

Article

Characterization of the Full-Length Transcriptome and Regulatory Networks During Quail Skeletal Muscle Development Using Nanopore Sequencing

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Abstract

The quail is a valuable model organism for avian developmental biology and genetic improvement; however, the full-length transcriptomic landscape and regulatory networks underlying critical embryonic developmental stages remain poorly characterized. In this study, we employed Oxford Nanopore Technologies full-length transcriptome sequencing to systematically analyze leg muscle tissues from quail embryos at embryonic day 8 (E8, pre-differentiation stage) and embryonic day 14 (E14, later differentiation stage), with four biological replicates per stage (eight libraries in total). Nanopore sequencing identified a total of 54,938 transcripts and 21,574 genes, including 49,678 known transcripts, 20,980 known genes, 5260 novel transcripts, and 594 novel genes. Analysis of transcriptome structure revealed that alternative first exon and exon skipping represented the predominant stage-specific alternative splicing events, and we identified 2692 lncRNAs. Differential expression analysis identified substantial transcriptome dynamics from E8 to E14, with 5934 differentially expressed transcripts and 3745 differentially expressed genes identified. Functional enrichment analyses, including KEGG, GSEA and PPI network analysis, revealed that the E8 transcriptome was predominantly enriched for pathways associated with cell proliferation, whereas the E14 transcriptome shifted toward the coordinated refinement of myofiber structural assembly and energy metabolism. Here, we present a high-resolution full-length transcriptional atlas of quail skeletal muscle development and identify candidate regulatory genes, offering a valuable theoretical framework and data resource for refining quail genome annotation.

Keywords: quail; skeletal muscle development; full-length transcriptome sequencing; alternative splicing; long non-coding RNA; differentially expressed genes



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1. Introduction

The quail (*Coturnix japonica*), with its short generation interval, high egg production rate, and high genomic synteny with the chicken, has emerged as an ideal model organism for avian developmental biology and genetic improvement research [1,2]. As a determinant of meat yield and quality, skeletal muscle development directly influences

the growth performance and carcass quality of meat-type poultry [3]. Elucidating the molecular mechanisms governing skeletal muscle development is therefore of considerable practical importance, as it directly determines meat yield, carcass quality, and overall growth performance in quail [4,5]. Although our understanding of the molecular basis of avian skeletal muscle development has advanced considerably, significant gaps persist in characterizing the transcriptional regulatory networks operating during critical embryonic stages in quail. These gaps are particularly evident when compared with the extensively studied chicken. However, full-length and isoform-level transcript annotation in quail remains limited compared with that in chicken, which has hindered efforts to elucidate the regulatory mechanisms governing muscle growth.

Avian skeletal muscle development is a highly ordered biological process encompassing key stages such as myogenic progenitor cell proliferation, myoblast differentiation, myotube fusion, and myofiber maturation [6]. This process is orchestrated by core transcription factors, including Pax3/Pax7 and the myogenic regulatory factor (MRF) family, together with conserved signaling pathways such as Wnt, Notch, and Hedgehog that have been implicated in vertebrate skeletal muscle development, including in birds [7–9]. Furthermore, non-coding RNAs (ncRNAs) play critical roles at the post-transcriptional and epigenetic levels, fine-tuning myogenesis by constructing complex competing endogenous RNA (ceRNA) networks or participating in chromatin remodeling [10,11]. However, studies of quail skeletal muscle development have largely relied on short-read sequencing [4]. While short-read approaches can accurately quantify gene expression when a reference transcriptome is available, they offer limited resolution for assembling complex transcript structures, detecting alternative splicing (AS) events, and characterizing full-length transcript isoforms. Consequently, numerous low-abundance or structurally complex transcripts have likely been overlooked.

Full-length transcriptome sequencing technologies, particularly long-read sequencing based on Oxford Nanopore Technologies (ONT), offer a powerful means of overcoming these limitations. By directly capturing full-length transcript sequences, this technology enables precise definition of transcription start sites and termination sites, resolves complete exon-intron structures, and circumvents the assembly biases inherent to short-read sequencing [12]. Full-length transcriptome sequencing has already demonstrated considerable utility in muscle development studies across various species. In pigs, ducks, and chickens, it has enabled the discovery of novel transcripts, improved resolution of alternative splicing events, and better characterization of dynamic transcriptomic changes during myogenesis [13–15]. However, its application to quail skeletal muscle development remains unexplored, underscoring the pressing need to construct a high-resolution transcriptional atlas using this approach.

To address this gap, we collected leg muscle tissues from quail embryos at critical developmental stages (E8 and E14) and performed full-length transcriptome sequencing using the ONT platform. We selected embryonic day 8 (E8, pre-differentiation) and embryonic day 14 (E14, later differentiation stage) based on our prior systematic histological and molecular characterization of Korean quail leg muscle development [4]. In that study, tissue section analysis and expression of myogenic differentiation markers demonstrated that the majority of myoblasts remain undifferentiated at E8, whereas myofiber differentiation and structural assembly are largely completed by E14. Building on our previous short-read transcriptome analysis of quail skeletal muscle development [4], the present study employs Oxford Nanopore long-read sequencing to generate a high-resolution, full-length transcriptomic atlas. Leg muscle samples were newly collected from the same Korean quail population under matched feeding, incubation, and sampling protocols, ensuring biological comparability while providing an independent cohort. The specific objectives

were to: (1) comprehensively characterize transcript structures and gene expression profiles in developing quail skeletal muscle; (2) identify alternative splicing events and novel transcripts associated with myogenesis; and (3) integrate differentially expressed transcript (DET) and differentially expressed gene (DEG) datasets to delineate candidate signaling pathways and genes potentially involved in skeletal muscle differentiation. This work provides a high-resolution full-length transcriptomic resource and identifies candidate regulatory factors, offering a foundation for future mechanistic studies on quail skeletal muscle development.

2. Materials and Methods

2.1. Sample Collection and Processing

Healthy Korean quail (*Coturnix japonica*), a breeding line maintained at the Poultry Institute of Jiangxi Agricultural University (Nanchang, China), were used in this study, and all experimental procedures were performed in accordance with a previously published protocol [4]. Briefly, 80 fertile eggs from the same batch (with no significant differences in egg weight) were incubated under standardized conditions (38.0 °C, 60% relative humidity, 12 turns per day). Leg muscle tissues (including thigh and shank muscles) were collected from 20 embryos at each of E8 (pre-skeletal muscle differentiation stage) and E14 (later differentiation stage). After sex identification by PCR amplification of the *CHD1* gene [4], leg muscle samples from four female embryos per stage (E8 and E14) were used for Nanopore full-length transcriptome sequencing. The two embryonic stages (E8 and E14) were chosen based on prior histological validation in Korean quail showing that these time points effectively capture the transition from the proliferative to the differentiation/maturation phase of leg muscle development [4]. Collected samples were immediately snap-frozen in liquid nitrogen and subsequently stored at −80 °C.

2.2. RNA Extraction and Quality Assessment

Leg muscle tissue was thoroughly homogenized under liquid nitrogen using TRK Lysis Buffer (R6834, Omega Bio-tek, Wuhan, China). The lysate was transferred to a homogenizer spin column and centrifuged at $14,000\times g$ for 2 min at room temperature to remove insoluble debris. The filtrate was collected, mixed thoroughly with an equal volume of 70% ethanol, and transferred to an RNA binding column. After centrifugation at $10,000\times g$ for 1 min, the flow-through was discarded. The binding column was washed sequentially with RNA Wash Buffer I and II, followed by a dry spin at $10,000\times g$ for 2 min to remove residual ethanol. Finally, RNA was eluted using Nuclease-free Water. RNA purity and concentration were assessed using a Nanodrop one spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and Qubit 3.0 fluorometer (Life Technologies, Carlsbad, CA, USA), and integrity was evaluated by gel electrophoresis to ensure samples met the requirements for subsequent library construction.

