

Article

Transcriptome Sequencing Reveals Candidate Genes and Signalling Pathways for Skeletal Muscle Development in Ningxiang Pig

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ABSTRACT: Ningxiang pigs are resistant to roughage, have good meat quality, and are rich in polyunsaturated fatty acids. However, their growth rate is slow, and meat yield is low; therefore, it is necessary to analyse the molecular mechanisms of their muscle growth and development. Genes are important molecules that are involved in muscle growth and development. In this study, the transcripts of difference tables of Ningxiang pigs at two developmental stages (30 and 150 days after birth) were identified by transcriptome sequencing, and the molecular mechanism of their regulation of muscle development was further explored. A total of 22,411 transcripts were identified by transcriptome sequencing, and 9021 transcripts were obtained after screening with TPM > 1. Differential expression analysis revealed 1322 differentially expressed transcripts, 978 of which were up-regulated and 344 were downregulated. There were 1125 known and 197 novel identified differentially expressed transcripts, 823 upregulated and 302 downregulated known transcripts, and 155 upregulated and 42 downregulated novel transcripts. Quantitative PCR verification was consistent with the sequencing results. After gene ontology (GO) enrichment analysis of the differentially expressed genes, 15 known genes (15 transcripts) and six new genes (8 transcripts) related to muscle were screened. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis revealed that muscle-related genes were significantly enriched in the FoxO, MAPK, cAMP, IL-17, and PI3K-Akt signalling pathways. The prediction of PPI and transcription factors of 15 known genes showed that there were significant interactions among nine proteins (ATF3-EGR1-EGR2-FOS-HOMER1-KLF5 and KLHL40-LMOD2-MYOZ2) and eight transcription factors (*KLF5*, *COPS2*, *FOXN2*, *EGR2*, *ATF3*, *EGR1*, *FOS*, *PAX7*) among the 15 proteins. Furthermore, we elucidated the role of candidate gene *HOMER1* in myoblast proliferation and differentiation. These results provide a theoretical basis for studying the mechanisms underlying muscle development in Ningxiang pigs.

Keywords: Ningxiang pig; Transcriptome; Skeletal muscle development; Candidate gene; Signal pathway; PPI

1. Introduction

Intramuscular fat content is an important factor affecting meat quality and can affect the flavour, tenderness, juiciness, and fatty acid content of muscles [1–3]. The Ningxiang pig is a fat-type native pig breed in China that has the advantages of high intramuscular fat content [4] and significantly higher arachidonic acid and docosahexaenoic acid contents than foreign pig breeds [5]. However, the slow growth rate of Ningxiang pigs and the unclear molecular mechanism of muscle development have seriously restricted the development and utilisation of Ningxiang pigs; therefore, it is urgent to analyse the molecular mechanism of muscle development in Ningxiang pigs.

Muscle development is regulated by several genes. Although candidate genes such as *PAX3/PAX7* [6], *MyoD*, *Myf5*, *Myog*, *MRF4* [7–9], *MSTN*, and *MyHC* [10] have been identified, there may be differences in the genes that play major roles among different species. Transcriptomes can explain the functional elements of the genome, reveal the molecular composition of cells and tissues, and explore the molecular mechanisms of complex traits [11,12]. With rapid developments in science and technology, transcriptomes have been widely used for disease diagnosis [13,14], organ growth and development [15], and genetic improvement [16]. Ma et al. assembled the reference genome of Ningxiang pigs through *de novo* genome sequencing, which provided a reference for further studies of gene function and molecular breeding of Ningxiang pigs [17]. Li et al. found that 1171 circRNAs (circular RNA) were significantly differentially expressed in the *longissimus dorsi* (LD) muscle of Ningxiang pigs over four periods, and further analysis revealed that the host genes of these circRNAs were significantly enriched in AMPK and other signalling pathways regulating muscle development [18]. Liufu et al. sequenced the transcriptome of the LD muscle of Ningxiang pigs over three time periods (1, 60, and 210 d), and identified candidate genes affecting muscle growth and development, including *RIPOR2*, *MEGF10*, *KLHL40*, *PLEC*, *TBX3*, *FBP*, and *HOMER1* [19]. Pig-specific non-core genes may exist in the development of different pig breeds [20,21]; therefore, it is not sufficient to mine the key genes involved in muscle development in different pig breeds using published public reference genomes. Therefore, the reassembled genome of this species could be used as a reference genome for identifying specific genes. Xu et al. identified the structural variation gene *MYL4* in the LD muscle of Ningxiang pigs, which inhibits myoblast proliferation and promotes apoptosis and differentiation [22]. Raza et al. found that *KLF6* promotes the proliferation of bovine preadipocytes [23]. *GPX2* regulates the development of porcine preadipocytes and myoblasts through the MAPK signalling pathway [24], *PDLIM5* negatively regulates the proliferation and differentiation of porcine myoblasts [25], *CDC23* promotes the proliferation and differentiation of porcine myoblasts [26], *SATB2* mediates histone acetylation as a transcription factor to promote the proliferation of porcine myoblasts [27], and *ZBED6* deletion alleviates muscle atrophy induced by sepsis [28]. Ai et al. identified differentially expressed circRNAs (circTTN) in Ningxiang pig muscle tissue and further verified circTTN's role in the development of skeletal muscle cells. The results showed that circTTN inhibits host gene transcription and myogenesis by binding to the PURB protein [29]. However, the functional verification of the genes and circRNAs identified in Ningxiang pigs is still not sufficiently deep, and further research is needed. Our previous study showed that Ningxiang pigs grew rapidly from weaning to pre-fattening, and the diameter of the muscle fibres did not change significantly after pre-fattening, which suggests that it is very important to analyse the key genes in these two stages to understand the molecular mechanism of skeletal muscle development in Ningxiang pigs [30]. Our previous research mainly analysed the molecular mechanism of the ceRNA network in muscle development in Ningxiang pigs, but it was not sufficient to mine the key genes in the rapid growth stage of muscle development; therefore, this study focused on mining key genes during the critical period of muscle development.

The key period from weaning to early fattening is the rapid muscle development in Ningxiang pigs. The weaning period for Ningxiang pigs is 30 days after birth, and the early fattening period is 150 days

after birth. In this study, the LD muscles of 30- and 150-day-old Ningxiang pigs were collected, and candidate genes and signalling pathways related to muscle development were mined by transcription sequencing to provide a reference for comprehensively analysing the mechanism of muscle development in Ningxiang pigs.

2. Materials and Methods

2.1. Experimental Animals and Sample Preparation

Three healthy 30-day-old (named N30D_M1-N30D_M3) and three 150-day-old (named N150D_M1-N150D_M3) Ningxiang boars with similar body weight were selected. Ningxiang pigs of the same age were full siblings, and Ningxiang pigs of different ages were half-siblings. Experimental pigs were obtained from the Dalong Pig Farm of Hunan Chu WeiXiang Agriculture and Animal Husbandry Co., Ltd (Changsha, China). After slaughter, the LD muscle tissue of the fourth to seventh ribs was collected in a sterile and enzyme-free EP tube, and sampling was completed within 1 h. The collected samples were temporarily stored in liquid nitrogen and then transferred to a -80°C refrigerator within 2 h for long-term preservation for follow-up experiments.

2.2. Histomorphological Analysis of LD

The LD muscles of Ningxiang pigs were fixed with an environmentally friendly muscle fixing solution (Service, Wuhan, China), dehydrated, and waxed in a dehydrator (DIAPATH, Milan, Italy). The tissues were embedded in an embedding machine (WHJJ, Wuhan, China). The embedded wax was repaired and sliced into a slicer (Leica, Vizlal, Germany) to a thickness of $4\text{ }\mu\text{m}$. In the spreading machine (KEDEE, Jinhua, China), the cut wax slices were flattened and spread on the glass slides, baked in an oven at 60°C , dried with water, dyed according to the instructions of the haematoxylin-eosin staining kit (Service, Wuhan, China), sealed, and placed in a tissue section digital scanner (3DHISTECH, Budapest, Hungary) to collect images. The long and short diameters of the muscle fibres were measured using CaseViewer 2.4 (3DHISTECH, Budapest, Hungary). Muscle fibre diameter = (long diameter + short diameter)/2. The number of muscle fibres in the images was measured using the Image J software (version 1.51). Muscle fibre density = number of muscle fibres per area of the image (mm^2).

2.3. Transcriptome Sequencing (RNA-Seq)

Total RNA from the LD was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration and purity of RNA were detected using a Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA). The integrity of RNA was detected by agarose gel electrophoresis, and the RNA integrity number (RIN) was determined using an Agilent 2100 (Agilent, Santa Clara, CA, USA). The sequencing library requires a total RNA of $5\text{ }\mu\text{g}$, a concentration of $\geq 250\text{ ng}/\mu\text{L}$, and an OD260/280 of 1.8–2.2. A TruSeqTM Stranded Total RNA Library Prep Kit (Illumina, San Diego, CA, USA) was used to construct a chain-specific sequencing library. An Illumina PE150 library was constructed and sequenced using the Illumina HiSeq 4000 sequencing platform (Shanghai Major Biomedical Technology Co., Ltd., Shanghai, China).

2.4. Read Mapping and Differential Expression Analysis

Upstream processing of the original data was performed on the server, and the sequenced original images were converted into FASTQ format data by base calling. The converted data were controlled using Fastp (version 0.17) software [31], and the quality control data were counted using SeqPrep (version 1.2) (<https://github.com/jstjohn/SeqPrep>, accessed on 10 August 2023) and Sickle (version 1.33) (<https://github.com/najoshi/sickle>, accessed on 10 August 2023). Quality control data were mapped to the

Ningxiang pig reference genome (accession number: PRJNA531381) [17] using the Hisat2 software (version 2.1.1) [32]. The compared data were assembled using StringTie (v2.2.0) [33], and a new transcript was obtained using the GFFcompare (version 1.12.6) software [34]. The results were converted and sorted using Samtools (version 0.0.9) software [35], clean reads were counted using Htseq software (v2.0.3) [36], the TPM value of each transcript was calculated using RSEM (version 1.3.5) software [37], and genes with TPM > 1 were screened for follow-up function enrichment analysis. The R software (version 3.6.3) was used to analyse downstream transcriptome data, and the DESeq2 package was used to analyse differences in gene expression [38]. The differentially transcripts/genes were screened according to the results of $\text{P}_{\text{adjust}} < 0.05$ and $|\log_2(\text{fold change})| \geq 2$ [39].

