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FULL LENGTH ARTICLE

MiR-743a-5p regulates differentiation of myoblast by targeting *Mob1b* in skeletal muscle development and regeneration

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Abstract   The microRNAs (miRNAs) play an important role in regulating myogenesis by targeting mRNA. However, the understanding of miRNAs in skeletal muscle development and diseases is unclear. In this study, we firstly performed the transcriptome profiling in differentiating C2C12 myoblast cells. Totally, we identified 187 miRNAs and 4260 mRNAs significantly differentially expressed that were involved in myoblast differentiation. We carried out validation of microarray data based on 5 mRNAs and 5 miRNAs differentially expressed and got a consistent result. Then we constructed and validated the significantly up- and down-regulated mRNA-miRNA interaction networks. Four interaction pairs (miR-145a-5p-*Fscn1*, miR-200c-5p-*Tmigd1*, miR-27a-5p-*Sln* and miR-743a-5p-*Mob1b*) with targeted relationships in differentiated myoblast cells were demonstrated. They are all closely related to myoblast development. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis indicated cell cycle signals important for exploring skeletal muscle development and disease. Functionally, we discovered that miR-743a targeting gene Mps One Binder Kinase Activator-Like 1B (*Mob1b*) gene in differentiated C2C12. The up-regulated miR-743a can promote the

differentiation of C2C12 myoblast. While the down-regulated *Mob1b* plays a negative role in differentiation. In addition, the expression profile of miR-743a and *Mob1b* are consistent with skeletal muscle recovery after Cardiotoxin (CTX) injury. Our study revealed that miR-743a-5p regulates myoblast differentiation by targeting *Mob1b* involved in skeletal muscle development and regeneration. Our findings made a further exploration for mechanisms in myogenesis and might provide potential possible miRNA-based target therapies for skeletal muscle regeneration and disease in the near future.

Introduction

Skeletal muscle is the dominant organ system of animals in locomotion, energy storage and metabolism.[1] It originates from the gastrula, undergo the mesoderm, somite and myogenic stage, and then form trunk and limb muscles. Myoblast proliferation and differentiation is crucial for skeletal muscle development and regeneration after injury. These events were highly coordinated multistep process in which myoblasts terminal withdrawal from cell cycle, activation of muscle-specific genes, transcriptional regulation and fusion of myoblasts into multinucleated myotubes.[2] Skeletal muscle development was elaborately controlled by the myogenic regulatory factors (MRFs), which are part of a super-family of basic helix-loop-helix transcription factors,[3] including myoblast determination protein (MyoD),[4] myogenin (Myog),[5] myogenic factor 5 (Myf5)[6] and myogenic regulatory factor 4 (MRF4).[7] MRFs promote myoblast differentiation by binding to a conserved consensus sequence, termed E-box (CANNTG), which present in the regulatory regions of many muscle-specific genes.[3] C2C12, an immortalized mouse myoblast cell line, was extensively used as a model to study the molecular mechanism of myogenesis.[8] Up to now, however, knowledge about myogenesis was still mysterious. Except for protein coding genes, non-coding RNAs including miRNAs also significantly contribute to myogenic differentiation and skeletal muscle development.

MiRNAs, highly conserved and endogenous short (~22 nt) non-coding RNAs, play important regulatory roles in many biological processes including development by targeting mRNAs of protein-coding genes (PCGs) for cleavage or translational repression.[9,10] In 1993, Lee et al described that lin-4, first miRNA discovered in larval development, contains sequences complementary to the untranslated region (UTR) of lin-14 mRNA and regulates lin-14 translation via an antisense RNA—RNA interaction.[11] Many studies have revealed that miRNAs play important roles in cell differentiation[12] and skeletal muscle development.[13,14] The miRNAs, specifically expressed in muscle, were called myomiRs.[15] The miR-1 and miR-133 were abundantly expressed in cardiac and skeletal muscle, and miR-206 was specifically expressed in skeletal muscle.[16,17] The expression of miR-1 and miR-206 significantly increased during myoblast differentiation. The miR-29 regulated myogenesis via directly targeting *Rybp* gene at 3′UTR binding sites.[18] MicroRNA-148a promotes myogenic differentiation by targeting the *ROCK1* gene.[14] Myoblast differentiation is a complex process conveying a large number of mRNAs[19] and miRNAs.[20] However, it is still indeed to understand more about the role of miRNAs in myogenic differentiation and skeletal muscle development.