2.3. Full-Length Transcriptome Library Construction and Sequencing

Total RNA (250 ng) was used for library preparation with the SMART cDNA kit (BN-RTS110, Benagen, Wuhan, China). Reverse transcription was performed using Maxima H Minus Reverse Transcriptase (EP0752, Thermo Fisher, Waltham, MA, USA) at 42 °C for 30 min (1 cycle). No size selection was applied. The resulting cDNA was purified with AMPure XP beads (Beckman Coulter, Brea, CA, USA), and sequencing adapters were ligated using the Rapid Adapter (Oxford Nanopore Technologies, Oxford, UK). The final library was sequenced on a PromethION platform (Oxford Nanopore Technologies) with R9.4 flow cells (Oxford Nanopore Technologies) for 48–72 h. Base-calling was performed with dorado (v1.4.0) using model V5.0.0. Raw reads were filtered with NanoFilt (v2.8.0)

using a minimum quality threshold of $Q > 7$. Each sample was individually barcoded and then pooled for sequencing on the same flow cell. No polishing or error correction was applied to the raw reads; however, racon was used during the consensus transcript identification step. Library construction and sequencing were performed by Benagen.

2.4. Bioinformatics Analysis

Raw reads were filtered using NanoFilt (v2.8.0) [16], and full-length transcripts were identified using Pychopper (v2.2.2). Default parameters were used for NanoFilt, gffcompare (v0.12.1) and Pychopper unless otherwise specified. Clean reads were aligned to the quail reference genome (*Coturnix japonica* 2.1, https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_001577835.2/, accessed on 9 January 2024) using Minimap2 (v2.24) [17] with the command *minimap2 -ax splice -uf -k14* and sorted using Samtools (v1.15.1). Transcript reconstruction and novel transcript discovery were performed using StringTie (v2.2.1) and Gffcompare. Minimap2, StringTie and Transcript quantification with Salmon (v1.9.0) were run in long-read mode. Transcript quantification was performed by first aligning the full-length ONT reads to the assembled transcripts with Minimap2 using *minimap2 -a -k14 -uf --secondary=no*, followed by Salmon quantification on the resulting BAM files with the command *salmon quant --writeUnmappedNames --libType A --noErrorModel*. The transcriptome FASTA used to build the Salmon index was derived from the merged GTF file generated by StringTie. Per-sample transcript assemblies were generated with StringTie using *stringtie -p 16 -R -G ref.gtf -L --conservative -o sample.uniq_isoforms.gtf sample.pass_isoforms.sorted.bam*, after which redundant transcripts were removed by merging all per-sample GTF files with *stringtie --merge -G ref.gtf gtf.list -o merged.gtf*. Transcripts Per Million (TPM) values were calculated for quality control, including density distribution plots and Pearson correlation analysis between samples. Principal component analysis (PCA) was performed on the normalized gene or transcript expression data using the R v4.2.0 to visualize the overall transcriptomic differences between E8 and E14 samples.

Gene-level estimated counts were obtained from Salmon and used as input for differential expression analysis with DESeq2 (v1.26.0). Prior to analysis, low-expression genes and transcripts were filtered by retaining only those with counts per million (CPM) > 1 in at least two samples. DESeq2 performed its default normalization using size factors, independent filtering, and shrinkage. Adjusted p -values were calculated using the Benjamini–Hochberg method. Genes or transcripts with $|\log_2(\text{fold change})| > 1$ and adjusted p -value < 0.05 were considered differentially expressed. In this analysis, positive $\log_2(\text{fold change})$ values indicate higher expression at the E14 stage compared to E8, while negative values indicate higher expression at E8 compared to E14. DESeq2 was applied at both the transcript level (to identify differentially expressed transcripts, DETs) and the gene level (to identify differentially expressed genes, DEGs), with transcript counts aggregated to gene level for the latter analysis. Functional annotation and enrichment analysis were conducted using ClusterProfiler (v4.2.2), integrating GO, KEGG (KofamScan v1.3.0), and Uniprot databases. Additionally, alternative splicing analysis was performed using SUPPA2 (v2.3), alternative polyadenylation (APA) analysis was performed using Tapas (v2018.5.26) with default parameters. Candidate long non-coding RNAs (lncRNAs) were identified using CPC2, CNCL, and PLEK. Only transcripts predicted as non-coding by all three tools were retained as high-confidence lncRNAs. Default parameters were used for DESeq2, SUPPA2, and Tapas. Coding potential prediction was performed using TransDecoder, CPC2, CNCL, and PLEK with default settings. Protein–protein interaction (PPI) network analysis was performed using the STRING database (Organisms: *Coturnix japonica*) and visualized using Cytoscape v3.10.4. Specific parameter configurations were adopted from relevant literature [13,18]. To construct the PPI network, the top 200 DEGs with the smallest adjusted p -values were

selected. This threshold was applied to focus on the most statistically reliable DEGs, reduce potential noise from marginally significant genes, and maintain a manageable network size for visualization and biological interpretation. Software versions used in the bioinformatics analysis are provided in Supplementary Table S1.

2.5. RNA Extraction, cDNA Synthesis, and qRT-PCR Validation

To validate the reliability of the full-length transcriptome sequencing data, several key DEGs were selected for quantitative qRT-PCR analysis. Total RNA was extracted using TRIzol Reagent (TaKaRa, Tokyo, Japan) strictly following the manufacturer's instructions. Total RNA was extracted from the same leg muscle tissue samples and biological replicates ($n = 4$) that had been used for Nanopore sequencing. RNA concentration and purity were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher). cDNA synthesis was performed using a reverse transcription kit (Takara), with specific reaction conditions as previously described by our laboratory [19].

The qRT-PCR reaction mixture contained ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China), forward and reverse primers, and template cDNA. *GAPDH* was used as the internal reference gene. All reactions were performed on a Bio-Rad CFX Duet system (Bio-Rad, Hercules, CA, USA) with three technical replicates. Data were analyzed using the relative quantification method ($2^{-\Delta\Delta C_t}$ method). All the primers utilized in this study can be found in Table 1.

Table 1. Primer information for qPCR validation.

Primer Name	Sequence (5'–3')	Tm (°C)	Product Length (bp)
qquTCAP-F	CGAGGATGGGGTGATCTGAG	59	117
qquTCAP-R	TCCAGGCTGCACCTTTTATTG	59	
qquSYPL2-F	GGAGTGTGATGGTGAATCGG	58	171
qquSYPL2-R	AACGGGAGCTTCTTGATGTC	58	
qquSLN-F	CTCTCTAGTCTCCGTTGCCT	58	175
qquSLN-R	TACCCTGCCCTGATACCAAA	58	
qquMYOZ1-F	CTGGGAACGAGCAATGGGTA	60	158
qquMYOZ1-R	AGGTCATTCGTTTGGCTGCT	60	
qquCRABP1-F	TCCGCACCACAGAGATCAAC	60	91
qquCRABP1-R	TCCCAAGTTGCCAAACTCCT	60	
qquPENK-F	GATGACGATGCCCTGGCTAA	60	127
qquPENK-R	CGCCGTATCTTTTGCTGACG	60	
qquGAS2-F	GTCGGTTAGCTGAGACCCTG	60	175
qquGAS2-R	ACCCCTACATCTCTGCACCA	60	
qquNES-F	TACGTAGCACACCCGTTTCC	60	132
qquNES-R	GGAGCCTGGTTTCTTGCTCT	60	
quGAPDH-F	TGCCGTCTGGAGAAACC	55	160
quGAPDH-R	CAGCACCCGCATCAAAG	55	

2.6. Data Analysis of qRT-PCR Data and AS Event

Quantitative data are presented as mean $\pm$ standard error of the mean (SEM). Comparisons between the E8 and E14 groups were performed using a two-tailed Student's *t*-test based on the mean values obtained from four biological replicates per group. Significance thresholds were set at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). Data analysis and visualization were primarily performed using GraphPad Prism 9.0. For AS event proportions, the proportion of each event type was calculated within every individual sample. Differences between E8 and E14 were assessed using two-sided Wilcoxon rank-sum tests ($n = 4$ biological replicates per stage). *p*-values were adjusted for multiple testing across

the seven event types using the Benjamini–Hochberg false-discovery-rate (FDR) procedure. Significance thresholds were set at $FDR < 0.05$ (*). The proportions are compositional (they sum to one within each sample) and are therefore not statistically independent; this limitation is acknowledged, and a fully compositional analysis would be desirable in future studies with larger sample sizes. Transcript-level and gene-level differential expression analyses of the Nanopore sequencing data were performed using DESeq2 as described in Section 2.4.