2.5. Functional Analysis of Differentially Expressed Transcripts

BioDBnet (<https://biodbnet-abcc.ncifcrf.gov/>, accessed on 10 August 2023) was used to convert gene ID [40]. The AnnotationHub R package was used to obtain annotation files, and the clusterProfiler R package was used for gene ontology (GO) enrichment analysis of the differentially expressed transcripts. The KOBAS website was used to analyse the Kyoto encyclopedia of genes and genomes (KEGG) pathways of the differentially expressed transcripts [41]. The significant enrichment standards of GO and KEGG were corrected $p < 0.05$ ($\text{P}_{\text{adjusted}} < 0.05$). Visualisation of the protein-protein interaction network was performed using the Cytoscape StringApp software (version 3.10.1). Transcription factor prediction was conducted using AnimalTFDB4.0 [42].

2.6. Cell Culture

C2C12 cells were purchased from Anwei Biotechnology Co., Ltd. (Shanghai, China). The cells were cultured in DMEM (Gibco, Carlsbad, CA, USA) containing 10% foetal bovine serum (Gibco, Carlsbad, CA, USA) and 1% penicillin-streptomycin (Gibco, Carlsbad, CA, USA) under standard culture conditions (humid environment containing 5% CO₂ and 95% air at 37 °C). To induce differentiation, the culture medium was replaced with DMEM containing 2% horse serum (Gibco, Carlsbad, CA, USA) when the cell confluence reached 80–90%. The induction period lasted 7 days.

2.7. RNA Extraction and Quantitative PCR (qPCR)

The mRNA sequence was obtained from the sequencing platform, and the primers for qPCR were designed using the NCBI Primer blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome, accessed on 6 September 2023). Primer sequences were synthesised by Beijing Tsingke Biotechnology Co., Ltd. (Beijing, China). The primer sequences and related parameters are listed in Table 1. Total RNA from the LD muscle and C2C12 cells was extracted using TRIzol reagent (Coolaber, Beijing, China). The concentration and purity of RNA were determined using a NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA). A RevertAid First Strand cDNA Synthesis Kit with a DNase I reverse transcription kit (Thermo Scientific, Waltham, MA, USA) was used to obtain cDNA following the TransStart[®]Top Green qPCR SuperMix (Transgen, Beijing, China) kit instructions. The reaction mix contained 0.5 µg cDNA, 0.4 µL forward and reverse primers (10 µM), 10 µL 2× PerfectStart[®]GreMen qPCR SuperMix, and added nuclease-free water to 20 µL. The cycling conditions were as follows: a pre-denaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min. The melting curve data were collected using the default parameters of the instrument. Data were collected using a CFX-96 manager (Bio-Rad). *GAPDH* was used as the internal reference. The relative expression of transcripts or genes was determined using the $2^{-\Delta\Delta\text{ct}}$ method [43].

Table 1. Oligonucleotide sequence and related parameters.

Gene	Forward Primer (5'–3')	Reverse Primer (5'–3')	Product Length/(bp)
<i>IGF2</i>	GACCGCGGCTTCTACTTCAG	CGGGGTATCTGGGGAAGTTG	190
MSTRG.14343	AACCCACTTCTTCCTGTGACC	CCTGTGTGGAGAGGGAATGTG	137
<i>ARFGEF1</i>	CCGCCTCCGATCAAGATGAA	CACCCACACCCTGTCTTCAG	284
<i>TEX9</i>	TTGAAGGCCAAACTCCGTGT	GGCACTTTGACTTGTGGCAG	294
<i>HSP90AB1</i>	ATCGCCCAGTTGATGTCTGTT	TATCGTGAGGGTCCGGTCTT	198
MSTRG.36875	TAAAGTCCCCTGTCTGCG	GACGCAGTCCGGCTCTTATT	253
<i>ASF1B</i>	CCTACCACGGACAGGAGTTC	GCCCTTGATAGGAGTGCAGC	251
<i>GAPDH</i>	AGGGCATCCTGGGCTACACT	TCCACCACCCTGTGTGCTAG	166
<i>Pcna</i>	GCCGAGACCTTAGCCACATT	GTAGGAGACAGTGGAGTGGC	229
<i>Ccna2</i>	GTCCTAACGCTCCCCTCTCC	TCGAAAGAGTGTGAGCCTC	72
<i>Ccnd1</i>	TCAAGTGTGACCCGGACTG	GCTCCTTCCTCTTTGCGGG	235
<i>Cdk4</i>	CGAGCGTAAGGCTGATGGAT	CCAGGCCGCTTAGAACTGA	177
<i>Bax</i>	CCAGGATGCGTCCACCAA	AAAGTAGAAGAGGGCAACCAC	196
<i>Casp3</i>	GCTTGGAACGGTACGCTAAG	CCACTGACTTGCTCCCATGT	112
<i>Bcl2</i>	GAAGTGGGGGAGGATTGTGG	GCATGCTGGGGCCATATAGT	194
<i>MyHC</i>	CGGTCGAAGTTGCATCCCT	GAGCCTCGATTGCTCCTTT	145
<i>Myod1</i>	AGTGAATGAGGCCTTCGAGA	GCATCTGAGTCGCCACTGTA	222
<i>MyoG</i>	CAATGCACTGGAGTTCGGT	CTGGGAAGGCAACAGACAT	134
<i>Myf5</i>	CAGGAATGCCATCCGCTACA	CCCGGCAGGCTGTAATAGTT	78
<i>Homer1</i>	GCTAGGGGAGGGGAAGGAAA	CTGAGGAACAACCCGGAGG	73
<i>Homer1</i> siRNA1	CAUUCAAUUCAGCAAUCAUGA	AUGAUUGCUGAAUUGAAUGUG	
<i>Homer1</i> siRNA2	CUCAGAAUGCAUUGCCAUUdTdT	AAUGGCAAUGCAUUCUGAGdTdT	
siNC	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT	

2.8. Small Interfering RNA and Plasmid Transfection

The function of Homer1 was verified via the gain/loss approach. Small interfering RNAs (siRNAs) and overexpression plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. The siRNA sequence for Homer1 is shown in Table 1. Oligonucleotides were synthesised by Beijing Tsingke Biotechnology Co., Ltd. (Beijing, China), and overexpression plasmids were purchased from Jintuosi Biotechnology Co., Ltd. (Wuhan, China).

2.9. Cell Counting Kit 8 (CCK-8) and 5-Ethynyl-20-Deoxyuridine (EdU) Staining Assay

Transfected cells were seeded in 96-well plates, and cell proliferation was detected using the CCK-8 [44] reagent (APExBio, Houston, TX, USA) and EdU [45] cell proliferation kit Alexa Fluor 555 (Meilunbio, Dalian, China) per the manufacturer's instructions. The CCK-8 reagent was added after incubation for 0 (cell adhesion), 12, 24, and 36 h. The absorbance was measured at 450 nm using a microplate reader (Thermo Scientific, Waltham, MA, USA). After the cells were cultured in 96-well plates for 20 h, EdU solution was added, and the proportion of cells labelled with EdU was detected using an Axio Vert A1 fluorescence microscope (Zeiss, Oberkochen, Germany). EdU-labelled cells were detected using Image J (version 1.51).

2.10. Western Blot Assay

Total protein was extracted by RIPA lysis buffer (Beyotime, Shanghai, China) containing 1% protease inhibitor (Beyotime). The protein concentration was detected using a BCA kit (Beyotime, Shanghai, China), and the proteins were separated by SDS-PAGE (Epizyme Biotechnology, Shanghai, China) and then

transferred to a PVDF membrane (Millipore, Boston, MA, USA). Subsequently, the PVDF membrane was incubated with the primary antibody at 4 °C overnight, and then with the secondary antibody at room temperature for 1–2 h. The antibody information is as follows: Anti-CDK4 (1:1000, AiFang, Changsha, China); Anti-PCNA (1:1000, Zenbio, Chengdu, China); Anti-CCNA2 (1:2000, Abcam, Cambridge, MA, USA); Anti- β -actin (1:20,000, TransGen, Beijing, China). The protein signal was detected by Meilunbio® fg super sensitive ECL luminescence reagent (Meilunbio, Dalian, China) according to the manufacturer's instructions, and then the images were obtained by Image Quant LAS 4000 mini chemiluminescence imaging system (GE, Boston, MA, USA). β -actin was used as an internal reference to calculate the relative expression level of protein, and the grey value of protein was detected by Image J (version 1.51) [46].

2.11. Immunofluorescence Staining

Immunofluorescence staining was performed as described by Y. Ogura [47]. Cells differentiated for 7 days were washed twice with PBS (Meilunbio, Dalian, China), fixed with 4% paraformaldehyde (Biosharp, Hefei, China) for 15 min, washed with 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) for 10 min, and washed with 5% BSA (Biofroxx, Cologne, Germany). They were then incubated in primary antibody (Anti-MyHC, 1:300, DSHB, Iowa, IA, USA) overnight at 4 °C, followed by incubation in fluorescent secondary antibody (Anti-mouse IgG, 1:1000, Abbkine, Wuhan, China) for 1 h at room temperature in the dark. Nuclei were stained with DAPI (Solarbio, Beijing, China). Finally, Antifade Mounting Medium (Beyotime, Shanghai, China) was added, and images were captured using an Axio Vert A1 fluorescence microscope (Zeiss, Oberkochen, Germany). Differentiated and fused myoblasts were counted using Image J (version 1.51).

The expression level of HOMER1 in the skeletal muscle of Ningxiang pig at different development stages was detected by immunofluorescence staining of LD tissue sections. The slice thickness was 5 μ m. After dewaxing and washing the tissue slices, the antigens in the tissue slices were repaired by antigen repair buffer (Beyotime, Shanghai, China) and sealed with 3% BSA (Biofroxx, Cologne, Germany). Add primary antibody (anti-HOMER1 Cat No.12433-1-AP, 1: 200, Proteintech, Wuhan, China) incubated overnight at 4 °C, add fluorescent secondary antibody (Anti-mouse IgG, 1:1000, Abbkine, Wuhan, China) and incubate at room temperature for 1 h, then add self-luminous fluorescence quencher (Beyotime, Shanghai, China) after DAPI (Solarbio, Beijing, China) staining, and seal the film with anti-fluorescence quencher (Beyotime, Shanghai, China). Then, put it in a slide digital scanner (AIFang, Changsha, China) for scanning and imaging. Three pigs at different stages were selected as slices, and five fields of view with the same size were randomly selected from each slice, and ImageJ calculated the mean fluorescence intensity.

2.12. Flow Cytometry Assay

Cell cycle [48] and apoptosis [49] were detected using flow cytometry (Becton Dickinson, Franklin, VA, USA). The cell cycle was evaluated using a Cell Cycle and Apoptosis Analysis Kit (Meilunbio, Dalian, China). Apoptosis was detected using an Annexin V-FITC/PI Cell Apoptosis Detection Kit (Meilunbio, Dalian, China). The procedure was performed according to the manufacturer's instructions. The data were further analysed using the FlowJo software (Version 9) (Becton Dickinson, Franklin, VA, USA).

