BMC Genomics

RESEARCH Open Access

LMOD2 interaction with *ACTC1* regulates myogenic differentiation

Kaiming Wang[1†], Caihong Liu[1†], Lei Yi[1], Sui Liufu[1], Wenwu Chen[1], Xiaolin Liu[1], Bohe Chen[1], Xin Xu[1], Jingwen Liu[1], Xibing Liu[1], Yulong Yin[3,4*] and Haiming Ma[1,2,3*]

Abstract

Background Skeletal muscle is the largest tissue in mammals, and it plays a crucial role in metabolism and homeostasis. Skeletal muscle development and regeneration consist of a series of carefully regulated changes in gene expression. Leiomodin2 (*LMOD2*) gene is specifically expressed in the heart and skeletal muscle. But the physiological functions and mechanisms of *LMOD2* on skeletal muscle development are unknown.

Results In this study, we examined the expression levels of the *LMOD2* in porcine tissues and C2C12 cells. *LMOD2* is mainly expressed in the heart, followed by skeletal muscle. The expression level of *LMOD2* gradually decreased with skeletal muscle growth, but increased after injury. *LMOD2* expression levels increased gradually with C2C12 cells proliferation and differentiation. In terms of function, the muscle fiber types were altered after *LMOD2* was knocked out in C2C12 cells, MyHC-I and MyHC-2b were inhibited, whereas MyHC-2a and MyHC-2x were promoted. *LMOD2* knockout has different effects on LMOD family, *LMOD1* expression level was promoted, while *LMOD3* was inhibited. Loss of *LMOD2* suppressed cell viability and PAX7 protein expression. At the transcriptome level, proliferation-related genes and muscle contraction-related genes were respectively inhibited after *LMOD2* knockout. In terms of molecular networks, a series of experiments have shown that MyoG is a transcription factor for *LMOD2*, while miR-335-3p can negatively regulate *LMOD2* expression. We screened *ACTC1* as a candidate interacting protein for *LMOD2* using protein prediction software and RNA-seq, and Co-IP experiments confirmed the relationship between *LMOD2* and *ACTC1*. In vivo, Lentivirus-mediated *LMOD2* knockdown reduces muscle mass. *LMOD2* knockdown inhibited MyHC-I mRNA expression, but had no effect on MyHC-2b. The protein expression of MyHC-I, MyHC-2x, and MyHC-2b was suppressed after *LMOD2* knockdown.

Conclusions Collectively, our data indicates that *LMOD2* knockout inhibits myoblast proliferation and alters muscle fiber types. MyoG is a transcription factor for *LMOD2*, while miR-335-3p can negatively regulate *LMOD2* expression. Moreover, *LMOD2* and *ACTC1* interact to regulate myogenic differentiation. Our study provides a new target for skeletal muscle development.

Keywords Skeletal muscle, Myoblast, *LMOD2*, *ACTC1*, Knockout

†Kaiming Wang and Caihong Liu contributed equally to this work.

*Correspondence:
Yulong Yin
yinyulong@isa.ac.cn
Haiming Ma
mahaiming2000@163.com

[1]College of Animal Science and Technology, Hunan Agricultural University, Changsha 410128, China
[2]Key Laboratory of Livestock and Poultry Resources (Pig) Evaluation and Utilization, Ministry of Agriculture and Rural Affairs, Changsha 410128, China
[3]Yuelushan Laboratory, Changsha 410128, China
[4]Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha 410125, China

Background

Skeletal muscle accounts for about 50% of adult body weight in mammals, and is one of the most dynamic and flexible tissues, playing a crucial role in metabolism and movement [1]. Embryonic skeletal muscle generation and postnatal regeneration depend on myogenesis [2]. However, myogenesis is a complex and rigorous process in which mononuclear myoblasts proliferate and differentiate to form multinucleated myofibers [3]. This process is tightly and sophisticatedly regulated by myogenic regulatory factors (MRFs), including *Myf5*, *MyoD1*, *MyoG* and *Myf6*. Skeletal muscle is composed of a series of muscle fibers. However, with the increasing global aging, muscle atrophy and muscle loss have become a common disease. It is significant to identify more key genes involved in skeletal muscle development.

Intricately designed striated muscles are composed of different cytoskeletons. Coordinated action between the sarcomere and the cytoskeletons is crucial for myogenesis. With huge advances in technology, numerous other significant muscle contraction proteins will certainly be discovered [4]. In 2001, Transcripts encoding the novel Lmod gene are present in fetal and adult heart and adult skeletal muscle, and it is named cardiac Leiomodin (*LMOD2*) [5]. It is worth noting that the *LMOD2* transcript was first detected in the hearts of chickens only after the heart began beating. Immunofluorescent staining of rat and chicken cardiomyocytes indicated that *LMOD2* localizes to actin filament pointed ends [6]. The increase in the expression level of *LMOD2* in the heart can lead to a 10% elongation of filaments [7]. Conversely, heart-specific conditional *LMOD2* knockout mice died with severe left ventricular systolic dysfunction, and the cardiac filaments of the knockout mice were uneven and significantly reduced in length [8]. These indicate that *LMOD2* plays an important role in regulating cardiac contractility and thin filament length [9]. So far, the focus on the *LMOD2* gene has mainly been on the heart, while its role in skeletal muscle has been ignored. However, recent sequencing results have revealed that *LMOD2* is differentially expressed at different stages of skeletal muscle development [10]. *LMOD2* is also highly expressed in injured and aged skeletal muscles [11, 12]. Compared with other muscle types, *LMOD2* is mainly expressed in the soleus muscle, and *LMOD2* is critical for specifying thin-filament length in skeletal muscle [13]. The soleus muscle of *LMOD2* knockout mice has shorter filaments, and the fiber type switched from fast muscle to the slow muscle [14]. However, the function of *LMOD2* in skeletal muscle myogenesis and its molecular mechanisms remain unclear.

In this study, we speculated that *LMOD2* gene could be a crucial factor in skeletal muscle myogenesis. This prompted us to conduct an in-depth study on the effect of *LMOD2* on myogenesis in this research. We found that the proliferation ability of *LMOD2* knockout cells was suppressed and the muscle fiber type was altered. We constructed the molecular network of *LMOD2* regulating myogenic differentiation. Our results demonstrate that *LMOD2* plays an important role in the regulation of skeletal muscle.

Result

In vitro and in vivo characterization of LMODs family expression in skeletal muscle

Pigs are often used as a research model. We detected the expression level of LMOD1-3 in pig tissues, and found that *LMOD1* was lowly expressed in heart and skeletal muscle (Fig. 1A), *LMOD2* was highly expressed in heart and skeletal muscle (Fig. 1B), and *LMOD3* was highly expressed in skeletal muscle and lowly expressed in heart (Fig. 1C). LMOD1 protein expression was not detected in pig skeletal muscle. The protein expression of LMOD2 and LMOD3 were upregulated at 1 and 4 days after skeletal muscle injury, then decreased to normal levels (Fig. 1D-E). The protein expression of LMOD2 and LMOD3 gradually decreased after birth in pigs (Fig. 1F-G). In vitro, we found that *LMOD1* gradually decreased with myoblasts proliferation, while *LMOD2* and *LMOD3* were the opposite (Fig. 1H). The mRNA and protein expression of LMODs increased gradually with the myoblasts differentiation (Fig. 1I-J). According to previous research, LMOD3 can promote differentiation and proliferation of myoblasts through the AKT and ERK pathways [15]. Considering that the expressions of *LMOD2* and *LMOD3* are similar, we speculated that *LMOD2* is also a crucial factor in skeletal muscle myogenesis. Therefore, *LMOD2* is selected as the object of further study.

The effect of *LMOD2* knockout on myogenic differentiation

To investigate the role of *LMOD2* in myogenesis, we constructed *LMOD2* knockout cell lines with CRISPR-Cas9 technology (Fig. 2A). The PCR and Sanger sequencing results showed that exon 1 of the *LMOD2* gene was deleted at 79 bp (Fig. 2B-C), and further verification results showed that *LMOD2* could not be detected at mRNA and protein levels (Fig. 2D-F). Functionally, the mRNA expression of *MyHC-I* and *MyHC-2b* was inhibited after *LMOD2* knockout, while the opposite results were observed for *MyHC-2a* and *MyHC-2x* (Fig. 2G-J). Immunofluorescence staining results showed that MyHC-I and MyHC-2b were rarely observed in *LMOD2* knockout compared to the wild type, whereas MyHC-2a and MyHC-2x were the opposite (Fig. 2K). Western blot results showed that after *LMOD2* knockout, MyHC-I and MyHC-2b protein expression was inhibited, MyHC-2a protein expression was promoted, and MyHC-2x protein expression was not statistically significant (Fig. 2L-M).