3. Results

3.1. Overview of Sequencing Information and Identification of Novel Transcripts

To comprehensively characterize the transcriptomic features of quail skeletal muscle at critical embryonic developmental stages (E8 and E14), we performed full-length transcriptome sequencing on eight samples using ONT nanopore sequencing. Raw sequencing data were filtered using NanoFilt (v2.8.0; parameters: -q 7 -l 50) to obtain clean data for subsequent analysis. Sequence statistics were generated using SeqKit (v0.12.0). The resulting data quality metrics are summarized in Table 2. Full-length sequences were identified using Pychopper (v2.4.0; parameters: -Q 7 -z 50), which orients, trims, and repairs fused Nanopore cDNA sequences. The resulting full-length sequences were further filtered with NanoFilt (v2.8.0; parameters: -q 7 -l 50). Summary statistics for the full-length sequences are presented in Table 3. Note that the per-sample sequence counts in Table 3 are distinct from the final set of 5260 non-redundant novel transcripts obtained after assembly and redundancy removal. The number of raw reads per sample ranged from 4.49×10^6 to 5.01×10^6 . After stringent quality control, the mapping rate of clean reads consistently ranged from 96% to 98%. Sequence statistics indicated that the mean sequence length per sample ranged from 1011 bp to 1176 bp, with N50 values distributed between 1370 bp and 1643 bp. The longest transcript observed in the filtered data reached 113,383 bp. These metrics affirm the capacity of ONT sequencing to generate high-quality full-length transcript data from quail skeletal muscle, providing a reliable foundation for subsequent transcript- and gene-level analyses.

By aligning the sequencing data to the reference genome, we generated a comprehensive transcriptome atlas of quail skeletal muscle. After removing redundant sequences, the length distribution of transcripts exhibited a broad continuum (Supplementary Figure S1A), with an N50 value reaching 5312 bp, further validating that full-length transcriptome sequencing can efficiently capture transcripts. To further characterize these novel transcripts, we performed classification annotation using the Gffcompare tool (Supplementary Figure S1B). The results showed that transcripts completely contained within the intronic regions of reference transcripts (class i) accounted for the highest proportion (3233), followed by exonic overlap on the opposite strand (class x, 972) and unknown transcripts (class u, 596). Coding potential of the 5260 non-redundant novel transcripts was assessed using TransDecoder, CPC2, CNCI, and PLEK. A total of 642 novel transcripts (12.21%) were predicted to have coding potential by at least one of the four tools and were therefore considered to possess coding capacity. These same 642 transcripts received functional annotation in public databases (Table 4 and Supplementary Table S2). The CDS length distribution of novel transcripts exhibited a clear right-skewed pattern, with an N50 of 606 bp (Supplementary Figure S1C).

To further elucidate the biological significance of these novel transcripts, we aligned them to multiple public databases for functional annotation (Table 4). After transcript assembly, comparison with the reference annotation using gffcompare and redundant transcripts clustering, a total of 5260 non-redundant novel transcripts were submitted to the functional annotation. Among the 5260 non-redundant novel transcripts, a total of

642 (12.21%) received functional annotation (Supplementary Table S2). The Uniprot (616, 11.71%) and Nr (613, 11.65%) databases yielded the highest annotation rates, while the GO and KEGG databases annotated 491 (9.33%) and 480 (9.13%) transcripts, respectively. Additionally, Pfam, Pathway, and transcription factor (TF) analyses provided important clues for deciphering the regulatory roles of these novel transcripts in skeletal muscle development.

Table 2. Sequencing data information for eight samples.

Sample	Raw Reads	Clean Reads	Mapped Reads	Mapping Rate	MaxLen	AvgLen
E141	4,861,221	4,819,660	3,684,656	97%	88,137	1271
E142	4,935,992	4,880,436	3,705,941	96%	57,394	1260
E143	4,980,057	4,913,608	3,726,091	97%	113,383	1251
E144	5,007,182	4,955,100	3,819,428	97%	45,316	1285
E81	4,739,321	4,697,290	3,640,080	98%	52,210	1377
E82	4,490,127	4,445,945	3,390,504	98%	42,628	1452
E83	4,567,160	4,524,282	3,440,226	98%	64,147	1445
E84	4,575,976	4,539,080	3,479,176	98%	36,809	1462

Note: Raw reads: number of raw sequencing reads; Clean reads: number of reads remaining after quality filtering with NanoFilt (v2.8.0; -q 7 -l 50); Mapped reads: number of reads successfully aligned to the reference genome; Mapping rate: percentage of Clean reads that were mapped; MaxLen and AvgLen: maximum and average lengths of the clean reads.

Table 3. Full-length sequence data statistics.

Sample Name	Seq Num	Mean Length (bp)	N50 (bp)	Max Length (bp)
E141	3,809,766	1016	1373	21,039
E142	3,849,092	1011	1376	19,459
E143	3,860,391	1028	1370	19,077
E144	3,945,353	1047	1384	21,264
E81	3,700,461	1126	1459	22,010
E82	3,464,122	1166	1639	20,464
E83	3,527,057	1170	1643	23,870
E84	3,555,531	1176	1637	21,140

Note: Seq Num: number of full-length sequences retained after Pychopper (v2.4.0; -Q 7 -z 50) processing for each sample; Mean Length, N50 and Max Length: length statistics of these full-length sequences. These per-sample counts are distinct from the final assembled, non-redundant transcript set reported elsewhere in the manuscript. The per-sample N50 values of the full-length sequences ranged from 1370 to 1643 bp, while the N50 of the merged, redundancy-filtered transcript set reached 5312 bp.

Table 4. Summary of functional annotation results for novel transcripts.

Item	Count	Percentage
All	5260	100.00%
Annotation	642	12.21%
KEGG	480	9.13%
Pathway	258	4.90%
Nr	613	11.65%
Uniprot	616	11.71%
GO	491	9.33%
KOG	5	0.10%
Pfam	390	7.41%
TF	99	1.88%

Note: The total number of non-redundant novel transcripts ($n = 5260$) is used as the denominator for calculating all percentages shown in the table.

3.2. Transcriptome Structural Analysis

Alternative splicing (AS) is a key mechanism that increases transcriptomic and proteomic diversity. This study identified a total of seven types of AS events, including

alternative 3' splice site (A3), alternative 5' splice site (A5), mutually exclusive exons (MX), alternative first exon (AF), alternative last exon (AL), retained introns (RI), and skipping exon (SE). As shown in Figure 1A, the E8 and E14 stages exhibited significant differences in the number of each AS event type, with AF and SE representing the predominant splicing modalities in quail skeletal muscle. Further proportion analysis (Figure 1B) revealed that the proportion of AF was significantly higher at E14 compared to E8 ($FDR < 0.05$), whereas SE showed a higher frequency at E8 ($FDR < 0.05$), suggesting that these specific splicing patterns may serve distinct regulatory functions during skeletal muscle differentiation. Additionally, analysis of simple sequence repeats (SSRs) within transcripts (Figure 1C) showed that mononucleotide repeats (p1) were predominant among all SSR types (57.40%), followed by complex repeats (c, 14.87%) and trinucleotide repeats (p3, 13.51%).

To assess transcriptional complexity in quail, we analyzed the distribution of transcripts and genes (Figure 1D). Among all detected transcripts, 49,678 corresponded to known transcripts and 5260 represented non-redundant novel transcripts. In addition, 594 novel genes were identified. Gene structural complexity analysis (Figure 1E) indicated that most genes tended to produce one to two transcripts, but a considerable proportion of genes exhibited highly complex transcriptional patterns. High-confidence lncRNAs were identified as those predicted to be non-coding by the intersection of CPC2, CNCI, and PLEK, yielding a final set of 2692 candidate lncRNAs (Figure 1F, Supplementary Table S3). The remaining 1926 transcripts produced discordant predictions across the tools and were not assigned to either category. These transcripts likely contain both potentially coding and non-coding sequences whose functional classification requires further experimental validation. Genomic location classification analysis of these lncRNAs (Figure 1G) showed that intronic lncRNAs were the most abundant (1924), followed by antisense lncRNAs (465) and intergenic lncRNAs (lincRNAs, 296), while sense lncRNAs were extremely rare. These results indicate the existence of a complex RNA regulatory network during quail skeletal muscle development.