2.13. Statistical Analysis

Excel was used to sort out the data. Data are presented as the mean $\pm$ SD (standard deviation). Statistical analysis was performed with IBM SPSS 22.0 (SPSS Inc., Chicago, IL, USA). The significance of intergroup differences was estimated with Student's *t*-test. Origin 2021 software (version 9.8.0.200) was used for graphic visualisation. Differences were considered significant at $p < 0.05$ and $p < 0.01$.

3. Results

3.1. Comparison of Muscle Fibre Diameter and Density of LD Muscle

Morphological analysis of the muscle fibre of the LD showed that the muscle fibre diameter of 150-day-old pigs ($53.76 \pm 2.09 \mu\text{m}$) was significantly higher than that of 30-day-old pigs ($23.04 \pm 0.47 \mu\text{m}$) ($p < 0.01$), and the growth rate reached 230% (Figure 1A,B). The muscle fibre density of 30-day-old pigs ($1445.04 \pm 80.61 \text{ fibers}/\text{mm}^2$) was significantly higher (4.25-fold) than that of 150-day-old pigs ($340.18 \pm 14.87 \text{ fibers}/\text{mm}^2$) ($p < 0.01$) (Figure 1C).

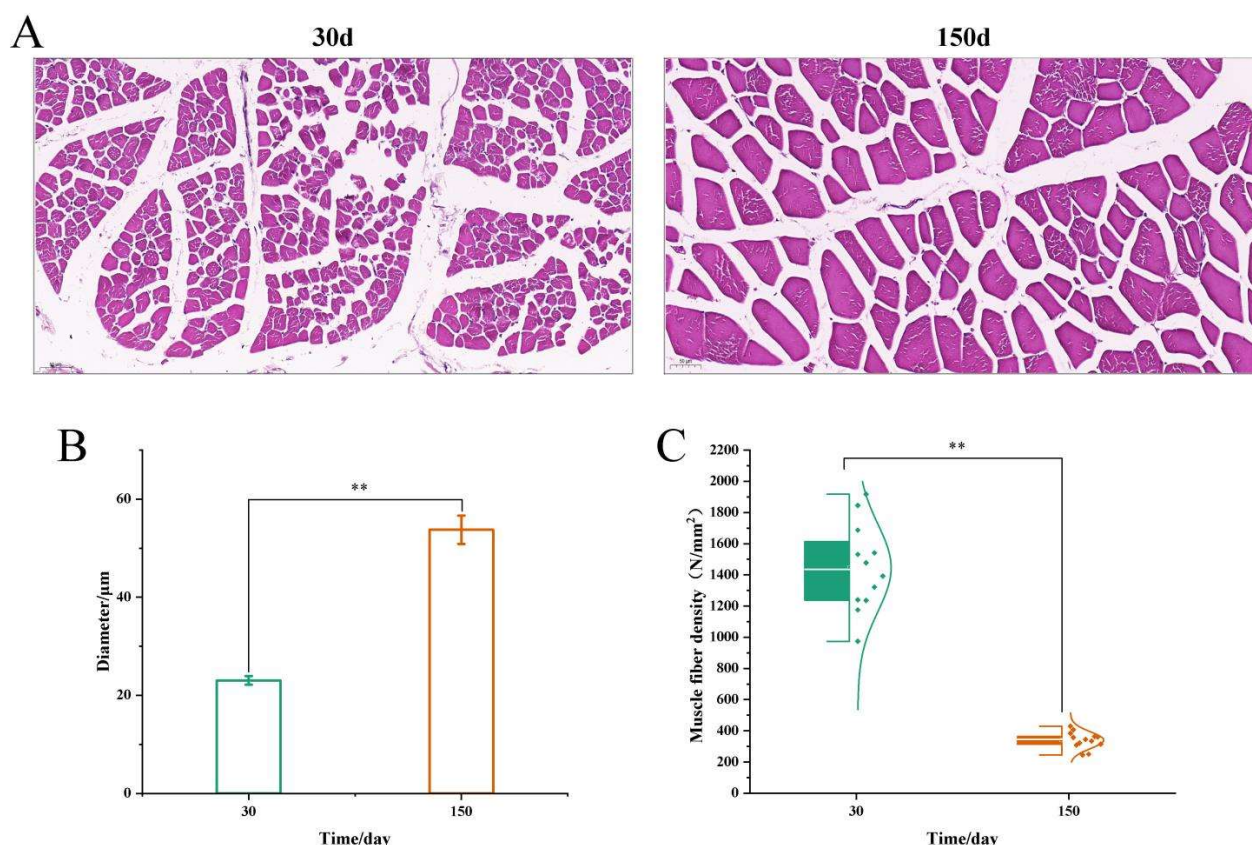


Figure 1. Histomorphological detection of skeletal muscle of LD of 30-day-old and 150-day-old Ningxiang pigs. (A) HE staining was used to detect the LD muscle; scale bar = $50 \mu\text{m}$. (B) Two-stage muscle fibre diameter analysis histogram. (C) Two-stage muscle fibre density analysis box chart (Mean number of muscle fibers) and normal curve plot (Normal distribution of muscle fiber density), ** $p < 0.01$.

3.2. Quality Control of Transcriptome Sequencing Data

As shown in Table 2, the average number of original sequencing fragments in 30-day-old and 150-day-old muscle tissue sequencing was 131,213,765.3 and 107,858,509.3, respectively, and the average amount of transformed data was 19,813,278,565 bp and 16,286,634,909 bp, respectively. After further quality control on the data of 30 days and 150 days, the average number of sequencing fragments of muscle tissue was 128,342,221.3 and 105,862,852 respectively, and the data volume was 16,991,744,589 bp and 14,340,131,010 bp, respectively. The Q30 values were 95.45% and 95.38%, respectively, and the GC contents were 55.51% and 52.47%, respectively. These results showed that the quality of the sequencing results met the requirements for further analyses.

Table 2. Quality control analysis table of sequencing data.

Sample	Raw Reads	Raw Bases	Clean Reads	Clean Bases	Error Rate (%)	Q30 (%)	GC Content (%)	Total Mapped	Unique Mapped
N30D_M1	125,643,820	18,972,216,820	122,988,528	16,416,455,345	0.0239	95.45	54.91	115,589,914 (93.98%)	106,744,173 (86.79%)
N30D_M2	131,506,100	19,857,421,100	129,299,096	17,379,678,801	0.0238	95.54	54.64	121,700,412 (94.12%)	112,264,242 (86.83%)
N30D_M3	136,491,376	20,610,197,776	132,739,040	17,179,099,621	0.024	95.35	56.97	124,599,625 (93.87%)	115,404,691 (86.94%)
N30D_M	131,213,765	19,813,278,565	128,342,221	16,991,744,589	0.0239	95.45	55.51	120,629,984 (93.99%)	111,471,035 (86.85%)
N150D_M1	105,975,290	16,002,268,790	104,317,724	14,219,362,833	0.0239	95.32	53.04	98,324,610 (94.25%)	90,999,613 (87.23%)
N150D_M2	109,666,184	16,559,593,784	107,345,576	14,444,210,597	0.0237	95.58	53.53	100,767,671 (93.87%)	91,601,719 (85.33%)
N150D_M3	107,934,054	16,298,042,154	105,925,256	14,356,819,599	0.024	95.24	50.85	100,986,824 (95.34%)	95,924,874 (90.56%)
N150D_M	107,858,509	16,286,634,909	105,862,852	14,340,131,010	0.0239	95.38	52.47	100,026,368 (94.49%)	92,842,069 (97.71%)

3.3. mRNA Identification and Expression Screening

A total of 22,411 transcripts—19,653 known and 2758 novel—were identified by transcriptome sequencing of the LD muscle at 30 and 150 days (Table S1). After TPM > 1 screening, 9021 transcripts were obtained, of which 8044 were known, and 977 were novel (Figure 2 and Table S2).

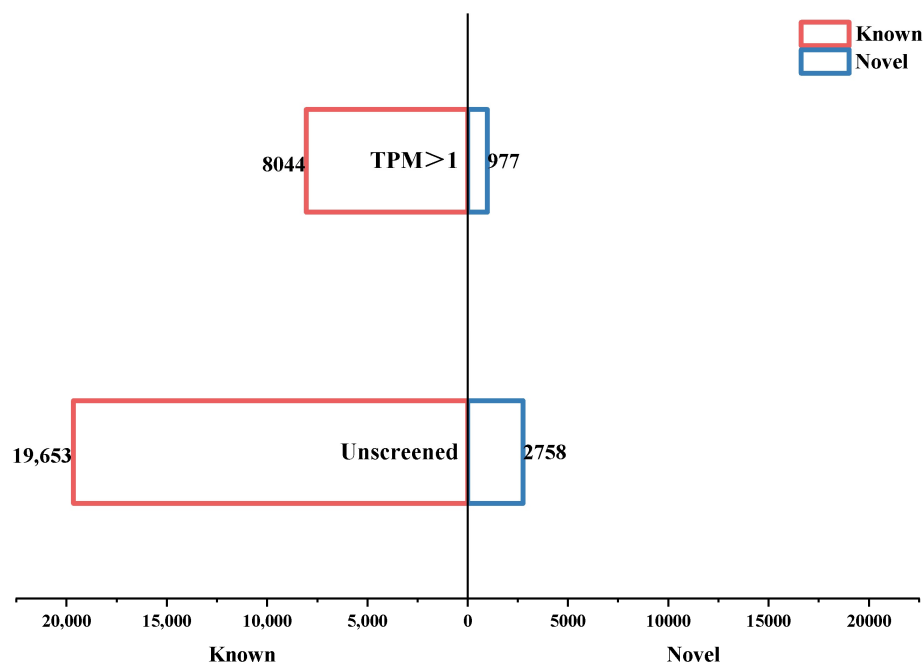


Figure 2. Bar chart depicting transcript statistical. The histogram below the X-axis is the unscreened transcript histogram, while the histogram above the X-axis is the histogram screened by TPM > 1. The red histogram on the left of the Y-axis indicates the known transcripts, and the blue histogram on the right of the Y-axis indicates the novel transcripts.

3.4. Differential Expression Analysis

Taking 30 d as the control group, differential expression analysis of the transcripts with TPM > 1 was performed, and 1322 differentially expressed transcripts—1125 known and 197 novel—were identified (Table S3). Of these, 978 were upregulated, and 344 were downregulated (Figure 3A and Table 2). Of the known genes, 823 were upregulated, and 302 were downregulated. Of the novel genes, 155 were upregulated, and 42 were downregulated (Figure 3B and Table S3).