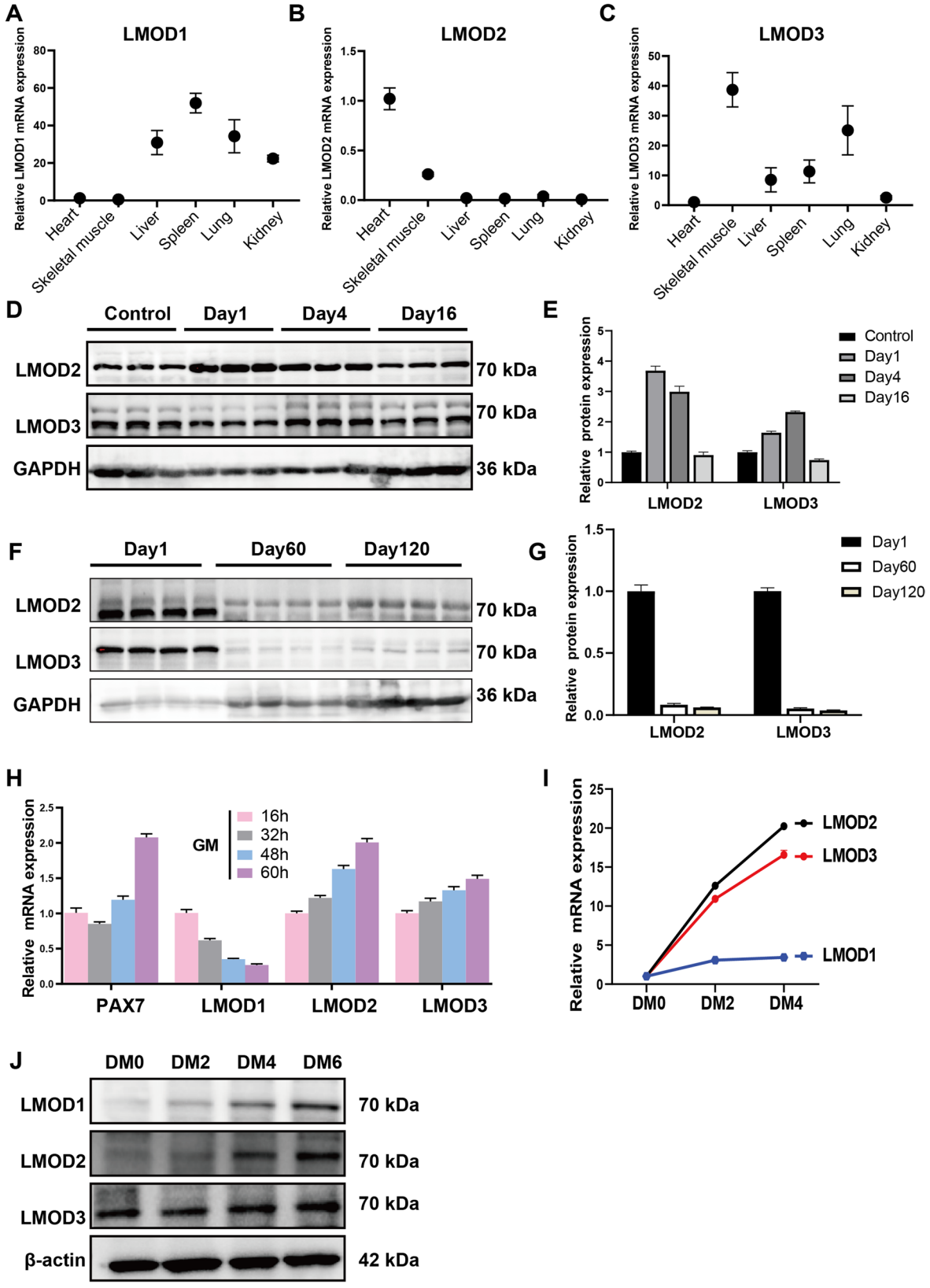

Fig. 1 In vitro and in vivo characterization of LMOD family expression in skeletal muscle. **A-C** The expression profile of LMOD1-3 in pig tissues was detected by qRT-PCR (n = 3). **D-G** The protein expression of LMOD2 and LMOD3 during pig skeletal muscle regeneration (n = 3), and development (n = 4), was detected by Western blot. **H** The mRNA expression of LMODs during C2C12 cells proliferation was detected by qRT-PCR (n = 3). **I-J** qRT-PCR and Western blot were used to detect the expression of LMODs during C2C12 cells differentiation. Values represent mean±SE. *, P<0.05; **, P<0.01

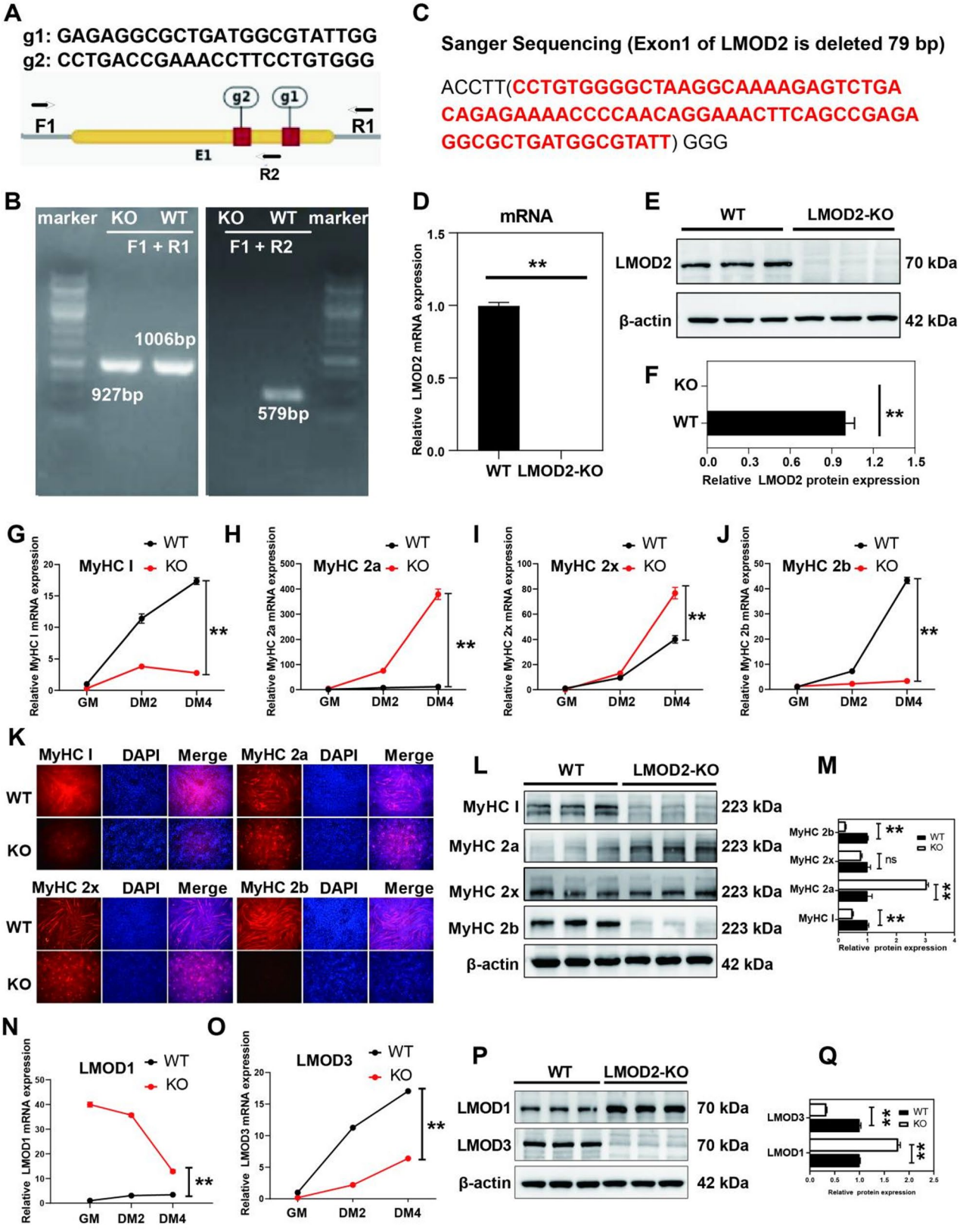

Fig. 2 (See legend on next page.)

(See figure on previous page.)