3.3. Comparative Transcript Analysis of Quail Skeletal Muscle Developmental Stages

To reveal the transcriptomic dynamics before and after quail skeletal muscle differentiation (E8 vs. E14), we first conducted a comprehensive assessment of transcript expression levels across all samples (Supplementary Table S4). It should be noted that the totals shown in Figure 1D represent all detected genes and transcripts. The downstream analyses (including differential expression, Venn diagrams, and functional enrichment) were performed exclusively on expressed genes and transcripts ($TPM > 0$). Box plots of TPM expression distributions (Figure 2A) displayed highly consistent expression distributions across the eight samples. PCA revealed clear separation between the E8 and E14 samples along the first two principal components. PC1 and PC2 explained 39.69% and 13.44% of the total variance, respectively, indicating that the major transcriptomic differences were captured by these two components (Figure 2B). Venn diagram analysis (Figure 2C) comparing transcripts detected in the two groups showed that 35,992 transcripts were shared between E8 and E14, while 6596 transcripts were specific to E8 and 2792 were specific to E14. These stage-specific transcripts may be involved in the precise regulation of different developmental phases during skeletal muscle differentiation. Based on the established differential expression screening criteria ($|\log_2(\text{fold change})| > 1$ and adjusted p -value < 0.05), we conducted an in-depth investigation of differentially expressed transcripts (DETs) between the two groups. The volcano plot (Figure 2D) illustrated the distribution pattern of DETs, identifying a total of 5934 significantly differentially expressed transcripts, including 3392 upregulated transcripts and 2542 downregulated transcripts, while 20,390 transcripts showed no significant difference between the two groups (Supplementary Table S5). The identification of a large

number of significantly differential transcripts reflects the complex molecular regulatory mechanisms underlying skeletal muscle development from E8 to E14.

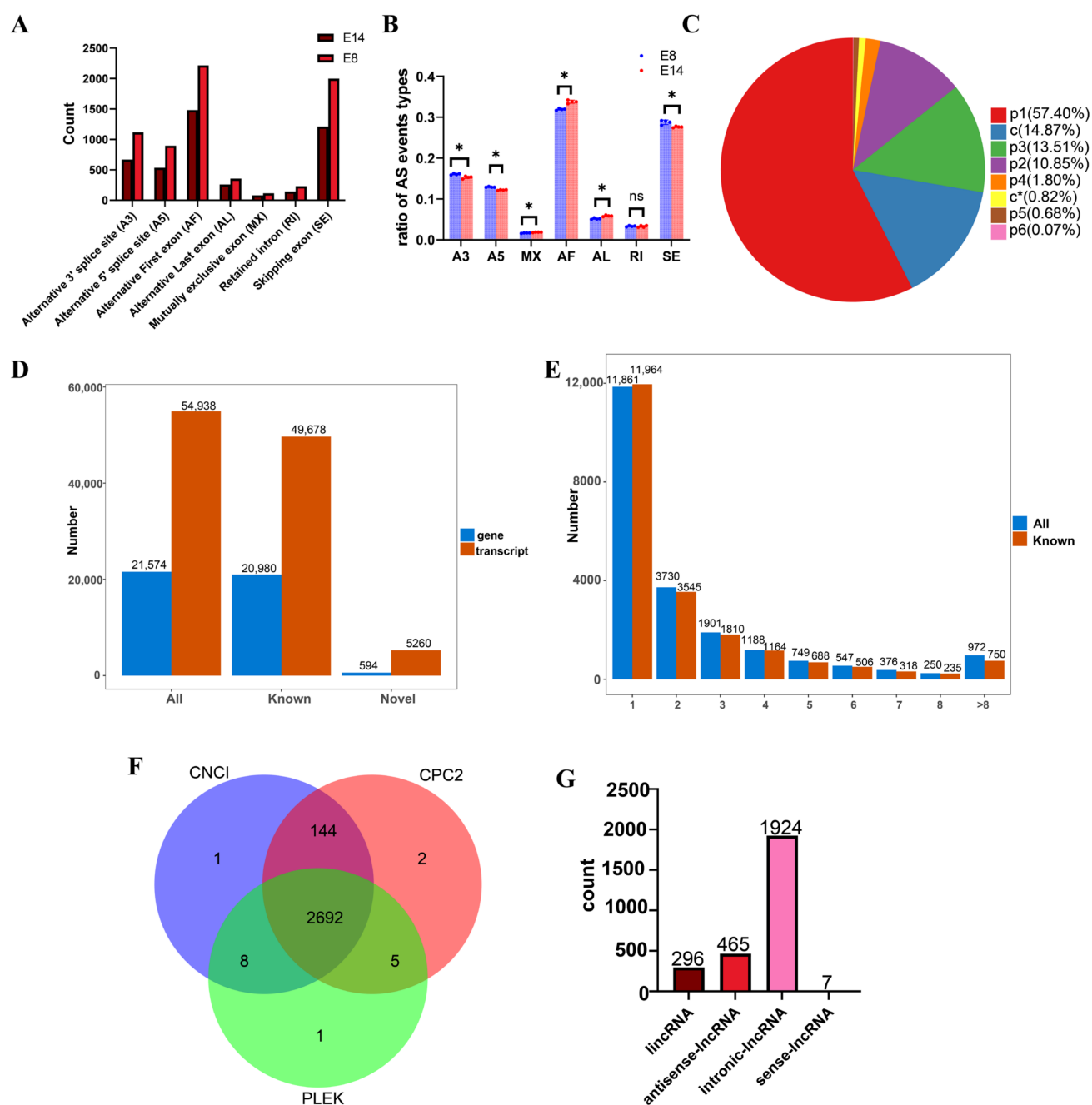


Figure 1. Analysis of transcriptome structural diversity during quail skeletal muscle development. (A) Quantitative statistics of seven types of alternative splicing (AS) events at E8 and E14 stages; (B) Comparison of the proportions of each AS event type between the two groups. Differences between E8 and E14 were assessed using two-sided Wilcoxon rank-sum tests ($n = 4$ biological replicates per stage). p -values were adjusted for multiple testing across the seven event types using the Benjamini–Hochberg FDR procedure. Significance thresholds were set at $FDR < 0.05$ (*). (C) Type distribution of simple sequence repeats (SSRs) in transcripts (c: complex repeat; p1–p4: mononucleotide to tetranucleotide repeats); (D) Statistical distribution of genes and transcripts at the total, known, and novel levels; (E) Distribution of the number of transcripts per gene; (F) Venn diagram of lncRNAs jointly predicted by three software tools (CNCI, CPC2, PLEK); (G) Statistics of different lncRNA types. Alternative 3' splice site (A3), alternative 5' splice site (A5), mutually exclusive exons (MX), alternative first exon (AF), alternative last exon (AL), retained introns (RI), and skipping exon (SE).