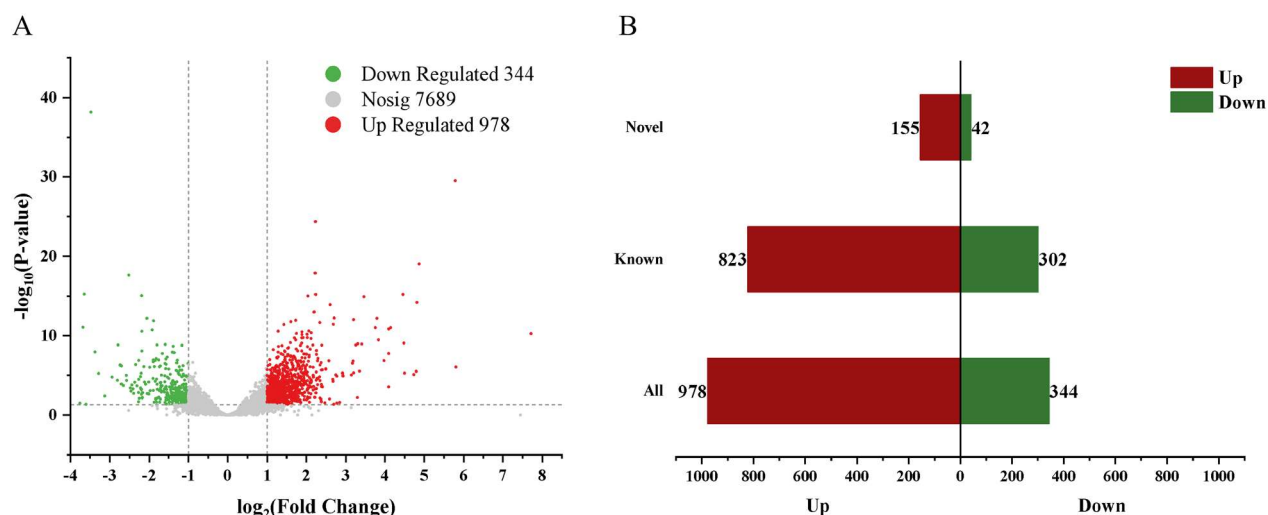


Figure 3. Differential expression analysis of transcripts of LD in two stages. (A) Volcano plot of transcripts. (B) Histogram showing the classification of differentially expressed transcripts.

3.5. GO Function Annotation and Enrichment Analysis of Differentially Expressed Transcripts

GO annotation and enrichment analyses were carried out on 1322 differentially expressed transcripts, and 22 biological processes, 16 cellular components, and 13 molecular functions were annotated (Table S4). The top 20 GO terms included nine biological processes (cellular process, biological regulation, metabolic process, cellular component organisation or biogenesis, response to stimulus, developmental process, localisation, multicellular organismal process, and immune system process), four molecular functions (binding, catalytic activity, transcription regulator activity, and molecular function regulator), and seven cell components (cell part, organelle, organelle part, protein-containing complex, membrane part, membrane, and extracellular region part) (Figure 4A). GO enrichment analysis revealed that 23 transcripts were enriched in 19 biological processes related to muscle development and function (Figure 4B and Table S5). The biological processes mainly involved skeletal muscle fibre adaptation, striated muscle contraction, muscle fibre development, skeletal muscle contraction, negative regulation of muscle cell apoptosis, skeletal muscle tissue regeneration, and skeletal muscle cell differentiation (Figure 4B). Twenty-three transcripts—17 known and six novel—were related to muscle development (Figure 4C), of which 19 were up-regulated and four were down-regulated (Table S6).

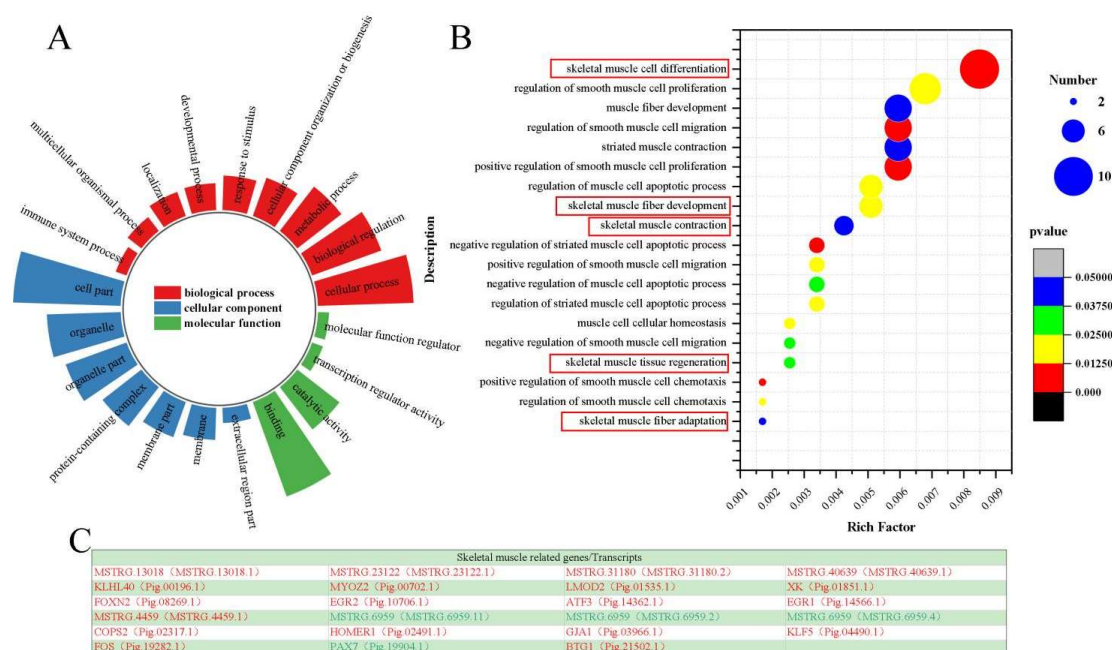


Figure 4. GO annotation and enrichment analysis. **(A)** The top 20 GO terms of differentially expressed transcripts were analysed by GO annotation. **(B)** Muscle-related GO (red frames) enrichment analysis bubble diagram. **(C)** Table presenting skeletal muscle-related genes/transcripts, red font means up-regulated, and green font means down-regulated.

3.6. KEGG Enrichment Analysis of Muscle-Related Differential Expression Transcripts

The KEGG enrichment results of the 23 muscle-related differentially expressed transcripts showed that they were mainly enriched in five processes: environmental information transduction processes, cell processes, genetic information processes, human diseases, and tissue systems (Table S7). The top 20 pathways were enriched in FoxO, MAPK signalling, gap connection, human T-cell leukaemia virus 1 infection, hepatitis B, protein disease, parathyroid hormone synthesis, secretion, and response, and glutamatergic synapses (Figure 5A).

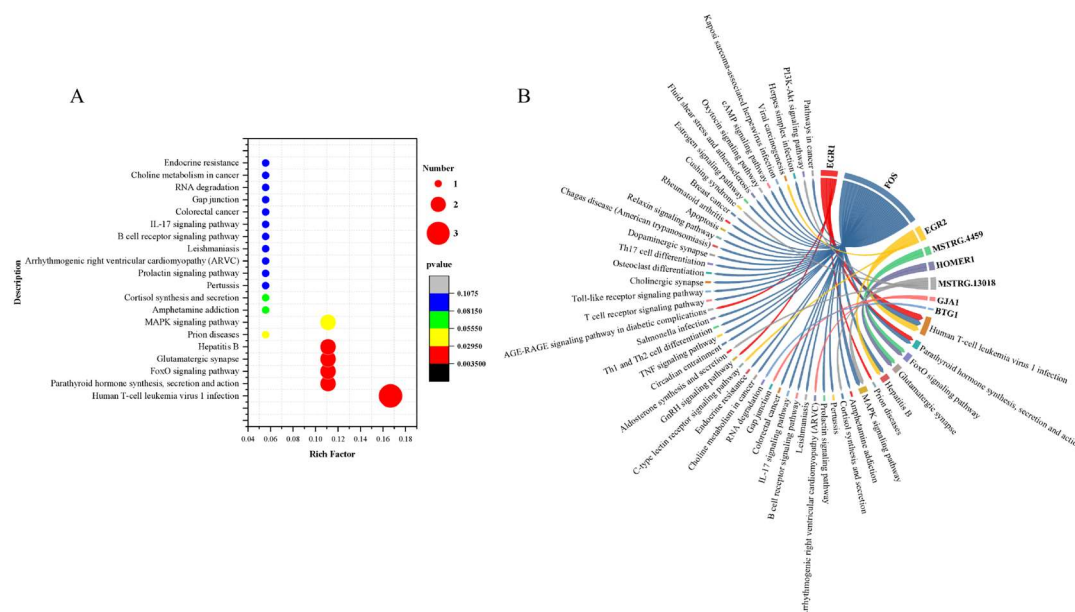


Figure 5. KEGG enrichment analysis of skeletal muscle-related differentially expressed transcripts. **(A)** Bubble diagram showing the top 20 results of KEGG enrichment. **(B)** Gene-pathway proportional chord diagram; the chord starting point is the gene, and the chord ending point is the pathway corresponding to the gene.

Further analysis showed that eight genes (*EGR1*, *MSTRG.4459*, *FOS*, *MSTRG.13018*, *GJA1*, *BTG1*, *EGR2*, *HOMER1*) were enriched mainly in the FoxO, MAPK, TNF, IL-17, cAMP, and PI3K-Akt signal pathways (Figure 5B). Therefore, they may regulate muscle development via these signalling pathways.

3.7. Analysis of Muscle-Related Proteins by PPI and Transcription Factors

To further explore the interactions of the 15 known muscle-related proteins, PPI analysis was performed in humans, mice, and pigs. The analysis yielded 11, 10, and 10 protein interactions in humans, mice, and pigs, respectively (Figure 6A–C). Among the three species, the most stable protein interactions were the EGR1-EGR2-FOS-ATF3-HOMER1-KLF5 and MYOZ2-KLHL40-LMOD2 interaction networks (Figure 6D).

To explore the regulatory mode among the 15 muscle-related genes, TF analysis was performed, and proteins encoded by eight genes were identified as transcription factors. These genes included *KLF5*, *COPS2*, *FOXN2*, *EGR2*, *ATF3*, *EGR1*, *FOS*, and *PAX7*. Further analysis revealed interactions between *KLF5*, *EGR2*, *ATF3*, *EGR1*, and *FOS* among the eight transcription factors (Figure 6E).