Fig. 2 The effect of *LMOD2* knockout on myogenic differentiation. **A** gRNAs of *LMOD2* gene were designed and synthesized. **B-C** PCR and Sanger sequencing techniques were used to identify whether the *LMOD2* gene was knocked out. The red word indicate the sequences in which *LMOD2* gene was deleted. **D-F** The efficiency of *LMOD2* gene knockout was detected by qRT-PCR and Western blot ($n = 3$). **G-M** qRT-PCR, Immunofluorescence staining and Western blot were used to detect the regulatory effect of *LMOD2* knockout on four types of muscle fibers ($n = 3$), scale bar = 200 μm. **N-Q** qRT-PCR and Western blot were used to detect the effect of *LMOD2* knockout on *LMOD1* and *LMOD3* ($n = 3$). Values represent mean ± SE. *, $P < 0.05$; **, $P < 0.01$

LMOD1 and *LMOD3* genes are homologous genes of *LMOD2*, After *LMOD2* knockout, *LMOD1* expression was enhanced, but *LMOD3* expression was inhibited (Fig. 2N-Q).

RNA-seq reveals the effect of *LMOD2* knockout on transcription levels

In order to understand the effect of *LMOD2* gene on myoblast proliferation, CCK-8 and Western blot experiments showed that cell proliferation was suppressed after *LMOD2* knockout (Fig. 3A-C). Then, during the cell proliferation phase, RNA-seq found that 1745 genes were down-regulated and 1631 genes were up-regulated after *LMOD2* knockout (Fig. 3D-E). Among the down-regulated genes contains some proliferative marker genes such as *Mki67* and *Pcna* (Fig. 3F). The GO, KEGG, and GSEA analyses of Down-regulated genes found that most of the genes were involved in DNA replication (Fig. 3G-J). During the cell differentiation stage, RNA-seq found that 1239 genes were down-regulated and 881 genes were up-regulated after *LMOD2* knockout (Fig. 3K-L). Down-regulated genes GO analyses found that most of the genes were related to muscle contraction (Fig. 3M). Down-regulated genes KEGG analyses found that most of the genes were related to hypertrophic cardiomyopathy and MAPK signaling pathway (Fig. 3N). Integration of transcriptome data showed that the *LMOD2* gene was highly correlated with muscle contraction genes (Fig. 3O).

LMOD2 is a target gene of *MyoG* and miR-335-3p

Myogenic regulatory factors (MRFs) are responsible for determining the fate of myoblasts. We examined the effects of *LMOD2* knockout on *MyoD1* and *MyoG* genes, and found that *MyoD1* expression was promoted, while *MyoG* expression was inhibited (Fig. 4A-C). Further, Compared with *MyoD1*, *MyoG* knockdown inhibited *LMOD2* expression at both proliferation and differentiation stages (Fig. 4D-E). This may be because *MyoD1* is mainly expressed in type II muscle fibers, while *MyoG* is mainly expressed in type I muscle fibers. Therefore, we speculated that *MyoG* is a transcriptional regulator of *LMOD2*. Further, this idea was confirmed by Double-luciferase reporter experiment (Fig. 4F). These results indicate that *MyoG* and *LMOD2* are mutually adjustable. Our results indicate that *LMOD2* is a positive regulatory factor for satellite cell differentiation, and *LMOD2* mainly regulates slow muscle phenotype.

MicroRNAs (miRNAs) usually act as negative regulators by influencing mRNA stability. To further understand the molecular regulatory network of *LMOD2* in myoblasts, we use miRNA-seq to screen the upstream miRNAs of *LMOD2*. Compared with the wild type (WT), 148 miRNAs were down-regulated and 118 miRNAs were up-regulated after *LMOD2* knockout (Fig. 4G). The correlation between samples showed good reproducibility in the group (Fig. 4H). Cluster heat maps showed up and down regulated miRNAs (Fig. 4I). The venny results from miRNA-seq and miRDB website indicated that miR-335-3p is a candidate miRNA for *LMOD2* (Fig. 4J). Further verification results showed that miR-335-3p expression was up-regulated after *LMOD2* knockout (Fig. 4L). The expression level of miR-335-3p gradually decreased with myoblasts differentiation (Fig. 4M). MiR-335-3p overexpression significantly suppressed the mRNA expression of *LMOD2*, but had no effect on the protein (Fig. 4N-P). Then, miR-335-3p overexpression decreased the luciferase activity of *LMOD2*-WT (Fig. 4Q). These results indicated that *LMOD2* is the direct target gene of miR-335-3p.

LMOD2 interacts with *ACTC1* to regulate myogenic differentiation

To further understand the mechanism by which *LMOD2* regulates myogenic differentiation, we used RNA-seq and STRING to screen the proteins that bind to *LMOD2* in myoblasts. The venn diagram results from RNA-seq and STRING showed that seven genes could possibly interact with *LMOD2*, namely *HSPB7*, *LMOD3*, *TRIM54*, *CASQ2*, *ACTC1*, *MYL1* and *MYH7* (Fig. 5A-B). It is worth noting that *ACTC1* (Actin alpha cardiac muscle 1), is an essential member of the actin family and is mainly expressed in heart tissue. Therefore, we speculate that ACTC1 is a potential binding protein of LMOD2. We found ACTC1 protein expression level was inhibited after *LMOD2* knockout (Fig. 5C-D). To confirm the LMOD2-ACTC1 association, we immunoprecipitated ACTC1 in myoblasts and found that both ACTC1 and LMOD2 were present in the precipitate (Fig. 5E). The above results validated the interaction between LMOD2 and ACTC1. We found that ACTC1 protein increased gradually with myoblast differentiation, suggesting that *ACTC1* may be involved in the regulation of myoblast differentiation (Fig. 5F). To clarify the function of *ACTC1*, we designed three pairs of small interfering RNA (siRNA) for *ACTC1*, and si-*ACTC1*–1073 was found to have the

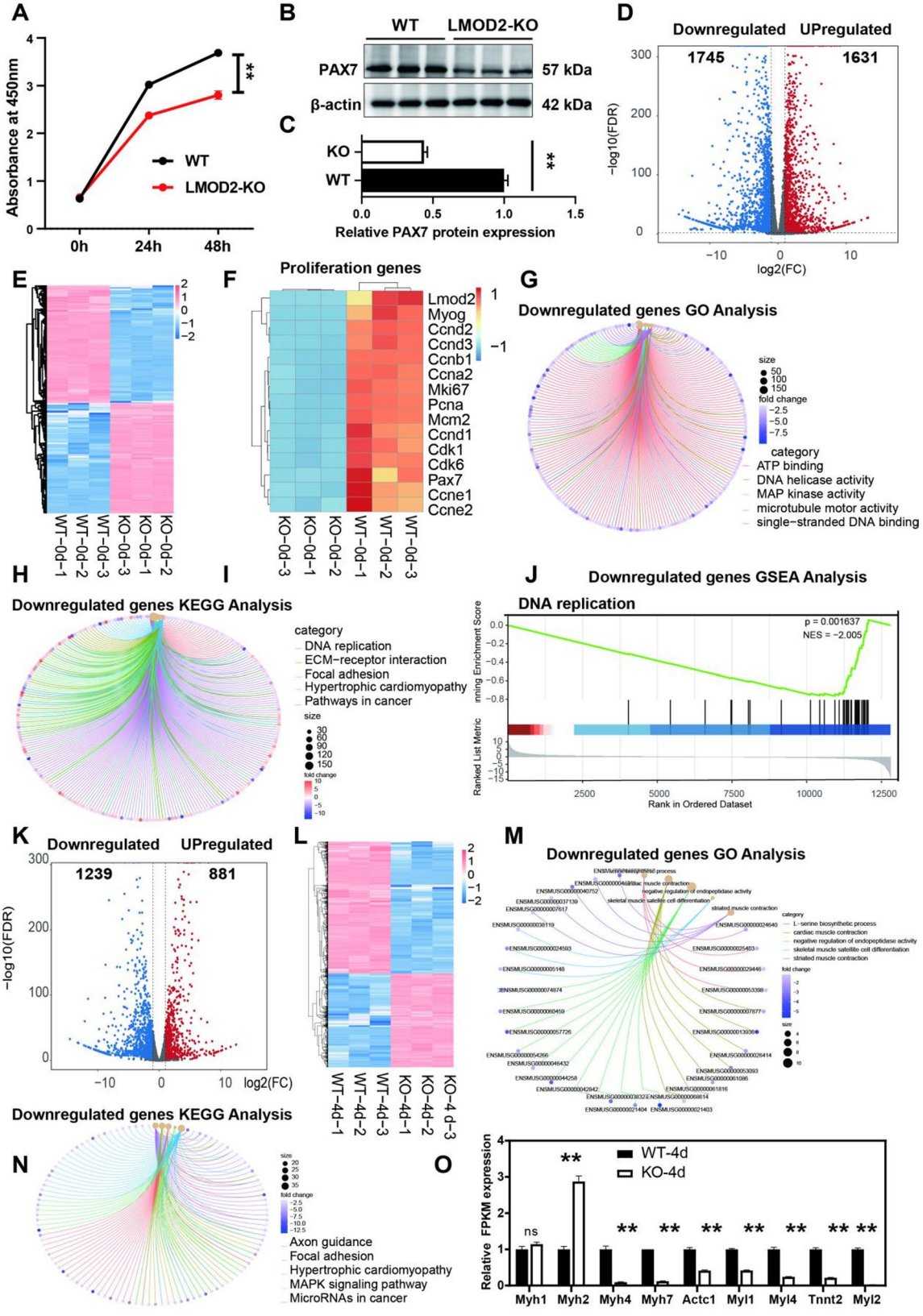

Fig. 3 (See legend on next page.)