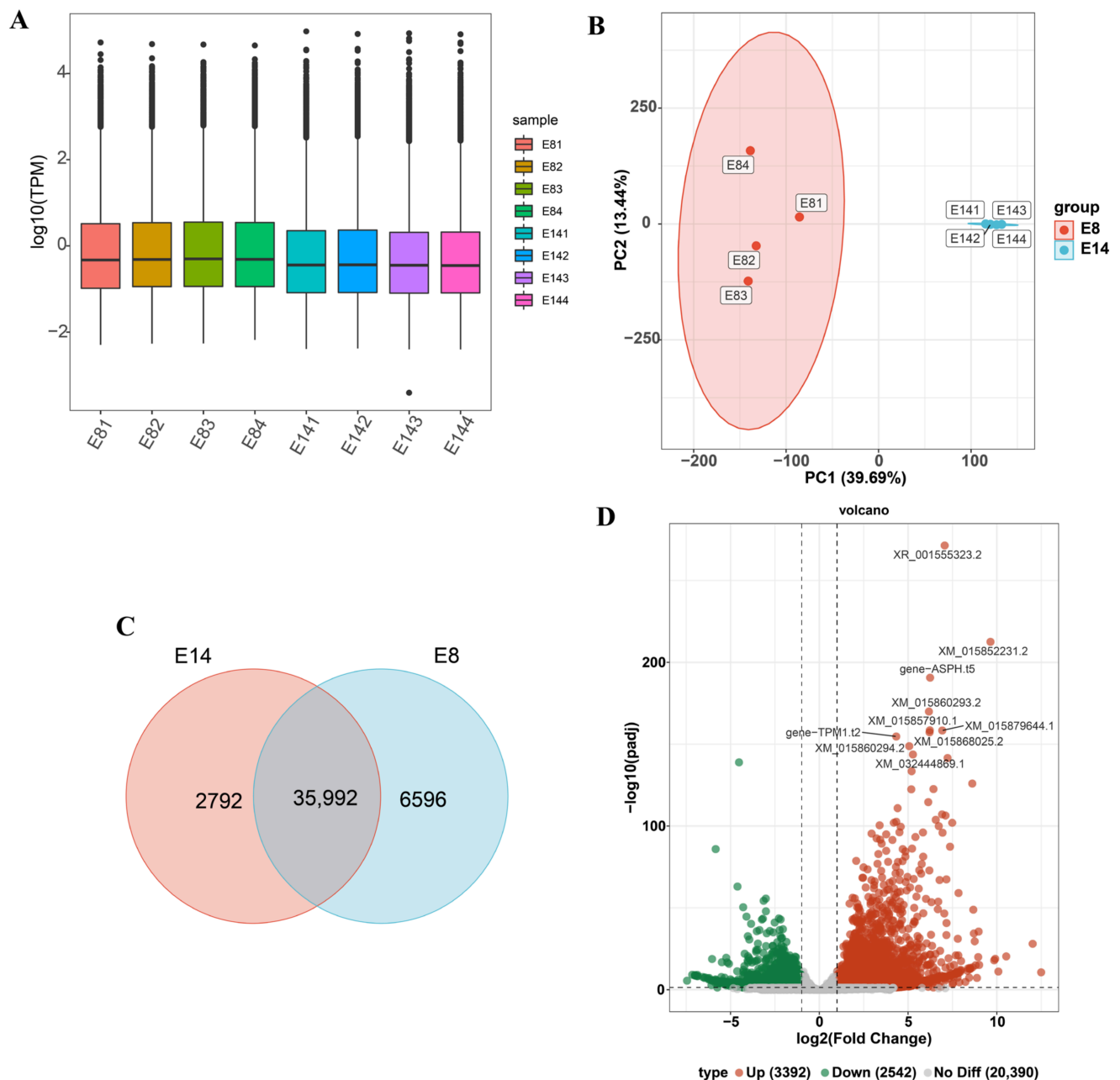


Figure 2. Comparison of transcript expression characteristics between E8 and E14 stages. (A) Box plot distribution of transcript expression levels ($\log_{10}(\text{TPM})$) for each sample; (B) Principal component analysis (PCA) plot at the transcript level; (C) Venn diagram of transcripts detected in the two groups; (D) Volcano plot of differentially expressed transcripts (DETs). Screening criteria: $|\log_2(\text{fold change})| > 1$ and adjusted p -value < 0.05 . Red represents upregulation, green represents downregulation, and gray represents no significant difference. Only expressed transcripts ($\text{TPM} > 0$) were included in this analysis.

3.4. Identification and Functional Enrichment Analysis of Differentially Expressed Genes During Skeletal Muscle Differentiation

To further elucidate the gene expression dynamics during quail skeletal muscle development, we performed PCA on the normalized gene expression data (Figure 3A). The results showed a clear separation between the E8 and E14 samples, with PC1 and PC2 explaining 50.43% and 10.79% of the total variance, respectively. Venn diagram analysis (Figure 3B) further revealed that 16,430 genes were co-expressed in both groups, while E8 and E14 possessed 1760 and 790 stage-specific expressed genes, respectively

(Supplementary Table S6). Based on differential expression analysis, we identified a total of 3745 significantly DEGs, including 2085 upregulated genes and 1660 downregulated genes (Figure 3C, Supplementary Table S7). The volcano plot showed that key genes related to muscle contraction and structural composition, such as *ACTA1*, *SLN*, *TPM1*, and *TNNT3*, exhibited significantly high expression at the E14 stage (positive $\log_2(\text{fold change})$), consistent with the terminal differentiation of skeletal muscle. In addition, the most significantly downregulated genes included *NES* (Nestin), *CRABP1*, *PENK*, *TNNT2*, and *MDK* (Figure 3C, Supplementary Table S7). These genes are consistent with the transition from the proliferative phase at E8 to the differentiation and maturation phase at E14. To elucidate the biological functions of these DEGs, we performed KEGG enrichment analysis (Figure 3D, Supplementary Table S8). The results showed that the differential genes were primarily enriched in pathways related to skeletal muscle development and energy metabolism. Among these, pathways related to muscle structure included Focal adhesion, Motor proteins, and Cytoskeleton in muscle cells, while pathways related to energy metabolism encompassed Oxidative phosphorylation, Metabolic pathways, and Citrate cycle (TCA cycle). These findings suggest that muscle structural remodeling and metabolic changes are coordinately regulated during skeletal muscle differentiation. GSEA enrichment analysis further validated this developmental dynamic (Figure 3E,F, Supplementary Table S9). The results showed that the Cell cycle pathway was significantly enriched at the E8 stage, suggesting that this period primarily involves cell proliferation processes. At E14, the 'Cardiac muscle contraction' (ko04260) pathway was significantly enriched. This enrichment was primarily driven by the strong upregulation of genes encoding contractile and structural proteins that are shared between cardiac and skeletal muscle, including *TPM1/2/3*, *TNNT3*, *MYL1/3*, *ACTA1*, and several calcium-handling genes. This indicates advanced myofibril assembly and maturation in skeletal muscle at this developmental stage. The above analysis indicates that quail skeletal muscle undergoes cell proliferation at E8 and completes skeletal muscle assembly and metabolic functional maturation at E14. Leading edge gene analysis further confirmed that several of these pathway genes, including *TPM1/2/3*, *MYL3*, *TNNC1* and *ATP2A3* in the Cardiac muscle contraction pathway, overlap with the significantly upregulated DEGs identified in Supplementary Table S7, reinforcing the coordinated structural and metabolic maturation at E14.

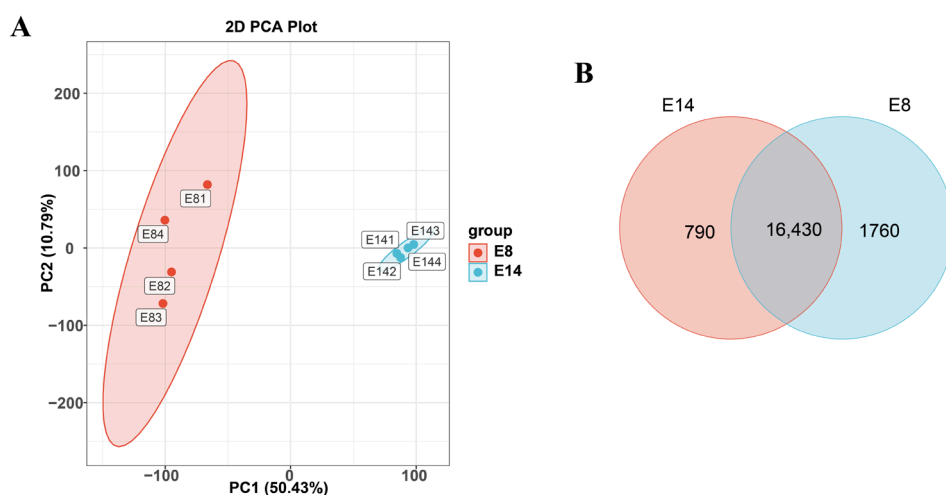


Figure 3. Cont.

