrin signaling, which has been implicated in the regulation of cell migration and cell cycle process [54]. Liu et al. reported that FAK is a negative regulator during the processes of myoblast migration and fusion [55]. This interesting observation indicates the diverse and complex functions of IncRNAs and deserves further investigation. Therefore, our ongoing efforts will focus on the function of those most likely IncRNAs to provide more fundamental information to further elucidate their regulatory mechanisms in skeletal muscle development at the molecular level.

Massive RNA-seq experiments comprising two or more time points that correspond to the development or distinct treatment are useful to unravel the intrinsic and dynamic regulatory mechanisms [56]. For genes with an explicit expression curve from a specific cluster, their similar expression trend can be a basis for the postulation that these genes share a common responsive behavior to a developmental or environmental stimulus [57,58]. Therefore, in this study, four clusters were defined according to the K-means results to further illustrate the relationships between DEGs with various expression patterns. GO analysis was performed for each co-expression gene cluster, which showed that the stage-specific clusters were enriched in terms of the expected functional gene sets. The K1 cluster exhibited a developmental trend with the highest expression during the proliferation stage, followed by a progressive decline when the cells underwent differentiation. As expected, these genes were enriched in the functional categories related to cell proliferation, such as the cell cycle, DNA replication, and mitosis. A group of DEGs were reportedly related to myoblast proliferation, which include YY1 [59], PAX3 [60], E2F3 [61], SIX1 [62], and MYF5 [63], among others. By contrast, the K2 and K3 clusters exhibited changes in the opposite direction, with an overall upregulated trend. Myoblast fusion is an important step in the formation of multinucleated fibers. Cell migration and adhesion are essential for cell-cell fusion of myoblasts. As we can imagine, the functional categories of the two clusters were mainly involved in cell migration, adhesion, and cell-extracellular interactions, suggesting possible roles over the entire myofibrillogenesis process. For example, IGF2 [64], FABP4 [65], KLF4 [66], FAK [67], and RAC2 [68] were recently reported to be involved in cell migration and adhesion. Notably, genes in the K4 cluster showed significant and specific up-regulation at day 1 of differentiation, indicating the importance of the contribution of these genes to the initiation of myoblast differentiation. Without question, these genes were mainly enriched in terms related to muscle development and muscle differentiation. Members of this cluster, including
MYOG, MEF2C, MEF2D, and TMEM8C [69], reportedly regulate myoblast differentiation. In fact, the fusion of myoblasts denotes that mononucleated myoblasts exit the cell cycle, begin differentiation, and fuse with one another to generate syncytial myofibers [52]. The functional categories of each stage-specific cluster are in accordance with the process of myogenesis. Moreover, this method might be a good choice for the discovery of putative genes as candidates for further experimental validation.

Furthermore, the accuracy of RNA sequencing was confirmed by qPCR in this study. Of the five lncRNA-mRNA pairs selected for testing, the results were consistent with the RNA-seq data, thereby confirming the reliability of RNA-seq and laying a solid foundation for further exploration.

5. Conclusions

In summary, the application of transcriptome analysis during myoblast proliferation and differentiation provided a catalog of lncRNAs and mRNAs to further understand the regulatory roles of these RNA molecules in chicken muscle development. In future studies, we plan to investigate the functions of some lncRNAs to further elucidate the regulatory mechanisms underlying chicken muscle development. Perhaps, the lncRNAs identified in this study can be utilized to improve the meat production in livestock.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4425/9/1/34/s1. Table S1. Primers for qPCR; Table S2. Summary of draft reads of 12 libraries by RNA-sequencing; Table S3. lncRNA targets predicted by cis- and trans-acting; Table S4. Functional enrichment analysis of protein-coding genes targeted by cis- and trans-acting lncRNAs; Table S5. KEGG analysis of protein-coding genes targeted by cis- and trans-acting lncRNAs; Table S6. Functional enrichment analysis of protein-coding genes co-expressed with lncRNAs; Table S7. List of differentially-expressed lncRNAs and mRNAs from four myoblast developmental stages; Table S8. Differentially-expressed genes for K-means analysis; Table S9. Functional enrichment analysis of DEGs involved in K1, K2, K3, and K4 clusters.

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