(See figure on previous page.)

Fig. 3 RNA-seq reveals the effect of *LMOD2* knockout on transcription levels. **A** Cell proliferation rate after *LMOD2* knockout was detected by CCK-8 ($n=3$). **B-C** The protein expression of proliferation marker gene PAX7 was detected by Western blot after *LMOD2* knockout ($n=3$). **D** During the cell proliferation phase, Volcanic maps of RNA-seq indicated differentially expressioned genes after *LMOD2* knockout ($n=3$). **E** Cluster heat maps showed up and down regulated genes ($n=3$). **F** Genes associated with proliferation were shown in picture after *LMOD2* knockout ($n=3$). **G-J** During the cell proliferation phase, Down-regulated genes of RNA-seq were analyzed by GO, KEGG and GSEA ($n=3$). **K** During the cell differentiation stage, Volcanic maps of RNA-seq indicated differential genes after *LMOD2* knockout ($n=3$). **L** Cluster heat maps showed up and down regulated genes ($n=3$). **M-N** During the cell differentiation stage, Down-regulated genes were analyzed by GO and KEGG ($n=3$). **O** The expression of muscle fiber MyHC type and muscle contraction-related genes was shown in picture after *LMOD2* knockout ($n=3$). Values represent mean ± SE. *, $P<0.05$; **, $P<0.01$

best inhibitory effect (Fig. 5G). Functionally (Fig. 5H-J), *ACTC1* knockdown inhibited the expression of myogenic differentiation marker genes MYHC and *LMOD2*. Our results indicate that *LMOD2* interacts with *ACTC1* to regulate myogenic differentiation.

Lentiviral-Mediated *LMOD2* knockdown reduced muscle mass

To verify the effect of *LMOD2* on muscle mass and myogenic differentiation in vivo, we injected LV-sh-NC and LV-sh-*LMOD2* intramuscularly into the left and right legs respectively of 4-week-old mice (Fig. 6A-B). Knockdown of *LMOD2* significantly reduced the weight of the hind limb, QU and GAS muscles, but it has no effect on the TA muscle (Fig. 6C-G). qRT-PCR showed that the mRNA expression of *MyHC I* was significantly decreased after *LMOD2* knockdown, and *MyHC 2b* had no effect (Fig. 6H). Western blot showed the protein expression of MyHC I, MyHC 2x, MyHC 2b were remarkably reduced by *LMOD2* knockdown, while that of MyHC 2a was no effected (Fig. 6I-J). These results show that the *LMOD2* gene mainly regulates MyHC I type muscle fibers.

Discussion

The process of myoblast proliferation and differentiation is called myogenesis, and it is not only important for muscle development and growth, but also necessary for muscle regeneration [16]. In this study, we revealed that *LMOD2* and *LMOD3* expression trends are highly similar in tissue, skeletal muscle development and regeneration, and myogenesis, suggesting that *LMOD2* may have the same function as *LMOD3.* Remarkably, *LMOD1* has long been known as a smooth muscle specific expression gene [17]. We first discovered that *LMOD1* is lowly expressed in skeletal muscle, but differentially expressed during myogenesis, suggesting that *LMOD1* may have regulatory function in myogenesis. After a comprehensive comparison, we chose *LMOD2* for further study.

CRISPR-Cas9 gene editing system is a common tool for studying gene function [18]. Thus, we successfully constructed the *LMOD2* knockout (KO) C2C12 cells using CRISPR-Cas9 in vitro, and found that *LMOD2*-KO inhibited *MyHC-I* and promoted *MyHC-2a* expression, which is inconsistent with a previous report in mice [19]. We speculate that this may be caused by differences in vivo and in vitro. Further, *LMOD1* expression was upregulated 10–40 times after *LMOD2* knockout compared to the wild type (WT). That may be because the CArG boxes for L*MOD2* and *LMOD1* are similar [20]. In addition, elevated *LMOD1* levels may be a compensatory mechanism for *LMOD2* deficiency. In contrast, the *LMOD3* expression was inhibited after *LMOD2* knockout. *LMOD3* may coordinate with *LMOD2* to regulate myogenic differentiation.

In fact, we observed functional and morphological differences between *LMOD2*-KO and WT. At the transcriptome level, KEGG of down-regulated genes such as *MYH7* and *ACTC1* are mainly enriched in hypertrophic cardiomyopathy. Interestingly, human *LMOD2* is located near the hypertrophic cardiomyopathy locus CMH6 on chromosome 7q3 [21]. Taking these results into consideration, *ACTC1*, a gene that is highly expressed in the heart and skeletal muscle [22, 23], caught our attention. ACTC1 expression in myoblasts differentiation is the same as that of *LMOD2*. Functionally, *ACTC1* knockdown suppressed myogenic differentiation, consistent with the previous report on bovine myoblast [24]. In addition, our study further found that *ACTC1* knockdown did not change muscle fiber types. In summary, *LMOD2* and *ACTC1* only synergistically act on myoblast differentiation, but not on muscle fiber type transformation.

Myogenesis is regulated by several crucial transcription factors, including myogenic differentiation 1 (*MyoD1*) and myogenin (*MyoG*) [25–27]. *MyoD1* is prevalent in fast muscles and *MyoG* in slow muscles [28]. *LMOD2* knockout results in up-regulation of *MyoD1* and down-regulation of *MyoG*. Compared with *MyoD1*, *MyoG* is highly positively correlated with *LMOD2* expression. Mice with *MyoD1* knockout display a slow muscle phenotype [29]. In contrast, low doses of *MyoG* promote a fast muscle phenotype [30]. We finally confirmed that *MyoG* is a true transcription factor for *LMOD2*. MicroRNA (miRNA) is a class of conserved small single-stranded RNA, generally act as a negative regulator of protein translation [31]. In order to construct the molecular network that *LMOD2* regulates myoblast differentiation, we performed miRNA-seq sequencing and found that 148 genes were down-regulated and 118 genes were up-regulated. The expression trend of miRNA was usually opposite to that of its target genes [32]. After *LMOD2* knockout, the miR-335-3p expression was up-regulated. On the other hand, contrary to *LMOD2*, miR-335-3p