In this study, we aimed at discovering miRNA and mRNA involved in myoblast differentiation. We firstly carried out profiling of miRNA and mRNA by microarray in differentiating C2C12 myoblast cells at D0 and D4. And then, we analyzed mRNA—miRNA interaction to illuminate their potential regulation role in myoblast myogenesis. Finally, we uncovered the functions of miRNA-743a in regulating myoblast differentiation by targeting *mob1b*. Our study not only provided abundant information of miRNAs and mRNAs involved in myogenesis, but also helpful to study their functions in gene therapy for muscle-relative disease.

Materials and methods

Cell culture and treatment

C2C12 cells were obtained from Peking Union Medical College Hospital and 293T cells were got from SUYAN BIOTECH company. They were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, USA) supplement with 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin-streptomycin (PS; Thermo, USA) at 37 °C in a 5% CO$_2$ incubator. To induce myogenic differentiation, the culture medium of C2C12 myoblasts was replaced with DMEM supplemented with 2% pregnant mare serum (Gibco, USA) and 1% PS at 80% DMEM confluence. And the cells were collected at the undifferentiated stages and at 4 days of differentiation. Transfection was conducted with reagent Lipofectamine™ 3000 (Thermo, USA) under instructions.

Microarray profiling and analysis

Total RNA was extracted from C2C12 cells at D0 and D4 after differentiation using 2% pregnant mare serum (Gibco, USA) and purified with RNeasy minit kit (QIAGEN, China) according to manufacturer's instructions. RNA quality and quantity were measured by Agilent 2100 bioanalyzer with Agilent 2100 RNA Nano 6000 Assay Kit conducted by Novogene company (Novogene, China). Agilent mouse miRNA microarray was used in detecting profiling of the miRNAs expression. Scanned images were imported into GenePix Pro 6.0 software (Axon) for grid alignment and data extraction. Replicated miRNAs were averaged and miRNAs

that intensities ≥ 30 in all samples were chosen for calculating normalization factor. Expressed data were normalized using the median normalization. After normalization, significant differentially expressed miRNAs between two groups were identified through Fold change and *P*-value (| Fold changed|≥ 2.0 and $P \leq 0.05$). Finally, hierarchical clustering was performed to show distinguishable miRNA expression profiling among samples.

GO and KEGG analysis

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were used to predict miRNAs potential functions with co-expressed genes. Enrichment of GO and KEGG were analyzed using the Database for Annotation, Visualization and Integrated Discovery (DAVID). Prediction terms and pathway with *P*-value ≤ 0.05 were selected as significant as and ranked by enrichment score (-log10(*P*-value)).

Network construction

Base on the significantly differently up- and down-regulated miRNAs and miRNA-target genes, the targeting interaction network was constructed. Besides, a measure with the number of common miRNAs, the target genes of known miRNAs were predicted by the online miRNA target prediction tools TargetScan (http://www.targetscan.org/), miRDB (http://mirdb.org/) and miRwalk (http://mirwalk. umm.uni-heielberg.de/). Moreover, the target genes of novel miRNAs were predicted by IntaRNA 2.0 (https:// github.com/BackofenLab/IntaRNA/) and DIANA (http:// diana.imis.athena-innovation.gr/DianaTools/index.php). Then, the overlapping genes especially highly expressed in C2C12 were selected as the final set of miRNA target genes. The networks between miRNAs and their target genes were constructed using software Cytoscape 3.6.0.