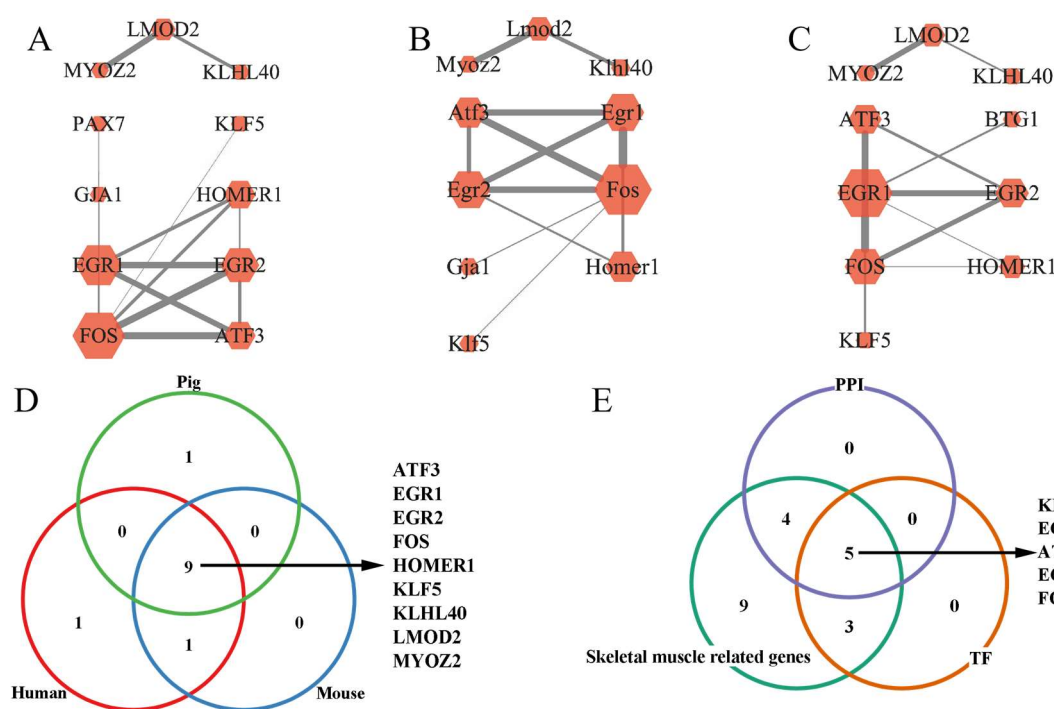


Figure 6. Results from PPI analysis of skeletal muscle-related differentially expressed genes. (A–C) are PPI networks of skeletal muscle-related genes in humans, mice, and pigs. (D) Venn diagram analysis of human, mouse, and pig PPI. (E) Venn diagram analysis of skeletal muscle-related genes, transcription factors, and proteins in PPI.

3.8. Verification of RNA-Seq by qPCR

The qPCR verification of five randomly selected up-regulated and two down-regulated genes was consistent with the sequencing trend (Figure 7). These results indicated that the sequencing data were highly reliable.

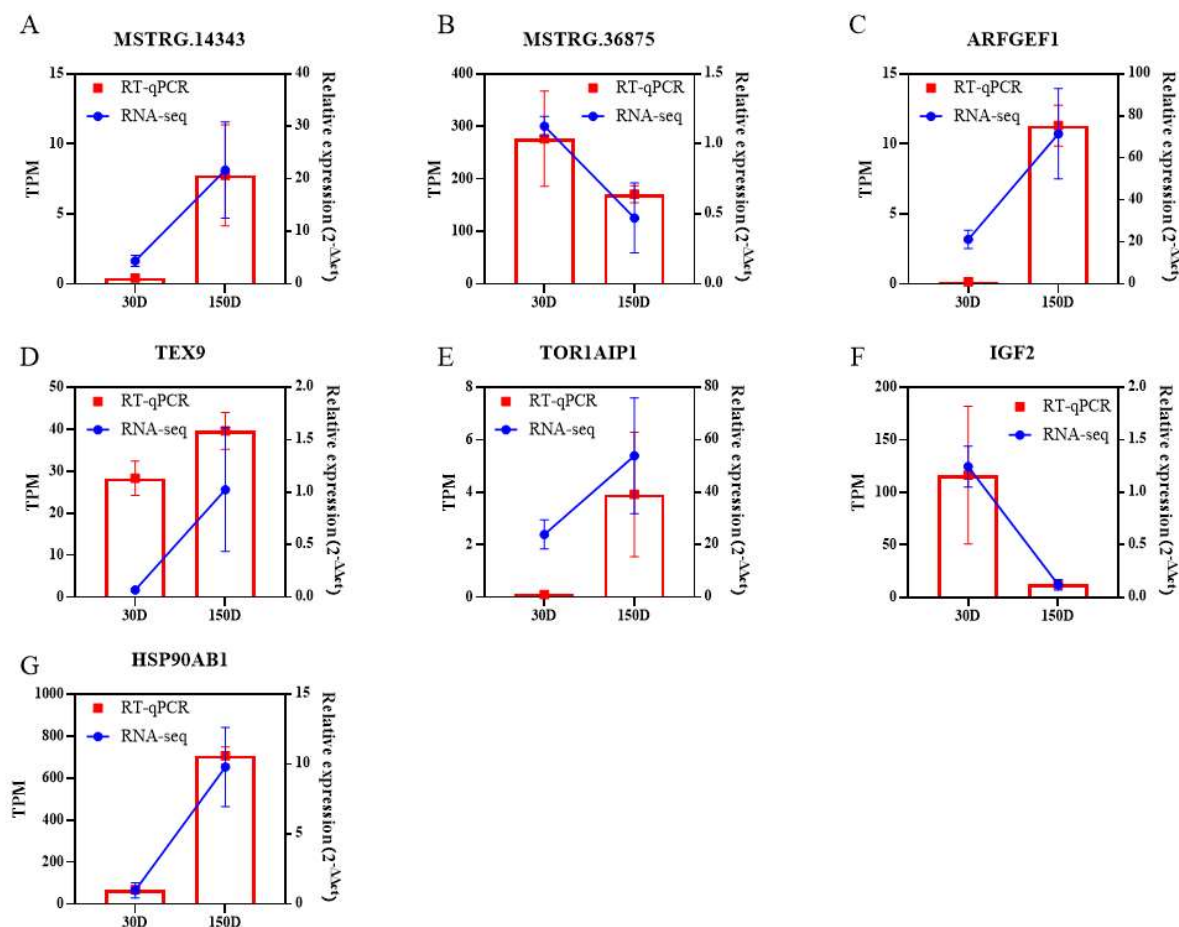


Figure 7. qPCR verification of RNA-seq differential expression genes. The histogram depicts the result of qPCR, and the line chart depicts the result of RNA-seq. The gene name is shown on the title of the X-axis. The expression levels yielded by RNA-seq and qPCR are shown on the left Y-axis and the right Y-axis, respectively. (A) MSTRG.14343; (B) MSTRG.36875; (C) ARFGEF1; (D) TEX9; (E) TOR1AIP1; (F) IGF2; (G) HSP0AB1.

3.9. Expression Pattern of Homer1

We detected the mRNA expression level of Homer1 in the longissimus muscle of Ningxiang pig at four development stages (30, 90, 150, 210 days after birth) by RT-qPCR. The results showed that the expression level of Homer1 increased gradually with the increase of age, and the expression level increased significantly at the age of 150 days ($p < 0.01$) (Figure 8A). The phenotypic analysis also found that the muscle fiber diameter increased significantly, which suggested that Homer1 might play an important regulatory role at this stage. To further clarify whether the expression of Homer1 is tissue-specific, we detected the mRNA expression level of homer1 in the heart, liver, spleen, lung, kidney, fat, and muscle of 150-day-old Ningxiang pigs. Compared with the heart, it was found that the expression level of Homer1 in liver and kidney was significantly higher than that in the heart ($p < 0.01$). There was no difference in the expression level in muscle and heart (Figure 8B). The expression of Homer1 in the LD was detected by immunofluorescence analysis, and it was found that the fluorescence of Homer1 labeled in the LD increased significantly at 150d compared with 30d (Figure 8C).

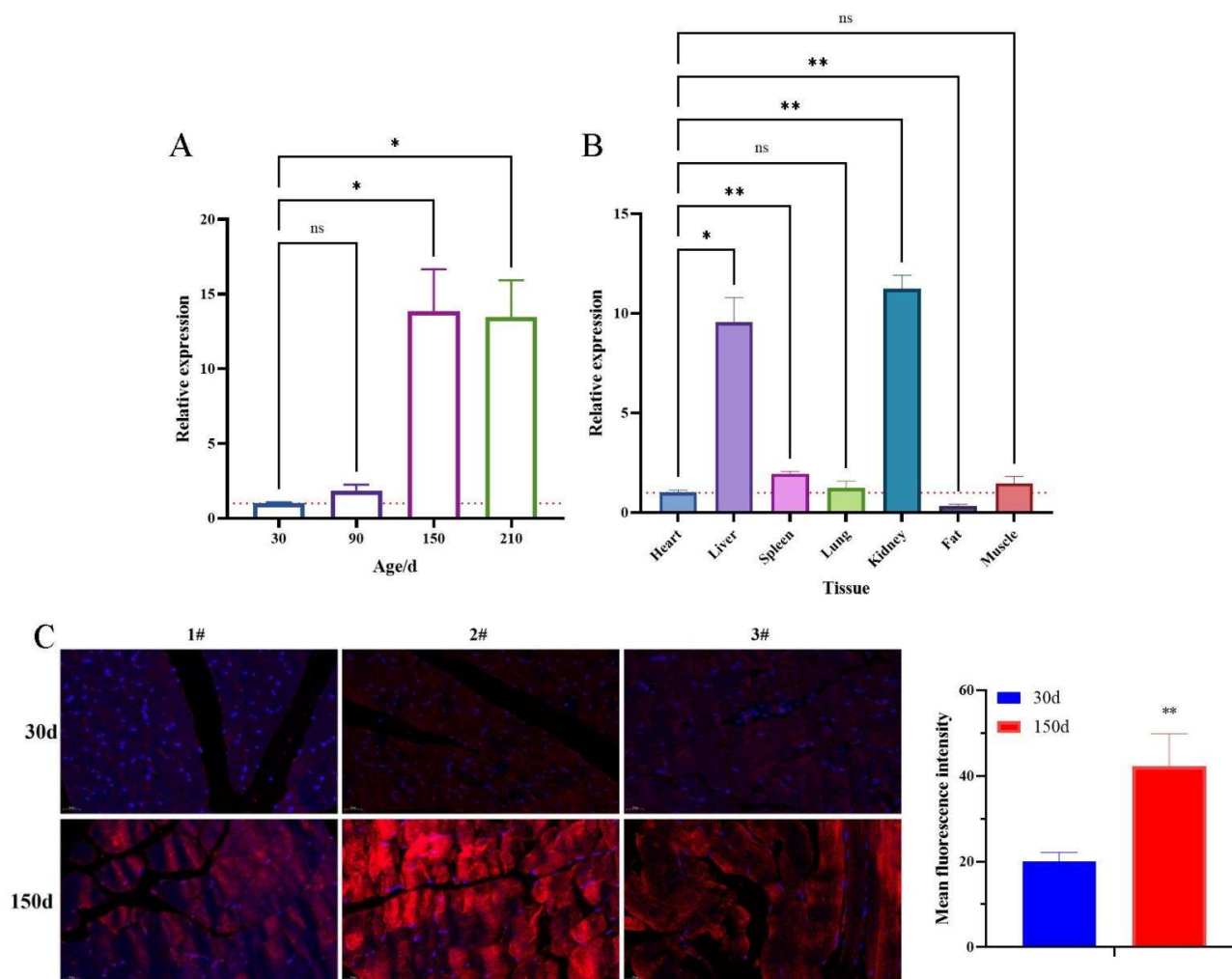


Figure 8. Pattern of time series (A,C) and tissue (B) gene expression of homer1 via RT-qPCR and IF, Homer1 labeled 570, scale bar is 25 μ m, 1-3# represent different Ningxiang pigs, * $p < 0.05$, ** $p < 0.01$, ns > 0.05 , $n = 3$.