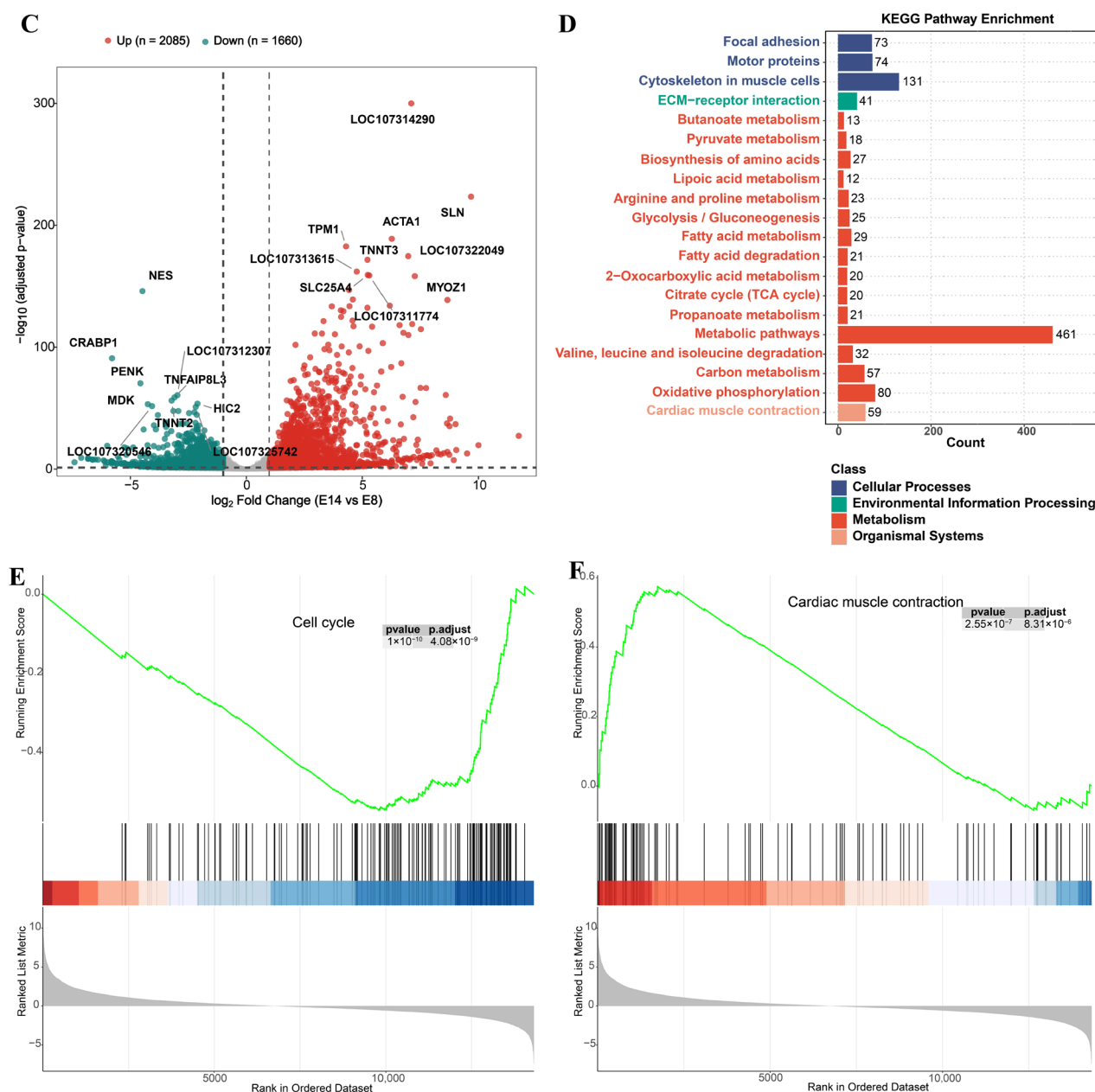


Figure 3. Functional enrichment analysis of differentially expressed genes. (A) PCA plot at the gene level; (B) Venn diagram of genes detected in the two groups; (C) Volcano plot of differentially expressed genes (screening criteria same as Figure 2); (D) KEGG pathway enrichment analysis of differentially expressed genes; (E) GSEA showing enrichment of the Cell cycle pathway (E8 stage). The leading-edge genes include multiple CCN family cell cycle regulators that are differentially expressed at E8; (F) GSEA showing enrichment of the Cardiac muscle contraction pathway (E14 stage). The leading-edge genes include key candidate genes such as *TPM1*, *TPM2*, *TPM3*, *MYL3*, *TNNT1* and *ATP2A3*, all of which are significantly upregulated DEGs at E14 (Supplementary Table S7). Only expressed genes (TPM > 0) were included in this analysis.

To identify highly connected genes in the interaction network during quail skeletal muscle differentiation, we selected the top 200 genes with the smallest adjusted *p*-value from the differential expression analysis to construct a PPI network (Figure 4). The network was mainly composed of two core functional clusters. The first cluster primarily consisted of genes related to skeletal muscle development, including members of the MYL family (e.g., *MYL1*, *MYL3*), *TNNT* family (e.g., *TNNT1*, *TNNT3*), *MYOZ1*, *MYO18B*, and the *TPM*

family (e.g., *TPM1*, *TPM3*). The second cluster was concentrated on energy metabolism pathways, containing multiple *NDUFA*, *NDUFB*, and *NDUFS* family members, which encode subunits of mitochondrial respiratory chain complex I and play a core role in maintaining muscle energy supply.

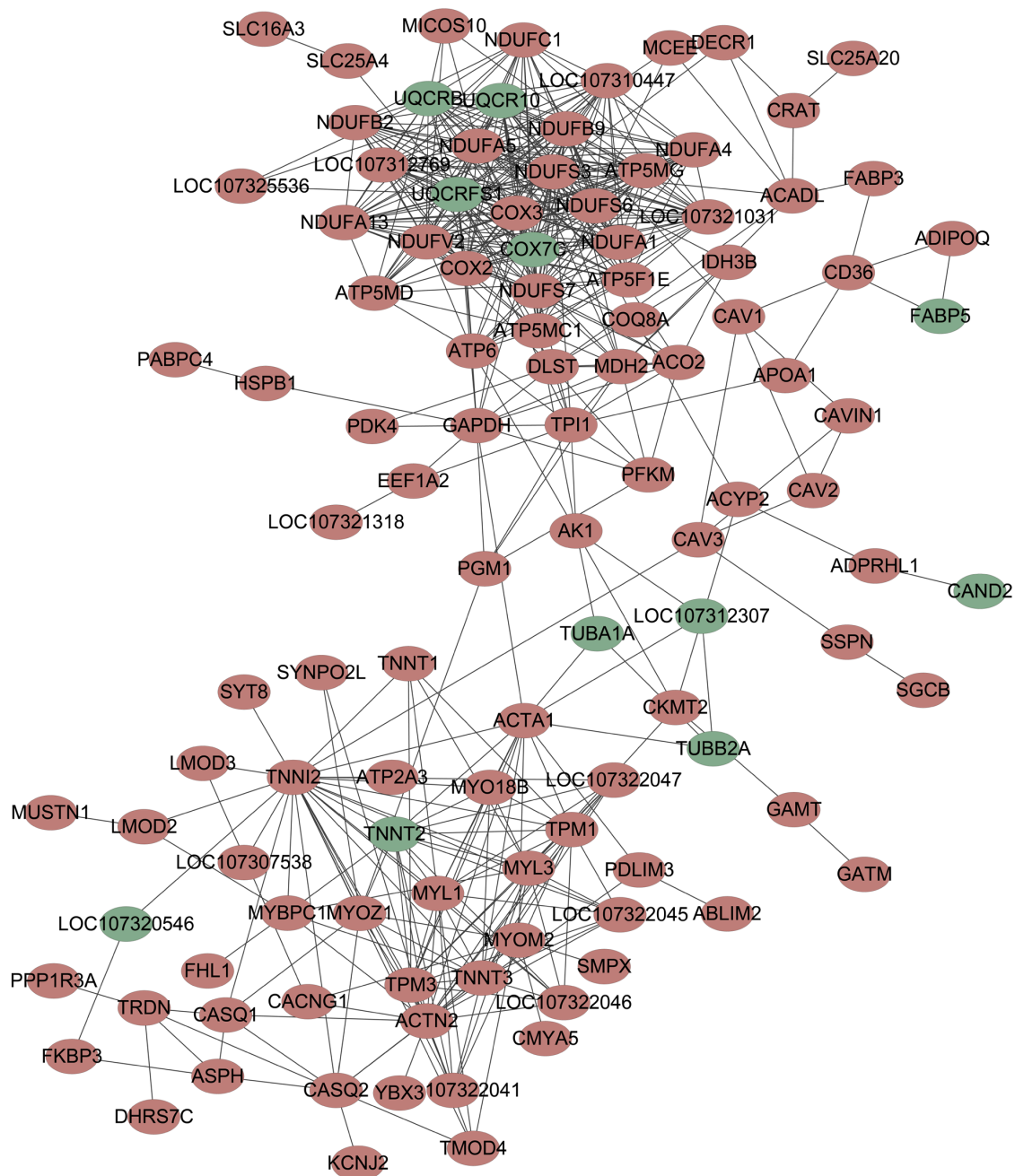


Figure 4. PPI network analysis of differentially expressed genes. The top 200 differential genes with the smallest adjusted *p*-value were selected to construct the PPI network; only genes with at least one interaction are shown (isolated nodes were removed). Red nodes represent genes upregulated at the E14 stage, and green nodes represent genes upregulated at E8. Neither node size nor edge thickness was used to encode additional information such as degree or interaction confidence.