Real-time quantitative PCR

Total RNA was extracted from C2C12 cells by TRIzol reagent (Invitrogen, USA) according to manufacturer's instructions. RNA quality and quantity were measured by using Nanodrop spectrophotometer (ND-2000C) and gel electrophoresis. For mRNA, single-stranded cDNA was reverse transcribed with random primers with HiScript III RT SuperMix (Vazyme, China). And qPCR was performed using Fast ChamQ Universal SYBR qPCR Master Mix (Vazyme, China) in a total reaction volume of 20 μl, including 10 μl 2 × SYBR Master Mix, 0.4 μl PCR Forward Primer, 0.4 μl PCR Reverse Primer, 2 μl cDNA and 7.2 μl double distilled water. For miRNA, single-stranded cDNA was reverse transcribed with stem-loop primers (Table S3) with miRNA 1st Strand cDNA Synthesis Kit (Vazyme, China). For miRNA qPCR, protocol is just the same except for using miRNA Universal SYBR qPCR Master Mix (Vazyme, China). The protocol was initiated at 95 °C (30 sec), then at 95 °C (10 sec), 60 °C (30 sec) for a total of 40 cycles. GAPDH was used as a reference for mRNA, U6 was used as a reference for miRNA. Primer sequences (TSINGKE, China) are listed in supplementary S5. The data were

analyzed using the cycle threshold (Ct) method and the relative expression level of each ncRNA and mRNA was calculated using $2^{-\Delta\Delta Ct}$. GraphPad Prism 5 software was applied to all statistical analysis. All data are performed in triplicate and are presented as mean $\pm$ standard error of mean (S.E.M.). The unpaired two-tailed Student *t*-test was used to test differences between two groups for statistical significance. A *P*-value≤ 0.05 was considered to be significant.

Western blot and cell immunofluorescence

Cell and tissue were lysed in RIPA buffer (Thermo, USA) supplemented with proteinase inhibitor cocktail (Roche) and phosphorylase inhibitor (Roche, USA). The extracts were boiled with 5 × SDS loading buffer (CWBIO, China) at 100 °C for 10 min and then 20 μg total proteins were loaded and separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels (Epi-Zyme, China), transferred to a 0.45 μm Hybridization Nitrocellulose Filter (NC) membrane (Merck, Germany). The member was blocked with 5% Albumin from bovine serum (BSA) (Biofroxx, Germany) in Tris Saline with Tween (TBST) buffer (EpiZyme, China) for 2 h at room temperature, and then incubated overnight with primary antibodies specific for anti-MyHC (DSHB, China), anti-MOB1B (CST, China), anti-GAPDH (FUDE BIOLOGICAL, China) at 4 °C. The membrane was washed with TBST (EpiZyme, China) and incubated with secondary antibody (KTSM, China). Finally, the membrane was placed into Super ECL Detection Reagent (YEASEN, China), and immediately exposed to X-ray film (Tanon 5200). The Western blot results (Objective protein & GAPDH) were quantified by using Image J each for 3 biological replicates.

Cell immunofluorescence was performed according to standard protocols. Briefly, cells were induced to differentiation in 6-well plate for indicated time period. Cells were fixed with 4% paraformaldehyde (Beyotime, China) for 20 min at room temperature. Permeabilized C2C12 was treated by 0.5% Triton-100 (Sigma, Germany) for 20 min and blocked with 5% bovine serum albumin (BSA) (Biofroxx, Germany). Primary antibody (mouse anti-myosin, MF-20-S, DSHB [1:500]) was incubated for 2 h at room temperature and secondary antibody (anti-mouse, CY3, Servicebio [1:1000]) was incubated for 1 h getting away from light. At last, DAPI (Beyotime, China) was used to stain nucleus for 10 min. Stained cells were imaged and analyzed using an OLYMPUS fluorescent microscope (OLYMPUS IX73) each for 5 random fields of vision.