3.10. Gene Overexpression and Interference Efficiency Detection

Gene overexpression and interference efficiency test revealed that among the three overexpression transfection schemes, 5 μ L Lipofectamine was used to transfect a 2.5 μ g overexpression plasmid. The mRNA expression level of HOMER1 was significantly increased ($p < 0.001$), with an overexpression efficiency of over 58-fold (Figure 9A). Among the four interference schemes, the mRNA expression level of HOMER1 was significantly reduced ($p < 0.001$) after transfection with 50 nM siRNA1, and the interference efficiency reached 79.64% (Figure 9B). The optimal transfection scheme involved transfecting 2.5 μ g of overexpression plasmid and 50 nM siRNA1 using 5 μ L lipofectamine. This setup was used for subsequent functional verification experiments.

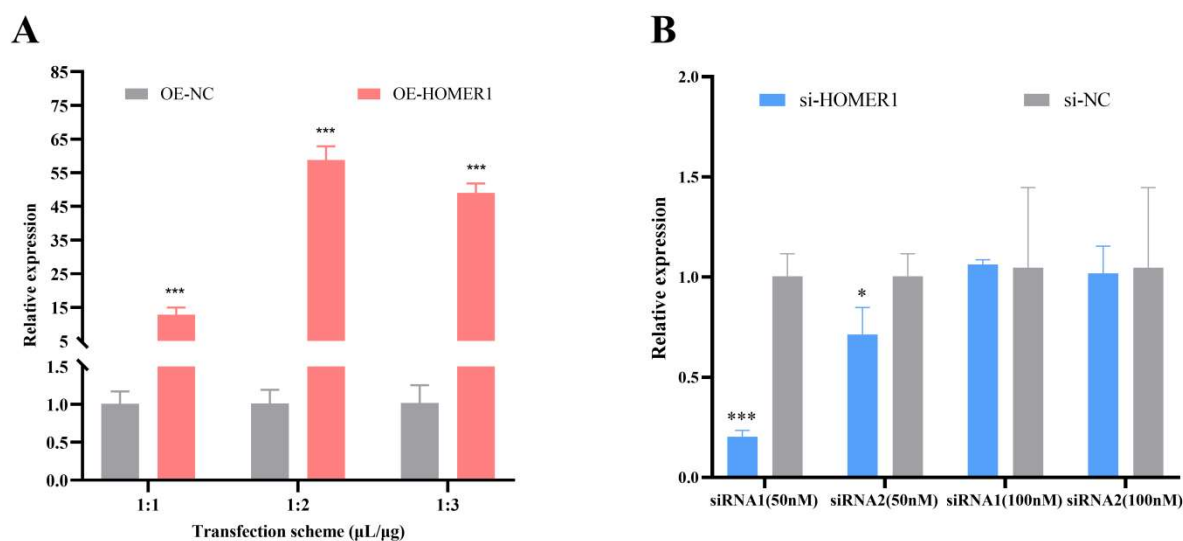


Figure 9. Analysis of overexpression (A) and interference efficiency (B) of *Homer1* gene. * $p < 0.05$, *** $p < 0.001$, $n = 3$.

3.11. *Homer1* Promotes Myoblast Proliferation

To verify the effects of HOMER1 on myoblast proliferation, HOMER1 siRNA1 (si-HOMER1), siRNA negative control (si-NC), pORP-HOMER1 (OE-HOMER1), and pORP (OE-NC) were transfected into myoblasts. The mRNA expression levels of proliferation marker genes (*Ccna2*, *Ccnd1*, *Cdk4*, *Pcna*) in the OE-HOMER1 group were significantly higher than those in the OE-NC group ($p < 0.05$) (Figure 10A), and the opposite result was obtained after knockdown *Homer1* (Figure 10B). Western blot analysis showed that the protein expression levels of PCNA, CCNA, and CDK4 were markedly higher in the OE-HOMER1 group than in the OE-NC group ($p < 0.01$). In contrast, the opposite results were obtained after knocking down *Homer1* (Figure 10G). The results of the CCK8 and EdU assays showed that the proliferation index and EdU positivity were markedly higher in the OE-HOMER1 group than in the OE-NC group ($p < 0.01$, Figure 10C,F), and the opposite was observed after the knockdown (Figure 10D,E). Flow cytometry analysis showed that the proportion of S-phase cells was significantly higher in the OE-HOMER1 group than in the OE-NC group ($p < 0.05$; Figure 10H). HOMER1 knockdown significantly increased the proportion of cells in the G1 phase ($p < 0.01$) and decreased the proportion of cells in the S and G2 phases. ($p < 0.05$, Figure 10I). In summary, HOMER1 promoted myoblast proliferation.

3.12. *HOMER1* Does Not Affect Myoblast Apoptosis

To verify the effect of *Homer1* on myoblast apoptosis, myoblasts were transfected with an overexpression plasmid and an interference sequence. qPCR results showed no significant differences in the mRNA expression levels of *Bax*, *BCL2*, and *Casp3* between the OE-HOMER1 and OE-NC groups ($p > 0.05$, Figure 11A), and knockdown of *Homer1* had no significant effect on myoblast apoptosis ($p > 0.05$, Figure 11B). Flow cytometry analysis showed no significant differences between the OE-HOMER1 and OE-NC groups in the cell proportion of Q1, Q2, Q3, and Q4 ($p > 0.05$, Figure 11C). There was no significant difference in the cell proportion of each region after knockdown ($p > 0.05$, Figure 11D). These results indicate that *Homer1* does not affect myoblast apoptosis.

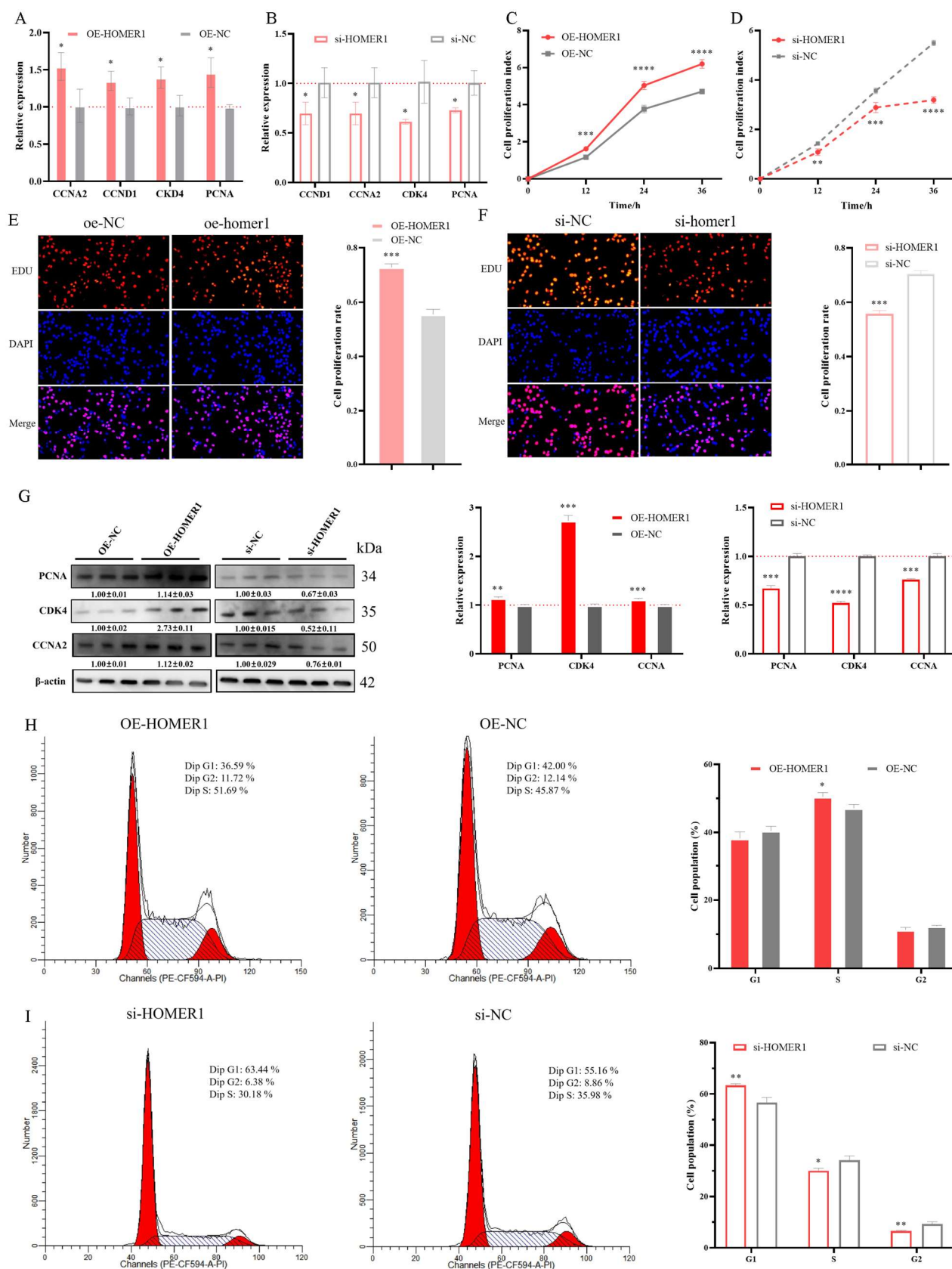


Figure 10. The effect of Homer1 on the proliferation of myoblasts. qPCR was used to detect the mRNA expression level of proliferation marker genes (*Ccna2*, *Ccnd1*, *Cdk4*, *Pcna*) after overexpression and knockdown of Homer1 (A,B). CCK8 was used to detect the cell proliferation rate (C,D). EdU staining was used to detect the positive cell rate; scale bar = 100 μm (E,F). Western blotting was used to analyse the proliferation marker proteins (Pcna, Cdk4, PCNA) after overexpression and knockdown

of Homer1. (H,I) The cell cycle of overexpressed or interfered with homer1 was detected by flow cytometry. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, $n = 3$.