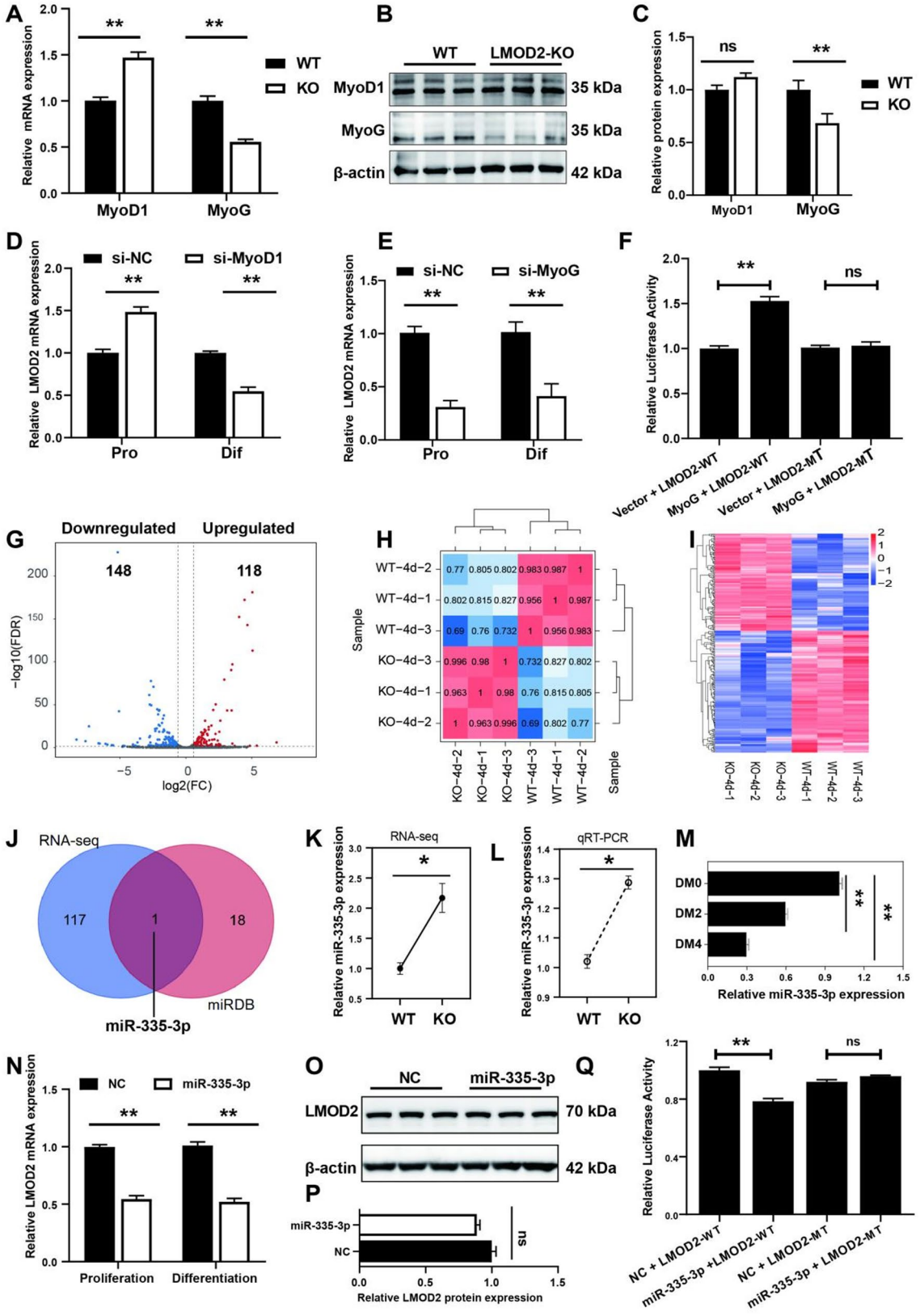

Fig. 4 (See legend on next page.)

(See figure on previous page.)

Fig. 4 *LMOD2* is a target gene of *MyoG* and miR-335-3p. **A-C** qRT-PCR and Western blot were used to detect the mRNA and protein expression of MyoD1 and MyoG after *LMOD2* knockout ($n=3$). **D** The effect of *MyoD1* knockdown on *LMOD2* gene expression in the proliferation (Pro) and differentiation (Dif) of C2C12 cells was detected by qRT-PCR ($n=3$). **E** The effect of *MyoG* knockdown on *LMOD2* gene expression in the proliferation (Pro) and differentiation (Dif) of C2C12 cells was detected by qRT-PCR ($n=3$). **F** In the Dual-luciferase reporter assay, the *LMOD2*-WT and *LMOD2*-MUT report vector was co-transfected with the *MyoG* or Vector into HEK293T cells ($n=3$). **G** Volcanic maps of miRNA-seq indicated up-regulated and down-regulated miRNAs after *LMOD2* gene knockout ($n=3$). **H** Correlation analysis between samples of miRNA-seq ($n=3$). **I** Cluster heat maps showed up and down regulated miRNAs ($n=3$). **J** The candidate miRNAs of *LMOD2* were screened by miRNA-seq and miRDB website. **K-L** The Effect of *LMOD2* gene knockout on miRNA-335-3p was detected by RNA-seq and qRT-PCR ($n=3$). **M** The expression trend of miRNA-335-3p in myogenic differentiation was detected by qRT-PCR ($n=3$). **N** The effect of miRNA-335-3p overexpression on *LMOD2* was detected by qRT-PCR during myoblasts proliferation and differentiation stages ($n=3$). **O-P**, During the differentiation of myoblasts, the effect of miRNA-335-3p on *LMOD2* protein was detected by Western blot ($n=3$). **Q** In the Dual-luciferase reporter assay, the *LMOD2*-WT and *LMOD2*-MUT report vector was co-transfected with the miRNA-335-3p mimic or mimic NC into HEK293T cells ($n=3$). Values represent mean ± SE. *, $P<0.05$; **, $P<0.01$

expression gradually decreased with myogenic differentiation. Similar to miR-100-5p [33], miR-335-3p may be negatively correlated with myoblast differentiation. Previous studies have shown that miR-335-3p regulates cellular biological processes by targeting different genes, but has not been reported in skeletal muscles [34–36]. At present, our study only proved that miR-335-3p has a targeting relationship with *LMOD2*, and the function of miR-335-3p still needs to be further explored.

Intramuscular injection of lentiviruses in mice has been widely used to study gene function [37]. We injected lentiviruses in vivo to silence *LMOD2* expression and found that *LMOD2* knockdown significantly reduced muscle mass. In more detail, *LMOD2* knockdown in vivo has a stronger inhibitory effect on slow muscles than on fast muscles. Previous studies have reported that the *LMOD2* gene is associated with muscle fiber hypertrophy in animals [38, 39]. Thus, it can be concluded that *LMOD2* mainly positively regulates the expression of slow muscle genes to affect the process of muscle differentiation.

Conclusions

Collectively, *LMOD2* knockout inhibits myoblast proliferation and alters muscle fiber types. In molecular networks, MyoG is a transcription factor for *LMOD2*, while miR-335-3p can negatively regulate *LMOD2* expression. Moreover, *ACTC1* and *LMOD2* interact to regulate myogenic differentiation. Our study provides a new target for skeletal muscle development.

Materials and methods

Sample Preparation

In this study, all the animals were euthanized. These pigs were stunned by electric shock and then bled to death. We collected longissimus dorsi muscle of pigs at different developmental stages (n=4). In terms of muscle injury and regeneration in pigs, 1 mL of 40 μM Cardiotoxin (9012-91-3, BOYAO, Shanghai, China) was injected into the longissimus dorsi muscle of 35-day-old pigs to induce muscle injury. The pigs were euthanized on the 1st, 4th, and 16th days after injection. Samples of the longissimus dorsi muscle were taken from the injection site.

Cell culture

C2C12 cells and 293 T cells (ATCC, New York, USA) were cultured in growth medium (GM). In short, the cells were cultured in DMEM (Gibco, CA, USA) containing 10% bovine serum (FBS, Gibco, CA, USA). For C2C12 cells differentiation, when the cell density reaches 80%, the cells were cultured in DMEM containing 2% Horse serum (HBS, Gibco, CA, USA). All cells were cultured in an incubator of humid environment at 5% CO_2 and 37 ℃.

Plasmids, sirnas, and transfection

The Plasmids and siRNAs in this paper were designed and synthesized by GenePharma (Suzhou, China). When the cell density reached about 35%, transient transfection was performed with lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the instructions. All ligonucleotide sequences are listed in Table S1.

Total RNA extraction and quantitative Real-time PCR

Total RNA was extracted from cells and tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, total RNA was reverse-transcribed into cDNA by using a kit (Invitrogen, Carlsbad, CA, USA). RT-qPCR was performed using SYBR Green Kit (TransGen, Beijing, China), and the relative expression of genes was cacu-lated by the $2^{-\Delta\Delta Ct}$ method. All primer sequences are listed in Table S2.

Generation of *LMOD2* knockout C2C12 cells

LMOD2 gene was knocked out using CRISPR-Cas9 system. In brief, a pair of guide RNA (gRNA) was designed and transfected into C2C12 cells. The deletion of target sites was verified by DNA sequencing and PCR.