Muscle injury models

In anesthetized (O_2/sevoflurane, 3% sevoflurane) mice (5-week-old) by injecting 20 μl of 10 μM cardiotoxin (CTX) (Sigma, Germany) in phosphate-buffered saline (PBS; Solarbio, China) into the left tibialis anterior (TA) muscle. Mice were sacrificed at day 0, day 1, day 3, day 5, day 7 and day 14 after the injury and TA muscles collected. 20 μl PBS was injected into the right TA muscle of each mouse as control.

Histochemistry by hematoxylin and eosin (H&E) staining

Formalin (Aladdin, China) fixed TA muscles were embedded in paraffin, sectioned (8 μm) and mounted on microscope slides (LEICA RM2235). The operation follows the consulted reference summarized by Ada T. Feldman and Delia Wolfe. The kit (Solarbio, China) was used for hematoxylin and eosin (H&E) staining of the TA muscles. The detail conduction follows the kit protocols. For light microscopy using an OLYMPUS microscope. Photographs were taken with a digital camera (OLYMPUS BX53) at random views and stored as.*tif files.

Plasmid construction

The 3′UTR fragments of *Mob1b* containing putative miR-743a-binding site was amplified by PCR. The reconstructed pmir-GLO Vector (Promega, USA) was made by homologous recombination TreliefTM SoSoo Cloning Kit Ver.2 (TSINGKE, China). Base mutation was conducted by using MutanBEST kit (TaKaRa, Japan). All constructs were confirmed by DNA sequencing (TSINGKE, China). Plasmid was extracted by using EndoFree Plasmid Kit (TIANGEN, China). The siRNAs, over-expression vectors and synthetic miRNA mimics were got from GenePharma company (GenePharma, China).
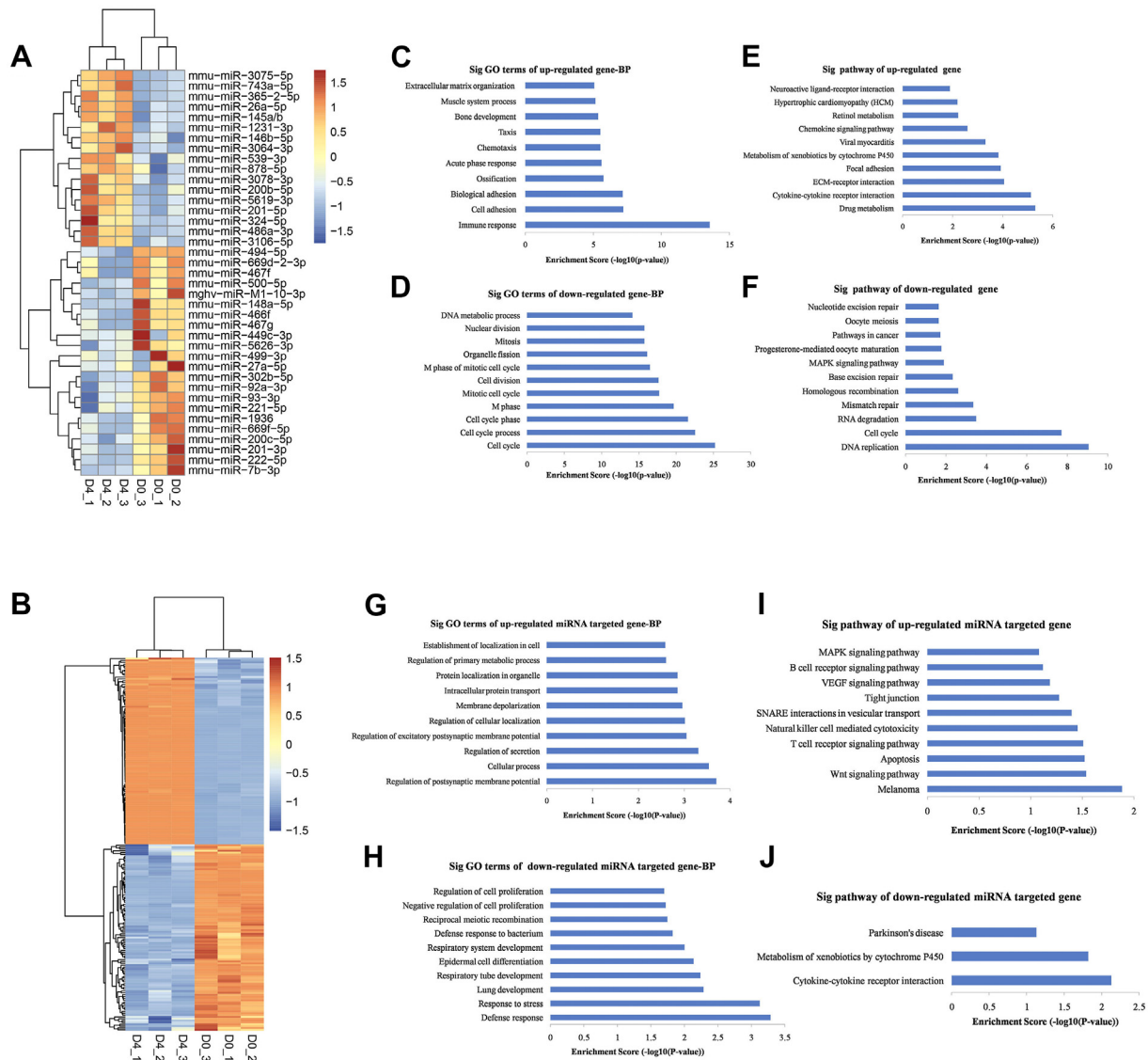