3.5. Quantitative Validation of Sequencing Data

To validate the reliability of the full-length transcriptome sequencing data, this study selected four significantly upregulated genes (*TCAP*, *SYPL2*, *SLN*, *MYOZ1*) and four significantly downregulated genes (*NES*, *GAS2*, *PENK*, *CRABP1*) from the E8 vs. E14 comparison group for qRT-PCR quantitative analysis. To assess the concordance between

RNA-seq and qRT-PCR results, we calculated the $\log_2$ (fold change) (E14 versus E8) for each of the eight selected genes from both platforms. Pearson correlation analysis of these fold-change values showed strong concordance between the two methods ($R = 1$, $p < 0.001$; Figure 5B). Individual biological replicate values are displayed in Figure 5A. It should be noted that these genes were selected based on the RNA-seq results; therefore, the analysis demonstrates concordance among the selected genes rather than independent validation of the differential expression calls.

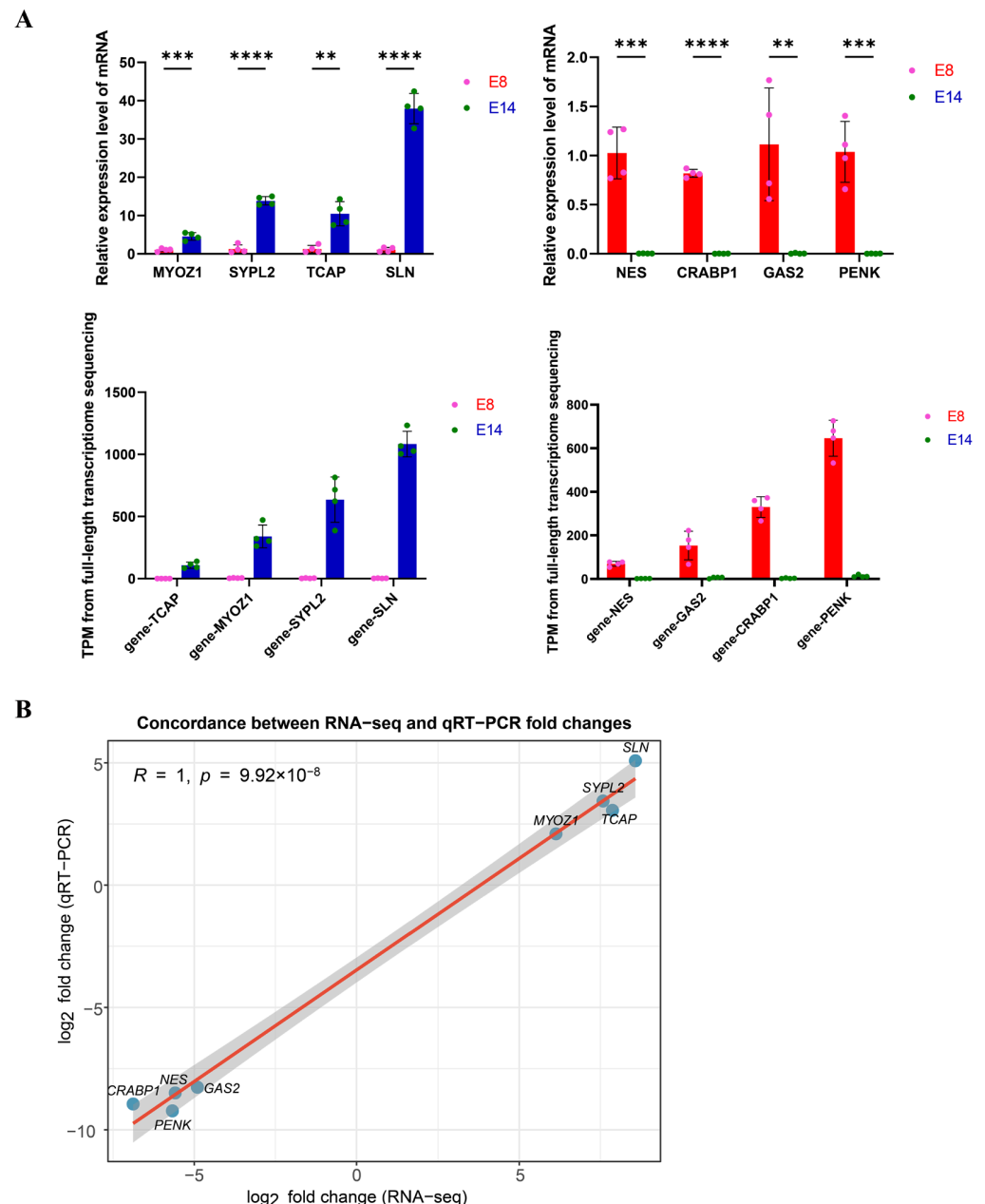


Figure 5. Correlation analysis between Nanopore RNA-seq and qRT-PCR data. (A) Relative expression levels of eight selected genes measured by qRT-PCR (upper panels) and corresponding TPM values from Nanopore full-length transcriptome sequencing (lower panels). Data are shown as mean $\pm$ SEM with individual biological replicates ($n = 4$ per stage). Statistical significance was determined by two-tailed Student's t -test (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). (B) Concordance between RNA-seq and qRT-PCR results. The $\log_2$ (fold change) (E14 vs. E8) calculated from RNA-seq TPM values was plotted against the $\log_2$ (fold change) calculated from qRT-PCR data for the eight selected genes. The black line represents the linear regression fit with 95% confidence interval (gray shading). Pearson's correlation coefficient (R) and p -value are indicated.

4. Discussion

The quail (*Coturnix japonica*), with its short reproductive cycle and high genomic synteny with the chicken, has emerged as an ideal model organism for avian skeletal muscle development and genetic improvement research [4,20]. Based on ONT full-length transcriptome sequencing, this study systematically characterized the key transcriptional features of quail leg muscle at E8 (pre-differentiation stage) and E14 (later differentiation stage). By constructing a high-resolution full-length transcriptional atlas, we identified a large number of novel transcripts and DEGs, providing insights into the transcriptome dynamics during the transition from cell proliferation to functional maturation. This study provides a high-resolution full-length transcriptomic resource for quail skeletal muscle development and helps address the current lack of isoform-level annotation in this species and offers new insights into stage-specific transcript diversity and regulatory candidates during myogenesis.

Transcriptome structural analysis demonstrated the significant advantages of ONT technology in capturing complex transcript structures. Among the novel transcripts identified in this study, intronic transcripts (Gffcompare class i) accounted for the highest proportion, underscoring the utility of long-read sequencing in refining genome annotations and identifying potential transcript isoforms [12]. According to gffcompare classification, class i transcripts are those that lie entirely within a reference intron. Only 12.21% of the 5260 non-redundant novel transcripts were functionally annotated, which is relatively low but not uncommon in long-read transcriptome studies of non-model avian species [21,22]. This low annotation rate likely reflects the substantial proportion of lncRNAs identified in this study (2692 candidates), many of which remain poorly annotated in current databases. However, it is also important to consider that some novel transcripts may represent assembly artifacts or false positives inherent to long-read sequencing technologies. Therefore, while these novel transcripts expand our understanding of quail transcriptome complexity, their biological authenticity and functional relevance require experimental validation in future studies.

AS, as a key link in post-transcriptional regulation, serves stage-specific regulatory functions in skeletal muscle development [23]. Our analysis identified AF and SE as the predominant splicing modalities, with their stage-specific enrichment patterns suggesting distinct regulatory roles during myogenesis. This pattern mirrors observations in muscle development of other poultry species such as chickens and ducks [15,24]. The predominance of alternative splicing events and the strong upregulation of contractile genes at later embryonic stages have also been observed in duck [22]. Additionally, the high proportion of intronic lncRNAs in our dataset aligns with reports in chicken skeletal muscle [25]. Furthermore, the 2692 lncRNAs identified through joint prediction further enriched the regulatory landscape of quail skeletal muscle. Intronic lncRNAs constituted the largest class in our dataset. This distribution is similar to that reported in chicken skeletal muscle, where intronic lncRNAs have been proposed to fine-tune host gene splicing or expression during myogenesis [25]. Compared to our previous study based on short-read sequencing [4], the present full-length transcript data substantially improves the completeness of lncRNA annotation. We hypothesize that the lncRNAs identified in this study, particularly the abundant intronic lncRNAs, may contribute to the regulation of quail skeletal muscle development through ceRNA networks or chromatin remodeling mechanisms [25]. While intronic lncRNAs have been reported to potentially regulate host gene expression or alternative splicing in some contexts, their functional relevance in avian skeletal muscle development should be interpreted with caution. Many intronic transcripts may represent transcriptional by-products or have limited functional activity [26]. Therefore, the biolog-

ical roles of these predominantly intronic lncRNAs in quail myogenesis warrant further experimental investigation.