RNA-seq and analysis

Total RNA was extracted from *LMOD2*-KO and C2C12 cells for sequencing. Sequencing and analysis were performed by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). In the process of differential expression gene detection, Fold Change was greater than 2 and FDR < 0.01 were used as screening criteria. During the detection of differentially expressed miRNA,|log2(FC)| is

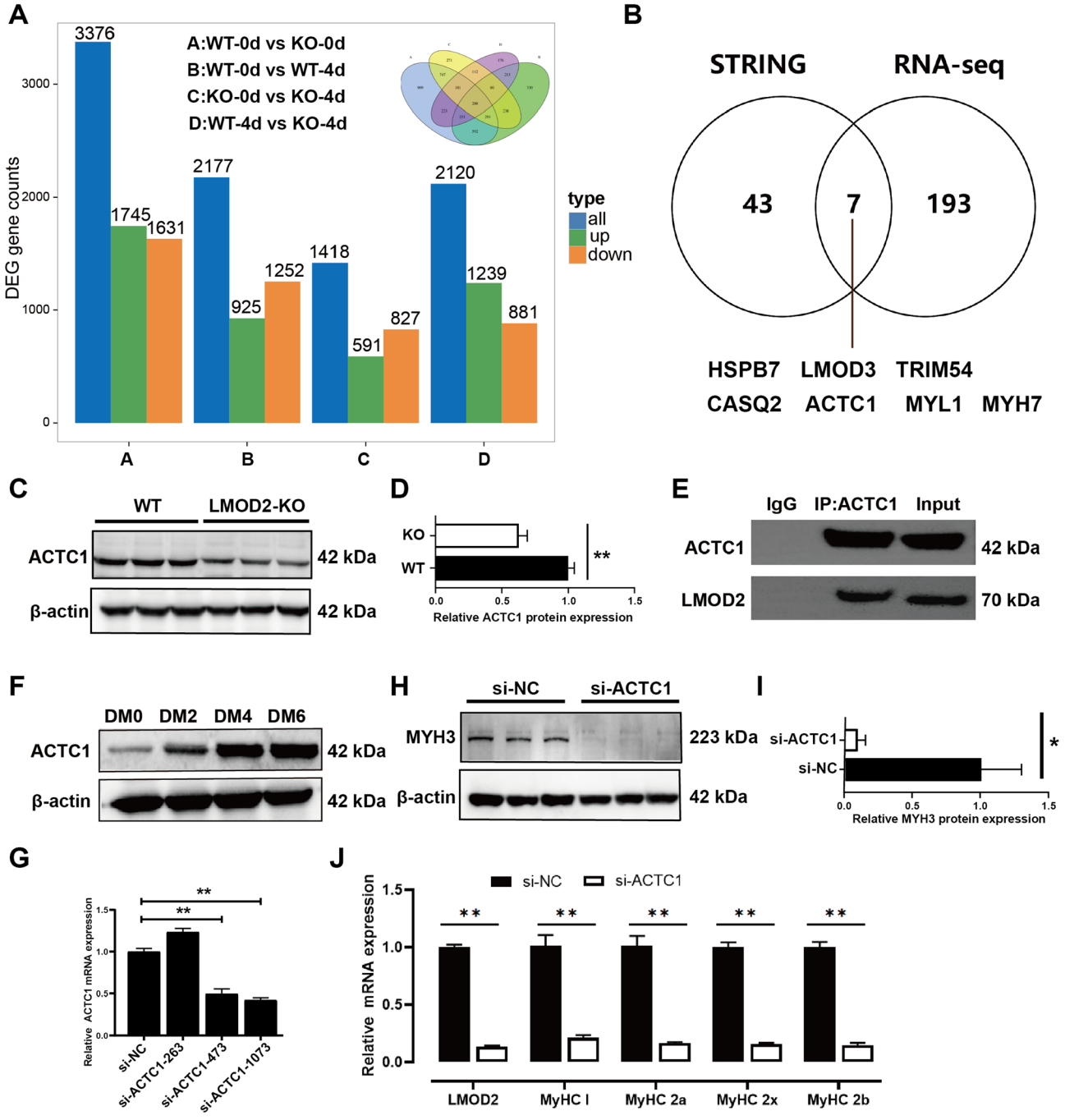

Fig. 5 *LMOD2* interacts with *ACTC1* to regulate myogenic differentiation. **A** RNA-seq screened differential genes in myoblasts proliferation (0d) and differentiation (4d) after *LMOD2* knockout (n = 3). **B** Venn diagram analyzed the intersection genes of RNA-seq and STRING. **C-D** *ACTC1* protein expression in *LMOD2* knockout cells was detected by Western blot (n = 3). **E** The C2C12 cells were Immunoprecipitated with *ACTC1* antibody and then Western blot with anti-ACTC1 and anti-LMOD2. **F** The protein expression of ACTC1 during myogenic differentiation was analyzed by Western blot (n = 3). **G** The knockdown efficiency of *ACTC1* gene in myoblasts by small interfering RNAs (siRNAs) was detected by qRT-PCR (n = 3). **H-I** Western blot analysis of the effect of ACTC1 knockdown on MYH3 protein (n = 3). **J** The effect of *ACTC1* knockdown on *LMOD2*, *MyHC I*, *MyHC 2a*, *MyHC 2x* and *MyHC 2b* was detected by qRT-PCR (n = 3). Values represent mean±SE. *, P<0.05; **, P<0.01

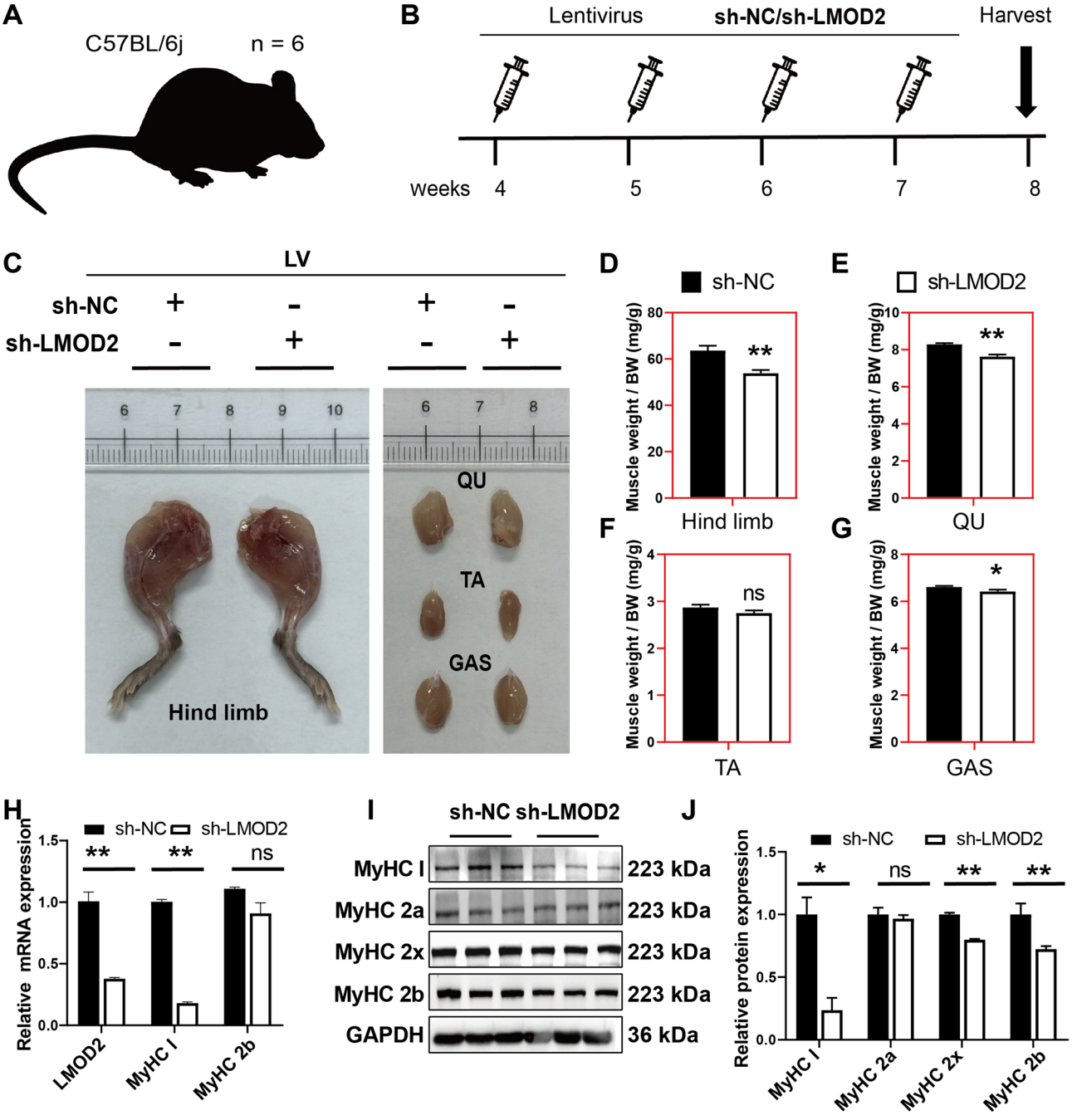

Fig. 6 Lentiviral-Mediated *LMOD2* knockdown reduces muscle mass and inhibits myoblast differentiation in vivo. **A-B** Injection diagram of the LV-sh-NC and LV-sh-*LMOD2* intramuscularly into the left and right legs, respectively (n = 6). **C-G** Representative pictures of the hind limb, QU (Quadriceps), TA (Tibialis anterior) and GAS (Gastrocnemius) muscles. Data are normalized to body weight (mg/g), (n = 6). **H** The mRNA expression of *LMOD2*, *MyHC I* and *MyHC 2b* after *LMOD2* knockdown was analyzed by qRT-PCR (n = 3). **I-J** The protein expression of MyHC I, MyHC 2a, MyHC 2x and MyHC 2b after *LMOD2* knockdown was analyzed by Western blot (n = 3). Values represent mean±SE. *, P<0.05; **, P<0.01

greater than 0.58 and FDR < 0.05 was used as screening criteria.