Figure 1 Differentially expressed mRNAs and miRNAs in differentiated C2C12. (**A, B**) Cluster heat maps showing up- and down-regulated miRNAs (A) and mRNAs (B) on D4 of C2C12 cell differentiation and in undifferentiated cells. (**C**) The top 10 up-regulated GO BP terms for the differentially expressed mRNAs, ranked by enrichment score. (**D**) The top 10 down-regulated GO BP terms for the differentially expressed mRNAs, ranked by enrichment score. (**E**) The 10 most significant KEGG pathways of the up-regulated mRNAs. (**F**) The 10 most significant KEGG pathways of the down-regulated mRNAs. (**G**) The top 10 up-regulated GO BP terms for the differentially expressed miRNAs targeted genes, ranked by enrichment score. (**H**) The top 10 down-regulated GO BP terms for the differentially expressed miRNAs targeted genes, ranked by enrichment score. (**I**) The 10 most significant KEGG pathways of the up-regulated miRNAs targeted genes. (**J**) The 10 most significant KEGG pathways of the down-regulated miRNAs targeted genes.

Dual-luciferase reporter assay

For the dual-luciferase reporter assay, 293T cells were co-transfected with each pmir-GLO 3′UTR construct, and either miR-743a or miRNA NC using Lipofectamine™ 3000 (Thermo, China). After 48 h, the cells were analysed using the Dual-Luciferase Reporter Assay System Kit (Promega, USA) on a GloMax™ 20/20 Luminometer (Promega). The same conduction was applied for miR-27a, miR-145a and miR-200c.

Statistical analyses

GraphPad Prism 5.0 software was applied to all statistical analyses. All data are performed in triplicate and are presented as means ± S.E.M. The unpaired two tailed Student t-test was used to test differences between groups for statistical significance. A P-value≤0.05 was considered to be significant.