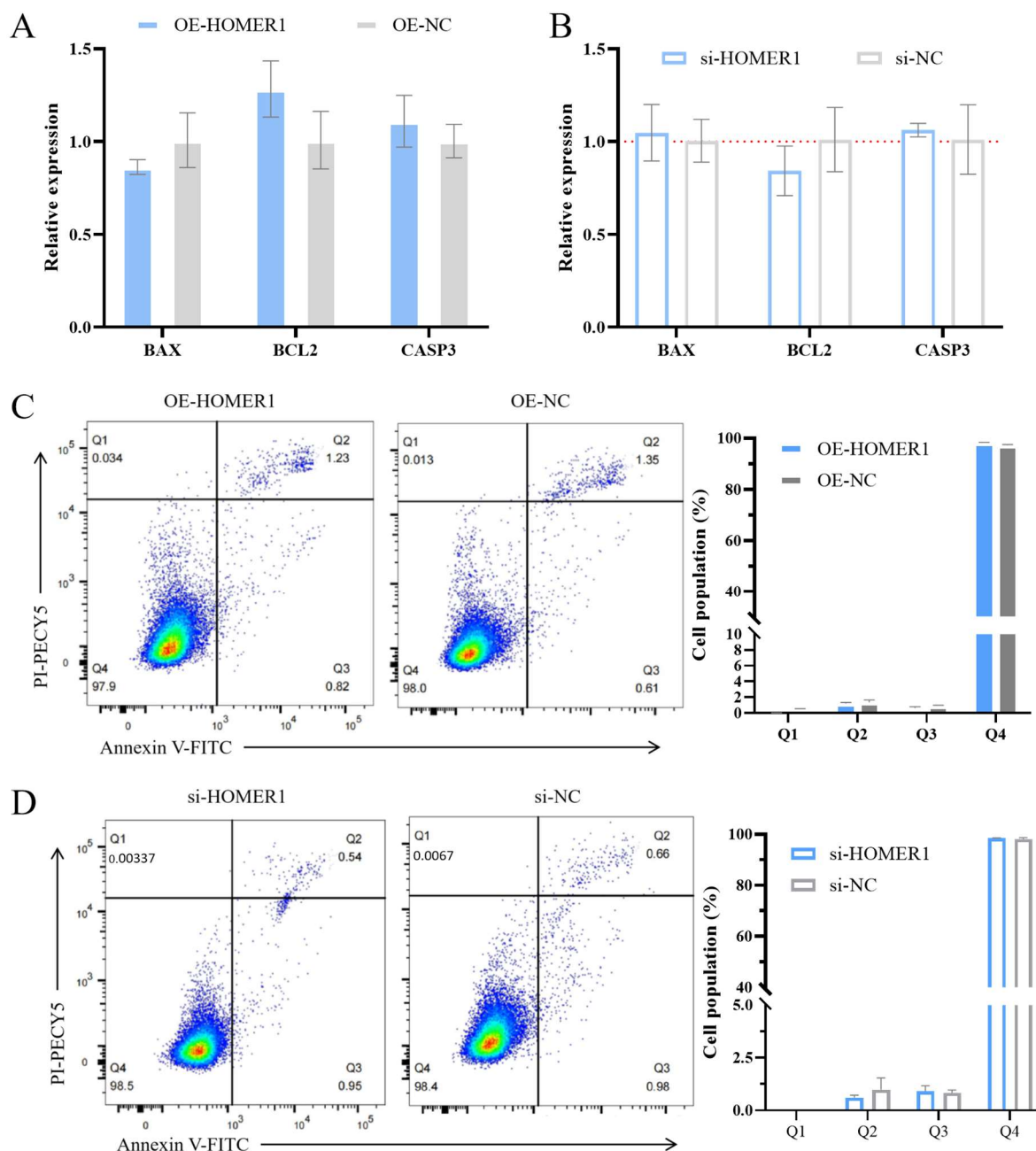


Figure 11. The effect of Homer1 on myoblast apoptosis. After overexpressing and knocking down Homer1, the mRNA expression level of apoptosis marker genes (*Bax*, *Bcl2*, *Casp3*) was detected (A,B) via qPCR, and the proportion of apoptotic cells was detected by flow cytometry (C,D). $n = 3$.

3.13. HOMER1 Promotes Myoblast Differentiation and Fusion

After transfection with the expression plasmid and a small interfering sequence, parameters related to differentiation marker genes and muscle fibre fusion were detected. Overexpression of *Homer1* significantly increased the mRNA expression levels of differentiation marker genes (*MyHC*, *Myod*, *Myf5*, *Moyg*) ($p < 0.05$, Figure 12A), and the opposite result was obtained after knocking down the *Homer1* gene ($p < 0.05$, Figure 12B). The analysis of induced differentiation showed that the differentiation index of the OE-HOMER1 group was significantly higher than that of the OE-NC group, while that of the si-HOMER1

group was significantly lower than that of the si-NC group ($p < 0.05$, Figure 12C–E). As shown in Figure 12C,D,F, the proportion of myotubes with fewer than three nuclei in the OE-HOMER1 group was significantly higher than that in the OE-NC group ($p < 0.01$), and the proportion of myotubes with 3–5 nuclei was significantly lower than that in the OE-NC group ($p < 0.01$); however, there was no significant difference in the proportion of myotubes with more than five nuclei ($p > 0.05$). The proportion of myotubes with fewer than three nuclei in the si-HOMER1 group was significantly higher than that in the si-NC group ($p < 0.05$), and the proportion of myotubes with to 3–5 nuclei and myotubes with more than five nuclei was significantly lower than that in the si-NC group ($p < 0.01$). These results indicate that HOMER1 promotes myodifferentiation and muscle fibre fusion.

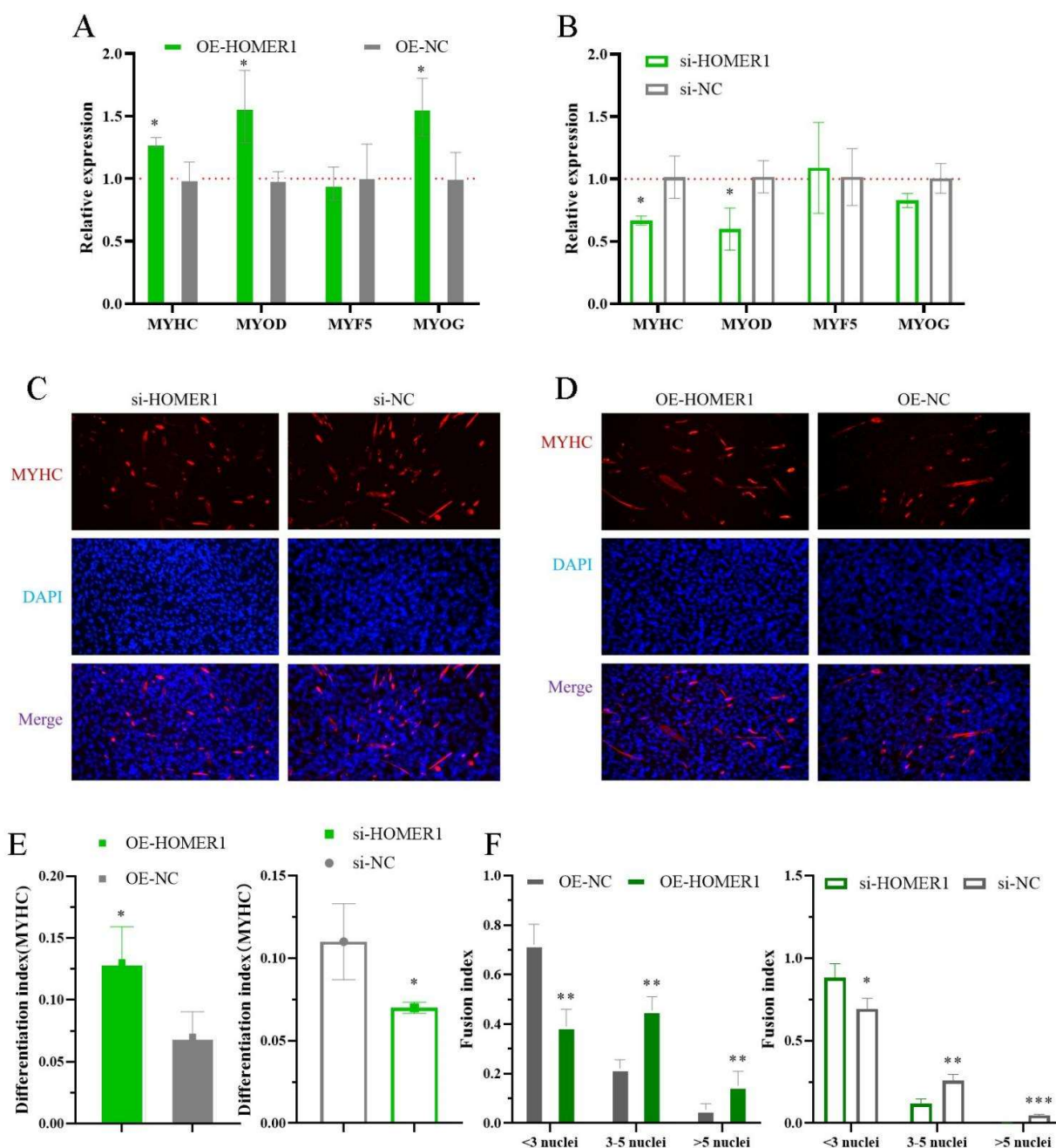


Figure 12. Homer1 promotes myoblast differentiation. After overexpressing and knocking down *Homer1*, the mRNA expression level of differentiation marker genes (A,B) was detected by qPCR, and the differentiation and fusion index of cells was detected by immunofluorescence staining; scale bar = 200 μ m (C–F). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 3$.

4. Discussion

In this study, we identified 15 known (*LMOD2*, *MYOZ2*, *FOS*, *COPS2*, *FOXN2*, *EGR1*, *ATF3*, *PAX7*, *XK*, *KLHL40*, *GJA1*, *KLF5*, *BTG1*, *EGR2*, and *HOMER1*) and six novel genes (MSTRG.13018, MSTRG.23122, MSTRG.31180, MSTRG.40639, MSTRG.4459, and MSTRG.6959) related to muscle development and function by transcriptome sequencing, differential expression analysis, GO annotation, and GO enrichment analysis. KEGG enrichment analysis revealed eight genes enriched in the FoxO, MAPK, GnRH, TNF, cAMP, PI3K-Akt, and other signal pathways, indicating that these pathways may be the key to regulating skeletal muscle development. Transcription factor analysis showed that *KLF5*, *COPS2*, *FOXN2*, *EGR2*, *ATF3*, *EGR1*, *FOS*, and *PAX7* are transcription factors, and PPI analysis revealed a significant interaction between *EGR1*-*EGR2*-*FOS*-*ATF3*-*HOMER1*-*KLF5* and *MYOZ2*-*KLHL40*-*LOMD2*, suggesting their participation in the growth and development of skeletal muscle by encoding transcription factors.