Cell counting Kit-8 (CCK-8) assay

LMOD2-KO and C2C12 cells were seeded at a density of 1×10^6 in 96-well plates. Then, CCK-8 (AFExBIO, USA) solution was added at 0 h, 24 h and 48 h after cell culture. The absorbance at 450 nm was detected by the instrument after incubation for 4 h.

Western blot analysis

The total proteins were extracted from cells and tissues using the RIPA (Beyotime). Antibody information is as follows: Anti-LMOD1 (15117-1-AP, 1:1000, Proteintech, China), Anti-LMOD2 (DF15006, 1:1000, Affinity Biosciences, China), Anti-LMOD3 (14948-1AP, 1:1000, Proteintech, China), Anti-MyHC-I (22280-1-AP, 1:1000, Proteintech, China), Anti-MYHC-2a (2F7, 1:500, DSHB, USA), Anti-MyHC-2x (67299-1-Ig, 1:1000, Proteintech, China), Anti-MyHC-2b (BF-F3, 1:500, DSHB, USA), Anti-PAX7 (20570-1-AP, 1:1000, Proteintech, China), Anti-MyoD1 (ER1913-45, 1:1000, HuaBio, China), Anti-MyoG (HA721688, 1:1000, HuaBio, China), Anti-ACTC1 (11032-1-AP, 1:1000, Proteintech, China), Anti-MYH3 (bs-10905R, 1:1000, Bioss, China), Anti-β-actin (66009-1-Ig, 1:20000, Proteintech, China), Anti-GAPDH (10494-1-AP, 1:20000, Proteintech, China), and two secondary antibodies (BF03001X and BF03008X, 1:10000, Biodragon) were used in this study. Finally, protein bands were measured by the ImageJ software.

Immunofluorescence assay

The differentiated *LMOD2*-KO cells and C2C12 cells were fixed with 4% formaldehyde, and permeabilized with Triton X-100 for 1 h. Subsequently, the cells were incubated with primary and secondary antibodies. Antibody information is as follows: Anti-MyHC-I (22280-1-AP, 1:200, Proteintech, China), Anti-MyH-2b (14-6503-80, 1:200, Invitrogen, USA), Anti-MyHC- 2x (67299-1-Ig, 1:200, Proteintech, China), Anti-MyHC-2a (2F7, 1:200, DSHB, USA). and two secondary antibodies (bs-0296G-Cy3, Bioss, 1:200; AF594, ZENBIO, 1:200) were used in this study. Finally, the cells were incubated with DAPI (1:1000). The images were obtained by a microscope (Leica SP8) and processed by ImageJ software.

Dual-Luciferase reporter assay

Dual-Luciferase Reporter Assay was applied to verify the targeting relationship between *LMOD2* and its upstream regulators (MyoG and miR-335-3p). In brief, MyoG and miR-335-3p overexpressed plasmids were synthesized. Next, wild and mutant sites of *LMOD2* were predicted by softwares (JASPAR and miRDB). Finally, these luciferase activities were detected by co-transfection experiment.

Identification of *LMOD2* interacting partners by Co-immunoprecipitation

For each Co-IP assay, proteins were extracted from cells that had differentiated for 4 days. The protein lysate was added to the culture plates for cell lysis. The protein lysate was centrifuged at 10,000 rpm for 15 min and then supernatant was collected. The ACTC1 antibody (11032-1-AP, Proteintech, China) was added to the protein lysate. Pierce™ Protein A/G agarose beads (Invitrogen, Carlsbad, CA, USA) were washed with the buffer. The microspheres were added to the cell lysate, incubated at 4℃ overnight, and centrifuged at 3000 rpm at 4℃ for 5 min. The supernatant was removed and the agarose beads were washed with buffer. Then 100 µl 2 × SDS loading buffer was added, and incubated at 90℃ for 10 min, and Western blot analysis was performed.

Lentiviruses mediated *LMOD2* knockdown in skeletal muscles

The sequence for the siRNA against mouse *LMOD2* was GGACCAAGAAUGAGUAUGATT. Mouse *LMOD2* siRNAs were designed as shRNA and cloned into the LV3 vector, and the virus titer is 10^9 (GenePharma, Suzhou, China). Four-week-old wild-type C57BL/6j male mice were injected with the lentivirus ($n = 6$). Concretely, the right and left muscles were injected with LV-shRNA-*LMOD2* and LV-shRNA-NC once a week for 4 weeks, respectively. Finally, the muscles at the injection site were collected.

Statistical assay

All the data were presented as the means ± standard error. Unpaired Student's t-test was performed between the treatment group and the control group using GraphPad prism 8. Values represent mean ± SE. *, $P < 0.05$; **, $P < 0.01$.

Abbreviations

SiRNA	Small interfering RNA
MiRNA	MicroRNA
MyHC	Myosin heavy chain
qRT-PCR	Quantitative Real-time polymerase chain reaction

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11897-z.

Supplementary Material 1

Supplementary Material 2

Acknowledgements

Not applicable.

Author contributions

KW and CL directed and supervised the study; KW, LY, and SL performed the experiments; WC, XL, amd BC collected the samples and analyzed the data;

KW, XX, JL, and XL wrote the manuscript; YY and HM provided resources. All the authors have read and approved the final manuscript.

Funding
This work was supported by the National Natural Science Foundation of China (grant number: U24A20445), the Biological Breeding-National Science and Technology Major Project (grant number: 2023ZD04046), the Major Science and Technology Special Plan of Yunnan Province (grant number: 202202AE090032), the Breeding of Lancang Black Pigs and its application (grant number: 2021kjc-js072), the Key technologies for the exploration of excellent genetic resources and their efficient farming and its application in Tibetan pigs (grant number: SNQYKJXT-01), the program of talent of science & technology and platforms of Yunnan Province (grant number: 202305AF150211).

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
All animals procedures in this study were approved by the Hunan Agricultural University Animals Care and Use Committee (permit number CACAHU-20230601). Moreover, All methods were preformed in accordance with this committee guidelines.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 6 March 2025 / Accepted: 9 July 2025
Published online: 31 July 2025