Results

### Profiling and interaction analysis of miRNAs and mRNAs in C2C12 cells

In this study, C2C12 cells were used as a model system to understand differentiation and myogenesis. Microarray profiling was performed based on C2C12 at differentiated D0 and D4 to systematically detect miRNAs and mRNAs involved in C2C12 myoblast differentiation. We found that 65 miRNAs and 1896 mRNAs were up-regulated, and 122 miRNAs and 2464 mRNAs were down-regulated on D4 (Fig. 1A, B and Table S1, S2). Of them, several miRNAs and mRNAs had been reported to participate in C2C12 differentiation, such as miR-133 and *Igf1*. We randomly selected five mRNAs (*Myh1*, *Myh3*, *Tnnt3*, *Myoz2*, *Igf1*) (Fig. S1A) and five miRNAs (miR-26a, miR-378a, miR-145a, miR-133b, miR-92a) to validate the microarray data. The results showed that the qPCR data was consistent with the analysis of microarray (Fig. S1B). This suggested that our microarray data was reliable for further analysis.

GO analysis revealed that up-regulated mRNAs were significantly enriched in single-organism process, response to extracellular matrix organization, muscle system process, and biological adhesion (Fig. 1C) while the down-regulated mRNAs were mainly involved in DNA metabolic process, nuclear division, mitotic cell cycle, single-organism cellular process, and DNA metabolic process (Fig. 1D). KEGG analysis suggested that