Several genes tightly regulate skeletal muscle development. With the progress in gene detection technology and biological analysis and the deepening of functional research, more genes related to muscle development have been identified. The spatiotemporal-specific expression of genes suggests that they play an important role in biological development. In the present study, we found that *LMOD2* was significantly upregulated in Ningxiang pigs at 150 days of age, suggesting that it may be involved in regulating muscle development. Kiss et al. reported that *LMOD2* regulates the length of thin filaments and participates in skeletal muscle energy regulation [50]. Larrinaga et al. showed that *LMOD2* is necessary for skeletal muscle contraction [51]. *MYOZ2* is highly expressed during the fattening period, indicating that it plays an important role in promoting muscle development. Wang et al. found that *MEF2A* regulates transcriptional activity by binding to the proximal promoter of *MYOZ2*, which promotes myogenic differentiation [52]. Zhang et al. reported that *MYOZ2* inhibits the proliferation of sheep myoblasts and promotes cell fusion [53]. The gene expression of *COPS2* during the fattening period was significantly higher than that during the weaning period, indicating that *COPS2* plays an important regulatory role in rapid muscle growth. Alves et al. found that *COPS2* was significantly decreased in rats with tumours, and overexpression of *COPS2* ensured myotube homeostasis and promoted the recovery of muscle contraction function [54]. This study found that *EGR1* may play an important role in muscle development in Ningxiang pigs. Previous studies have shown that *EGR1* can activate *SIRT1* transcription, thus eliminating the oxidative damage of reactive oxygen species to muscles [55,56]. The expression of *GJA1* was significantly increased in the muscle tissue of Ningxiang pigs at 150 days of age. Shen et al. found that *GJA1* deletion reduced fast muscle weight and grip strength in mice, thereby affecting the formation of the tendon intima in skeletal muscle [57,58]. Martins et al. found that *GJA1* participates in the nuclear shuttling of small molecules and regulates the myogenic differentiation of myocardial cells [59]. The expression of mRNA in *KLHL40* cells significantly increased during the late fattening period. Garg et al. found that *KLHL40* promotes the stability of nebulin and *LMOD3* and blocks their ubiquitination of *LMOD3*, thus inhibiting the deterioration of neuromuscular diseases [60]. Mansur et al. found that *KLHL40* regulates skeletal muscle development by mediating ubiquitination [61]. Rodier et al. found that *BTG1* is involved in the regulation of myoblast differentiation [62]. In this study, we found that the expression of *HOMER1* increased significantly, indicating that *HOMER1* plays a vital role in muscle development. Studies have shown that decreased or deleted *HOMER1* levels lead to skeletal muscle atrophy or overt myopathy in mice [63], indicating that *HOMER1* plays a crucial role in skeletal muscle. *XK* has rarely been reported in the study of muscle development. Whether the five novel identified genes participate in skeletal muscle development in Ningxiang pigs requires further verification at the cellular and individual levels.

Gene expression is the basis of gene function, and the initiation, extension, and termination of gene expression require the participation of regulatory factors, such as transcription factors. In this study,

transcription factor analysis showed that eight of the proteins encoded by 15 known genes were transcription factors. *FOS* is a transcription factor, and differential expression analysis showed that its expression level was significantly higher at 150 days than at 30 days. *FOS* participates in muscle cell differentiation [64], and when its expression level decreases, myotube formation is inhibited [65]. *EGR1/2* is an important member of the growth factor family and a transcription factor that is highly expressed in the muscle tissue of 150-day-old Ningxiang pigs. Studies have shown that it also regulates tendon formation [66]. The expression of *ATF3* was significantly increased in the muscle of Ningxiang pig aged 150 days. *ATF3* is involved in the regulation of muscle and fat development as a transcription activator [67,68]. *ATF3* also regulates the expression of chemokines in the skeletal muscles [69], regulating their adaptability during training [70]. *ATF3* can also activate the transcription of H2B protein, thus maintaining the stability of the genome and slowing down the premature activation of skeletal muscle satellite cells [71]. *PAX7* expression decreased significantly during the early stages of fattening in Ningxiang pigs. Studies have shown that *PAX7* is necessary to maintain the resting state of skeletal muscle stem cells in infancy [72], and *PAX7* plays an important role in the process of muscle growth after birth [73,74], suggesting that there may be some factors that negatively regulate the expression of *PAX7* in the muscle development of Ningxiang pigs. *KLF5* is a key transcription factor in pigs and is highly expressed in the early fattening period of Ningxiang pigs, indicating that it may affect the transcriptional activity of genes by binding to their promoters, thereby regulating muscle development. Hayashi et al. found that *Klf5* knockout specific to muscle satellite cells significantly reduced muscle regenerative activity and muscle atrophy [75,76]. The genes involved in the transcription of transcription factor *FOXN2* and their involvement in muscle development in Ningxiang pigs require further experimental verification.

Genes are not only expressed in cells but also participate in signal transduction between cells. To further clarify the action pathways of genes in the process of muscle development, KEGG function enrichment and PPI analyses were conducted on the muscle-related genes identified. MAPK and PI3K-Akt, among others, may be key pathways in muscle development. *EGR1-EGR2-FOS-ATF3-HOMER1-KLF5* and *MYOZ2-KLHL40-LOMD2* interact with each other. Studies have found that the PI3K/Akt [28,77] and MAPK [24,63] signalling pathways affect myoblast proliferation, and other studies have shown that the TNF signalling pathway affects muscle growth and development [78]. Zhang et al. found that the MAPK signalling pathway regulates fibre-type transformation in sheep skeletal muscles [79]. Yadav et al. found that the FoxO signalling pathway is involved in the formation of slow muscle fibres [80]. Franco-Romero et al. found that a new member of the FOXO signalling pathway regulates muscle atrophy [81]. Zheng et al. found that the IL-17 signalling pathway can promote the repair of sheep myoblast injury [82]. These signalling pathways play an important role in muscle development, but some of them have not been verified in muscle development, which also provides a direction for subsequent research. Molecular interactions during gene expression include transcription factor–transcription initiation factor, transcription factor–cofactor, cofactor–cofactor, translation initiation factor–cofactor, and modification factors, among others. The interaction between serpine 1 and THBS1 has been verified in gastric cancer and luteinization in endothelial cells [83,84]. *EGR1* can activate the expression of *ATF3* during cancer treatment [85]. Yuan et al. found that the activation of *EGF1* was regulated by *ATF3* [86]. Wang et al. found that *FOS* binds to the *EGR1* promoter to promote its expression and then promote the production of *IL4* [87]. Whether the above interactions in non-muscle development also exist in the muscle development of Ningxiang pigs requires further experimental verification.

Homer1 was first discovered in the brain tissue. As a scaffold protein, Homer1 can regulate the signal transduction of metabotropic glutamate to affect the development of the cortex and is mainly involved in the development of the nervous system [88–90]. Homer1 also relieves cerebral haemorrhage by promoting glial cell transformation [91]. Homer1 is expressed in skeletal muscle sarcoplasmic reticulum [92,93]. The EVH1 domain of Homer1 binds to the ryanodine receptor type 1 to regulate intracellular calcium concentration, thereby affecting muscle contraction [91]. Homer1 knockout mice show obvious skeletal muscle disease due to the

destruction of intracellular calcium homeostasis [94]. We found that *Homer1* overexpression significantly upregulated the expression of skeletal muscle differentiation marker genes and promoted muscle cell differentiation and muscle fibre fusion. *Homer1* likely influences the differentiation of muscle cells by regulating the calcineurin/NFAT signalling pathway [95,96]. In this study, we found that the overexpression of *Homer1* significantly promoted the proliferation of myoblasts, suggesting that *Homer1* may play an important role in muscle plasticity and adaptability. Bortoloso et al. also found that *homer* expression was positively correlated with muscle mass at the individual level [97], a result similar to that of this study. Schumacher et al. found that mutations in *Homer1* significantly affected myogenic differentiation in piglets [98].

5. Conclusions

In summary, we identified 15 known and six novel candidate genes related to muscle development through differential expression and functional annotation analysis of the Ningxiang pig muscle transcriptome after sequencing at the weaning and pre-fattening stages. Subsequent bioinformatics analysis revealed a significant interaction between 15 known genes related to muscle development, and that they also regulated muscle development in Ningxiang pigs through signalling pathways, such as FoxO, MAPK, PI3K-Akt, GnRH, and TNF. Proteins encoded by some genes act as transcription factors that regulate the expression of muscle-related genes, affecting muscle development in Ningxiang pigs. We verified that the candidate gene, *Homer1*, promotes the proliferation and differentiation of myoblasts. These results provide a reference for studying the molecular mechanisms of muscle development in Ningxiang pigs.

Supplementary Materials

The following supporting information can be found at: <https://www.sciepublish.com/article/pii/1267>, Table S1: Unscreened Transcript Details Table; Table S2: Details of transcripts screened with TPM > 1; Table S3: Differential expression details of transcripts with TPM > 1; Table S4: Detailed table for GO annotation analysis of differentially expressed transcripts related to muscles; Table S5: Detailed Table for GO Enrichment Analysis of Differentially Expressed Transcripts; Table S6: Detailed table of differentially expressed genes related to muscles; Table S7: Detailed table of KEGG enrichment analysis of muscle-related differentially expressed genes.

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Author Contributions

Conceptualization, H.M. and Z.Y.; Methodology, Z.Y.; Software, Z.Y.; Validation, Z.Y., L.X. and K.W.; Formal Analysis, X.L.; Data Curation, Z.Y.; Writing—Original Draft Preparation, Z.Y.; Writing—Review & Editing, H.M.; Visualization, Z.Y., J.T.; Project Administration, H.M.; Funding Acquisition, H.M.

Ethics Statement

The animal experiment of this study was carried out in accordance with ARRIVE guidelines (<https://arriveguidelines.org>, accessed on 10 July 2022). The experimental methods are consistent with the guidelines. All animal experiment procedures are approved by the Ethics Committee of the College of Animal Science and Technology, Hunan Agricultural University (No.2021-13, approved on 17 February 2021).

Informed Consent Statement

Not applicable.

Data Availability Statement

The datasets supporting the conclusions of this study are available in the manuscript and supplementary materials. The RNA-seq data used in the present study have been deposited in the NCBI database Sequence Read Archive (SRA) (<https://www.ncbi.nlm.nih.gov/sra>, accessed on 10 August 2023) (BioProject: PRJNA721288): SRR14211782-SRR14211784 (30 days after birth) and SRR14211767-SRR14211769 (150 days after birth).

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Declaration of Competing Interest

The authors declare that they have no competing interests.

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