References

1. Walter RF, Julien O. Skeletal muscle: a brief review of structure and function. CALCIFIED TISSUE INT. 2014;96(3):183–95.
2. Sander G, Anne Marie K, Ruurd T, Johannes WVDH. Skeletal muscle development and regeneration. STEM CELLS DEV. 2007;16(5):857–68.
3. Dingding Z, Xuan Z, Zhaojun L, Xiangfei M, Hongmin L, Ming S, Jie C, Honglin L. Diosmin Promotes Myogenesis via Activating the Akt/FOXO1 Pathway to Facilitate the Proliferation of C2C12 Myoblasts. J AGR FOOD CHEM. 2023;71(49):19705–16.
4. Christine AH, Christopher GG, Stefanie MN, Lei M, Carol C. G: Overview of the muscle cytoskeleton. COMPR PHYSIOL. 2017;7(3):891–944.
5. C, A C, K L F, A A, V M F. Leiomodins: larger members of the Tropomodulin (Tmod) gene family. Genomics. 2001;73(2):127–39.
6. Takehiro T, Christopher TP, Natalia M, Parker BA, Alla SK, Carol CG. Leiomodin-2 is an antagonist of tropomodulin-1 at the pointed end of the thin filaments in cardiac muscle. J CELL SCI. 2010;123(0):3136–45.
7. Lei M, Gerrie PF, Rachel MM, Joshua S, Miensheng C, Christopher TP, Carol C. G: In vivo elongation of thin filaments results in heart failure. PLoS ONE. 2020;15(1):e0226138.
8. Christopher TP, Gerrie PF, Rachel MM, John PK, Carol CG. Cardiac-specific knockout of Lmod2 results in a severe reduction in myofilament force production and rapid cardiac failure. J MOL CELL CARDIOL. 2018;122(0):88–97.
9. Christopher TP, Rachel MM, Christine H, Nima J, Cathleen C, Zachary H, Kirk RH, Miensheng C, Ki-Hwan N, Jose MV et al. Knockout of Lmod2 results in shorter thin filaments followed by dilated cardiomyopathy and juvenile lethality. P NATL ACAD SCI USA. 2015;112(44):13573–8.
10. Li D, Pan Z, Zhang K, Yu M, Yu D, Lu Y, Wang J, Zhang J, Zhang K, Du W. Identification of the differentially expressed genes of muscle growth and intramuscular fat metabolism in the development stage of yellow broilers. GENES-BASEL. 2020;11(3):244.
11. Comfort N, Gade M, Strait M, Merwin SJ, Antoniou D, Parodi C, Marcinczyk L, Jean-Francois L, Bloomquist TR, Memou A, et al. Longitudinal transcriptomic analysis of mouse sciatic nerve reveals pathways associated with age-related muscle pathology. J Cachexia Sarcopenia Muscle. 2023;14(3):1322–36.
12. Muhammad Farooq R, Lei C, Eric DT, Aaron C, Jay K. RNA-Seq analysis reveals sex-dependent transcriptomic profiles of human subacromial bursa stratified by tear etiology. J ORTHOP RES. 2022;40(12):2713–27.
13. Kiss B, Gohlke J, Tonino P, Hourani Z, Kolb J, Strom J, Alekhina O, Smith JE, Ottenheijm C, Gregorio C et al. Nebulin and Lmod2 are critical for specifying thin-filament length in skeletal muscle. SCI ADV. 2020;6(46):eabc1992.
14. Larrinaga TM, Farman GP, Mayfield RM, Yuen M, Ahrens-Nicklas RC, Cooper ST, Pappas CT, Gregorio CC. Lmod2 is necessary for effective skeletal muscle contraction. SCI ADV. 2024;10(11):eadk1890.
15. Fei-Hu L, Anmin W, Wuhou D, Song C, Yahui D, Ling VS. Lmod3 promotes myoblast differentiation and proliferation via the AKT and ERK pathways. EXP CELL RES. 2020;396(2):112297.
16. Florian C, Yu Xin B, Michael W. A R: Building muscle: molecular regulation of myogenesis. CSH PERSPECT BIOL. 2012;4(2):a008342.
17. Keqiang L, Lina L, Shanshan C, Beilin G, Hui C, Ying W, Wei C. Loss-of-function variants within LMOD1 actin-binding site 2 cause pediatric intestinal pseudo-obstruction by impairing protein stability and actin nucleation. FASEB J. 2022;36(3):e22194.
18. Ming-Ming C, Yue Z, Kun Y, Xue-Ling X, Xiao-Sheng Z, Jin-Long Z, Su-Jun W, Zhi-Mei L, Yi-Ming Y, Xiao-Fei G et al. A MSTN(Del73C) mutation with FGF5 knockout sheep by CRISPR/Cas9 promotes skeletal muscle myofiber hyperplasia. ELIFE. 2024;12(0):RP86827.
19. Tania ML, Gerrie PF, Rachel MM, Michaela Y, Rebecca CA, Sandra TC, Christopher TP, Carol CG. Lmod2 is necessary for effective skeletal muscle contraction. SCI ADV. 2024;10(11):eadk1890.
20. Vivek N, Joseph MM. Leiomodin 1, a new serum response factor-dependent target gene expressed preferentially in differentiated smooth muscle cells. J BIOL CHEM. 2011;287(4):2459–67.
21. Conley CA, Fritz-Six KL, Almenar-Queralt A, Fowler VM. Leiomodins: larger members of the Tropomodulin (Tmod) gene family. Genomics. 2001;73(2):127–39.
22. Jordan KB, Rhonda LT, Ramesh R, Elyshia M, Quang N, Hayley G, David C, Munish M, Lois B, Nigel GL et al. Variable cardiac α-actin (Actc1) expression in early adult skeletal muscle correlates with promoter methylation. BBA-GENE REGUL MECH. 2017;1860(10):1025–36.
23. Jessica XC, Matthew Carter C, Colby TM, Anthony JM, Hernan G, Lili-Naz H, James JD, Fatema AA, Yasemin A, Yolanda N et al. Variants in ACTC1 underlie distal arthrogryposis accompanied by congenital heart defects. HGG Adv. 2023;4(3):100213.
24. Anqi L, Xiaotong S, Yuan T, Guibing S, Linsen Z, Hongbao W. Effect of actin alpha cardiac muscle 1 on the proliferation and differentiation of bovine myoblasts and preadipocytes. ANIMALS-BASEL. 2021;11(12):3468.
25. Ping L, Xiaoyu Z, Liangliang T, Yahao Z, Yunqin Y, Shuang L, Shufeng L, Huili T. Vitamin C Promotes Muscle Development Mediated by the Interaction of CSRP3 with MyoD and MyoG. J AGR FOOD CHEM. 2022;70(23):7158–69.
26. Stephen JT. The circuitry of a master switch: Myod and the regulation of skeletal muscle gene transcription. DEVELOPMENT. 2005;132(12):2685–95.
27. Kulwant S, Jeffrey F. D: Differential modulation of cell cycle progression distinguishes members of the myogenic regulatory factor family of transcription factors. FEBS J. 2013;280(17):3991–4003.
28. S M H, J M T, S J T, C M G, W J C. C A P: Selective accumulation of MyoD and myogenin mRNAs in fast and slow adult skeletal muscle is controlled by innervation and hormones. DEVELOPMENT. 1993;118(4):1137–47.
29. Raymond M, Anthony O, Petr V, Ketan P. Neuromuscular junction morphology, fiber-type proportions, and satellite-cell proliferation rates are altered in MyoD(-/-) mice. MUSCLE NERVE. 2010;42(1):38–52.
30. Daisy VA, Tina C, Reza A, Stave D. K: Fiber-types of sarcomeric proteins expressed in cultured myogenic cells are modulated by the dose of Myogenin activity. CELL SIGNAL. 2008;21(1):128–35.
31. Naeli P, Winter T, Hackett AP, Alboushi L, Jafarnejad SM. The intricate balance between microRNA-induced mRNA decay and translational repression. FEBS J. 2023;290(10):2508–24.
32. Sheng-Zhong H, Kai G, Shuang-Yan C, Hak-Myong C, Hyo-Jin P, Biao-Hu Q, Xin-Yue L, Liu-Hui Y, Si-Tong L, Xi-Jun Y et al. miR-455-3p Is Negatively Regulated by Myostatin in Skeletal Muscle and Promotes Myoblast Differentiation. J AGR FOOD CHEM. 2022;70(33):10121–33.
33. Wang K, Liufu S, Yu Z, Xu X, Ai N, Li X, Liu X, Chen B, Zhang Y, Ma H et al. miR-100-5p regulates skeletal muscle myogenesis through the Trib2/mTOR/S6K signaling pathway. Int J Mol Sci. 2023;24(10):8906.
34. Zhu N, Wang D, Xie F, Qin M, Wang Y. MiR-335-3p/miR-155-5p involved in IGFBP7-AS1-Enhanced odontogenic differentiation. INT DENT J. 2023;73(3):362–9.

35. Zhang Q, He C, Li R, Ke Y, Sun K, Wang J. miR-708 and miR-335-3p inhibit the apoptosis of retinal ganglion cells through suppressing autophagy. J Mol Neuroscience: MN. 2021;71(2):284–92.
36. Han DH, Shin MK, Sung JS, Kim M. miR-335-3p attenuates transforming growth factor beta 1-induced fibrosis by suppressing thrombospondin 1. PLoS ONE. 2024;19(10):e311594.
37. Bai W, Zhang Y, Ma J, Du M, Xu H, Wang J, Zhang L, Li W, Hou Y, Liu X, et al. FHL3 promotes the formation of fast glycolytic muscle fibers by interacting with YY1 and muscle glycolytic metabolism. CELL MOL LIFE SCI. 2023;80(1):27.
38. Xin L, Quan L, Chong F, Mengxun L, Changchun L, Xinyun L, Shuhong Z, Zhuqing Z. Characterizing structural variants based on graph-genotyping provides insights into pig domestication and local adaption. J GENET GENOMICS. 2023;51(4):394–406.
39. Dongfeng L, Zaixu P, Kun Z, Minli Y, Debing Y, Yinglin L, Jiantao W, Jin Z, Kangning Z, Wenxing D. Identification of the differentially expressed genes of muscle growth and intramuscular fat metabolism in the development stage of yellow broilers. GENES-BASEL. 2020;11(3):244.

Publisher's note