the up-regulated mRNAs were significantly enriched in pathway for cytokine−cytokine receptor interaction, metabolism of xenobiotics by cytochrome P450 and chemical carcinogenesis (Fig. 1E); while the down-regulated mRNAs were enriched in cell cycle, DNA replication, and MAPK signaling pathway (Fig. 1F), implying expression of genes repressing
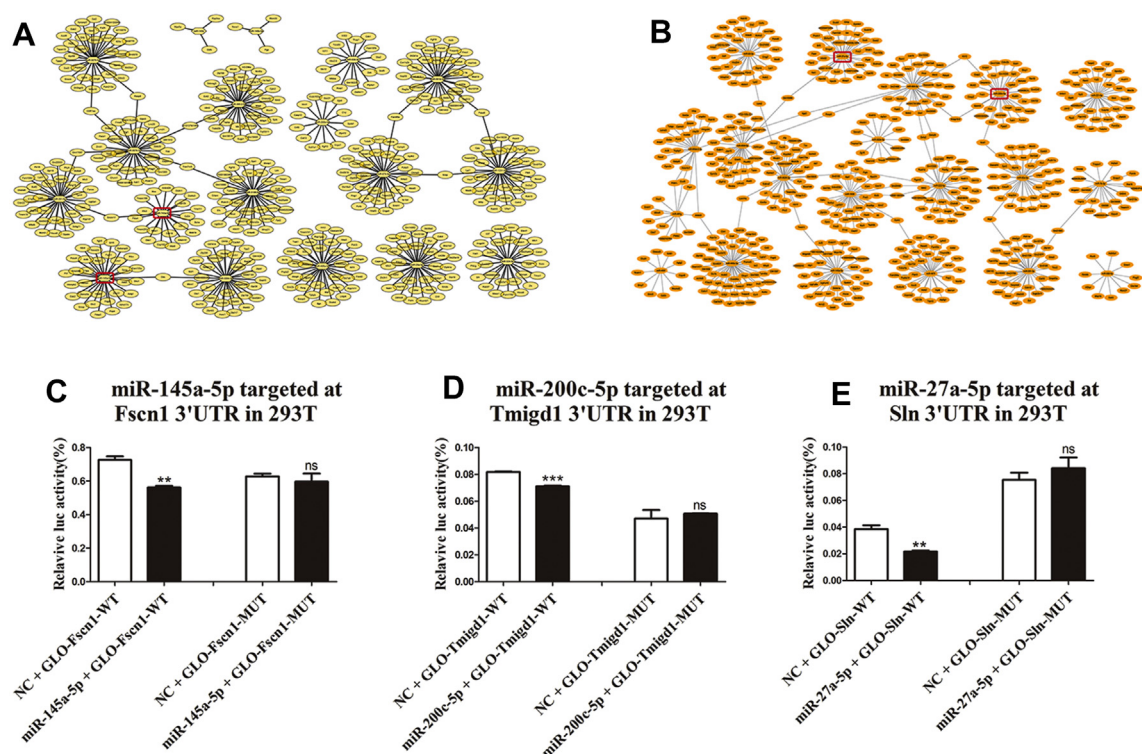
Figure 2 The interaction network validation between miRNAs and targeted mRNAs. (**A**) The up-regulated miRNAs and their targeted mRNAs. (**B**) The down-regulated miRNAs and their targeted mRNAs. (**C**) Dual Luciferase Reporter Assay to validate miR-145a-5p target at *Fscn1* 3′UTR in 293T. (**D**) Dual Luciferase Reporter Assay to validate miR-200c-5p target at *Tmigd1* 3′UTR in 293T. (**E**) Dual Luciferase Reporter Assay to validate miR-27a-5p target at *Sln* 3′UTR in 293T.
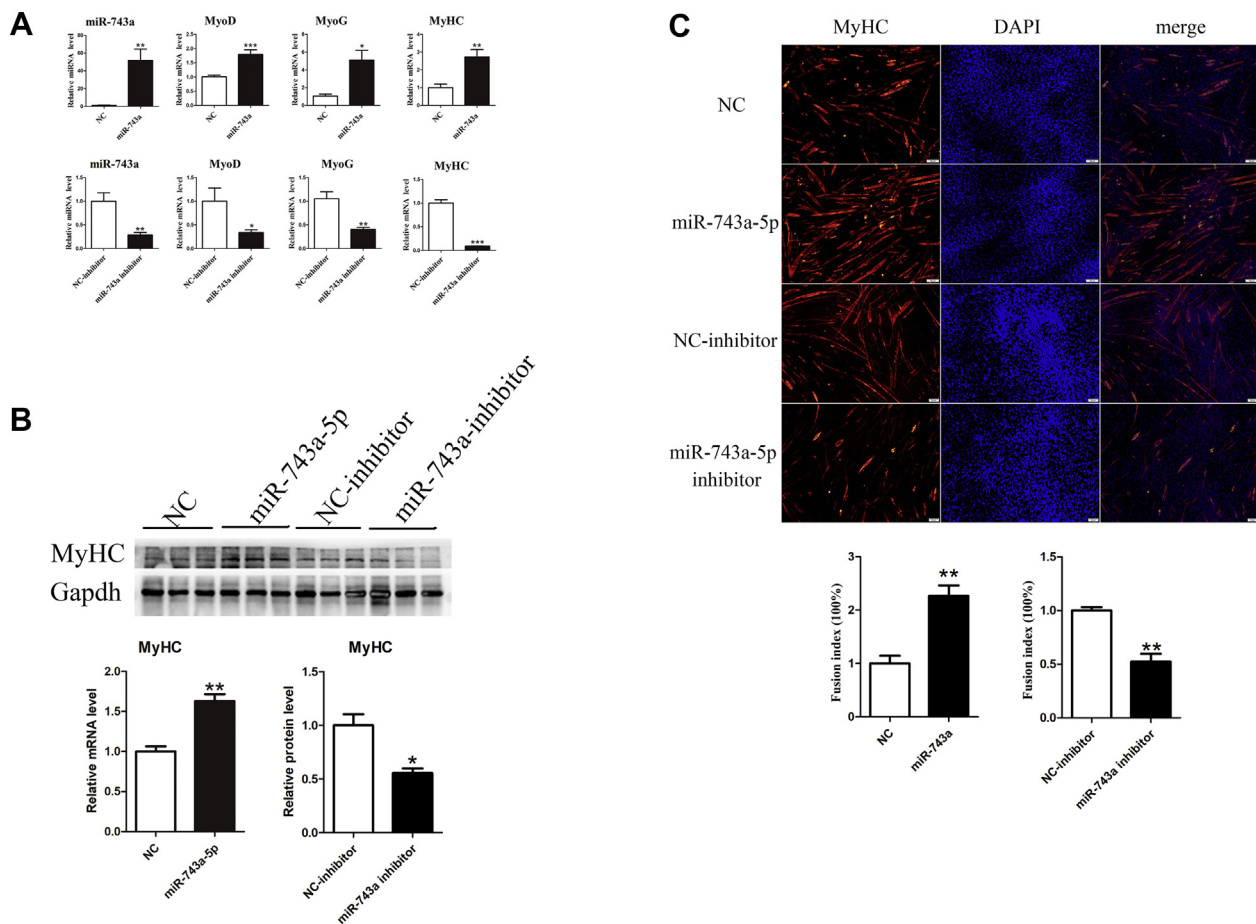
Figure 3   The function of miR-743a-5p in C2C12 differentiation. (**A**) The qPCR result showed that miR-743a-5p mimic could significantly promote the expression of marker genes for differentiation; while miR-743a-5p inhibitor on the contrary. (**B**) Western blot of MyHC showed a positive role of miR-743a-5p in differentiation. (**C**) Immunofluorescence result showed that over-expression of miR-743a-5p could effectively promote differentiation while inhibition on the contrary.