Transcriptomic dynamic analysis revealed the molecular trajectory of skeletal muscle transitioning from proliferation to maturation [27]. Consistent with our previous short-read transcriptome study in the same quail line [4], we observed a clear transcriptomic shift between E8 and E14 [6]. However, the long-read data additionally revealed a much higher resolution of alternative splicing events and novel transcript isoforms that were previously undetectable, highlighting the advantage of Nanopore sequencing in capturing stage-specific transcript diversity during avian myogenesis. KEGG and GSEA analyses further confirmed that the E8 stage was dominated by the Cell cycle pathway, while the E14 stage was significantly enriched in Cardiac muscle contraction and muscle structural assembly-related pathways. Although named after cardiac muscle, this KEGG pathway contains numerous genes encoding sarcomeric and contractile proteins that are functionally shared between cardiac and skeletal muscle. The observed enrichment at E14 largely reflects the coordinated upregulation of these shared structural genes during the final stages of myofiber maturation in skeletal muscle, rather than indicating cardiac-specific processes [28]. This interpretation is consistent with our previous histological observations in the same quail line, where E8 corresponded to active myoblast proliferation and E14 to advanced myofiber structural maturation [4]. This proliferative-to-differentiative transition aligns closely with muscle development patterns observed in species such as chickens and ducks [21,29,30]. PPI network analysis further supported this developmental dynamic. By analyzing core interaction modules, we found that structural gene families such as MYL, TNNT, and TPM [22,31,32], along with energy metabolism genes of the NDUF family [33,34], formed highly connected interaction modules. *TPM1/2/3* and *TNNT3* encode tropomyosin and fast skeletal troponin T, respectively. Their coordinated upregulation at E14 is consistent with the acceleration of myofibril maturation observed histologically in quail and chicken [4,24]. *MYOZ1*, encoding a Z-disc protein, was strongly upregulated at E14. Transcriptomic studies in ducks have identified *MYOZ1* as a key DEG during late embryonic and post-hatch skeletal muscle development, suggesting its important role in Z-disc organization and myofiber maturation in avian species [22,30]. Several genes showed strong downregulation at E14, notably *NES*, *CRABP1*, and *PENK*. *NES* is typically expressed in muscle progenitor and satellite cells and is known to decrease as myoblasts commit to terminal differentiation [35]. Its downregulation at E14 is therefore consistent with the completion of the proliferative phase and the progression toward mature myofibers. The coordinated upregulation of NDUF family genes at E14 suggests increased mitochondrial respiratory capacity, which is required for the metabolic transition toward oxidative phosphorylation during late-stage myofiber maturation [33,34]. This coordinated structural-metabolic remodeling not only corroborates the established paradigm that skeletal muscle functional maturation is accompanied by energy metabolism change but also offers a framework for identifying candidate genes potentially acting as regulatory nodes during myogenesis.

Although this study obtained rich high-resolution data, several limitations should be acknowledged. While these findings highlight key regulatory candidates and pathways, functional validation through gene editing or other experimental approaches will be required to confirm their roles in quail myogenesis. First, the samples only included leg muscle tissue; future investigations should encompass different muscle groups such as pectoral muscle to resolve tissue-specific heterogeneity. Second, functional validation of newly identified genes, transcripts, and lncRNAs through approaches such as gene knockdown/overexpression and luciferase reporter assays is warranted to confirm their regulatory roles. Third, the inherent base-calling accuracy limitations of ONT sequencing

may affect the precision of transcript identification, although this is partially mitigated by the high sequencing depth employed. Fourth, although we identified thousands of novel transcripts and 2692 candidate lncRNAs, the structures of these novel transcripts were not experimentally validated by RT-PCR or Sanger sequencing. Their functional roles in skeletal muscle development therefore remain to be confirmed in future studies. Fifth, while Oxford Nanopore sequencing enables full-length transcript detection, inherent base-calling errors may affect the accuracy of transcript boundaries and isoform identification. No short-read hybrid correction or consensus polishing was applied in this study. We instead relied on high sequencing depth combined with strict quality filtering to ensure the reliability of the identified full-length transcripts and novel isoforms. Future studies may benefit from hybrid correction approaches when short-read data are available. Only female embryos were used in this study after *CHD1*-based sex identification. Although this design improves biological consistency among samples and overt sexual dimorphism in skeletal muscle has not yet emerged at the E8–E14 stages, the findings cannot be directly generalized to male embryos. Future studies that include both sexes would help determine whether sex-specific transcriptomic differences exist during early skeletal muscle development in quail. Future research will integrate multi-omics data (e.g., proteomics, epigenomics) and combine techniques such as single-cell sequencing to further elucidate the fine regulatory landscape of quail skeletal muscle development, providing stronger theoretical support for precision breeding and production performance improvement in poultry.

5. Conclusions

In summary, this study presents the first high-resolution full-length transcriptional atlas of quail leg muscle from E8 to E14 embryonic stages. We identified a large number of known and novel transcripts and genes, revealed the dynamic alternative splicing landscape dominated by AF and SE, and identified a series of lncRNAs. Differential expression analysis and functional enrichment confirmed significant transcriptome dynamics during quail skeletal muscle development, accompanied by a coordinated transition from cell proliferation to structural assembly and metabolic maturation. This study provides a high-resolution full-length transcriptomic resource for quail skeletal muscle and identifies candidate genes and pathways involved in stage-specific myogenesis. These findings lay a foundation for future functional studies and may inform molecular breeding strategies aimed at improving growth and meat quality traits in quail.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agriculture16141554/s1>, Supplementary Table S1: Summary of software tools and databases with versions and main functions used in the bioinformatics analysis; Supplementary Table S2: Information of annotated novel transcripts; Supplementary Table S3: Information of identified lncRNAs; Supplementary Table S4: Information of all identified transcripts; Supplementary Table S5: Differentially expressed transcripts between E8 and E14. Supplementary Table S6: Information of all identified genes; Supplementary Table S7: Differentially expressed genes between E8 and E14; Supplementary Table S8: KEGG pathway enrichment analysis results of differentially expressed genes between E8 and E14; Supplementary Table S9: Gene Set Enrichment Analysis (GSEA) results of genes between E8 and E14. Supplementary Figure S1: Identification and structural characterization of the full-length transcriptome in quail skeletal muscle.

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Data Availability Statement: The raw sequencing data generated in this study have been deposited in the National Genomics Data Center (NGDC) Genome Sequence Archive (<https://ngdc.cncb.ac.cn/gsub/>, accessed on 17 July 2026). Accession number: CRA044793.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

MRF	myogenic regulatory factor
ncRNAs	non-coding RNAs
AS	alternative splicing
ONT	oxford nanopore technologies
DET	differentially expressed transcript
DEG	differentially expressed gene
E8	embryonic day 8
E14	embryonic day 14
GO	gene ontology
KEGG	Kyoto encyclopedia of genes and genomes
N50	the length of the shortest read in the group of longest sequences that together represent (at least) 50% of the nucleotides in the set of sequences
MaxLen	maximum length of the data
AvgLen	average length of the data
Seq Num	number of sequences
CDS	coding sequence
A3	alternative 3' splice site
A5	alternative 5' splice site
MX	mutually exclusive exons
AF	alternative first exon
AL	alternative last exon
RI	retained introns
SE	skipping exon
SSRs	simple sequence repeats
lncRNAs	long non-coding RNAs
lincRNAs	intergenic lncRNAs
TPM	transcripts per million
PCA	principal component analysis
PPI	protein–protein interaction
ceRNA	competing endogenous RNA
GSEA	gene set enrichment analysis
TF	transcription factor
ORF	open reading frame

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