proliferation and enhancing differentiation. Besides, GO analysis of miRNA targeted genes also displayed an association with cellular process involved in membrane depolarization and establishment of localization (Fig. 1G) and negative regulation of proliferation (Fig. 1H). KEGG analysis indicated that miRNA targeted genes are involved in MAPK and Wnt (Fig. 1I) and cytokine receptor interaction signaling pathway (Fig. 1J).

Mature miRNA 'seed sequences' usually recognize target sites in the 3′UTR of mRNA. Thus, we predicted target genes of differentially expressed miRNAs by miRBase, miRcode, miRWalk, and TargetScan programs, and then overlapped them with mRNAs from microarray data (Table S3, S4). We then constructed the up-regulated (Fig. 2A) and down-regulated miRNAs (Fig. 2B) together with their miRNA-mRNA interaction network based on 187 miRNAs and 4360 mRNAs that were differentially expressed and with the opposite trend of expression during the C2C12 myoblast differentiation. Subsequently, we validated prediction interaction based on four interaction pairs (MiR-145a-5p -*Fscn1*, MiR-200c-5p-*Tmigd1*, MiR-27a-5p-*Sln* and MiR-743a-5p-*Mob1b*) randomly using dual luciferase assay (Fig. 2C—E, 4C).

MiR-743a-5p promotes differentiation of C2C12 myoblast

Our microarray data showed that miR-743a-5p was up-regulated with 6 fold changes on D4 in C2C12 differentiation (Table S1). The qPCR result also revealed that miR-743a was up-regulated on D4 in differentiated C2C12 myoblast (Fig. S2C). These findings suggested that miR-743a-5p plays a potential role in myoblast differentiation. To unveil the function, we transfected synthetic miR-743a-5p mimics, inhibitor and scrambled negative control (NC) into C2C12, respectively. The marker genes (*MyoD*, *MyoG* and *MyHC*) for differentiation were up-regulated at mRNA level under over-expression of miR-743a-5p (Fig. 3A). While, they had an opposite expression trend under knockdown of miR-743a-5p (Fig. 3A). Western blot assays also indicated that miR-743a increased MyHC expression at protein level (Fig. 3B). Next, the cell immunofluorescence (IF) of MyHC in differentiated C2C12 conducted 5 days after induced by 2% pregnant mare serum suggested an efficient function of miR-743a-5p in promoting myoblast differentiation and myotubes formation (Fig. 3C). These results suggested
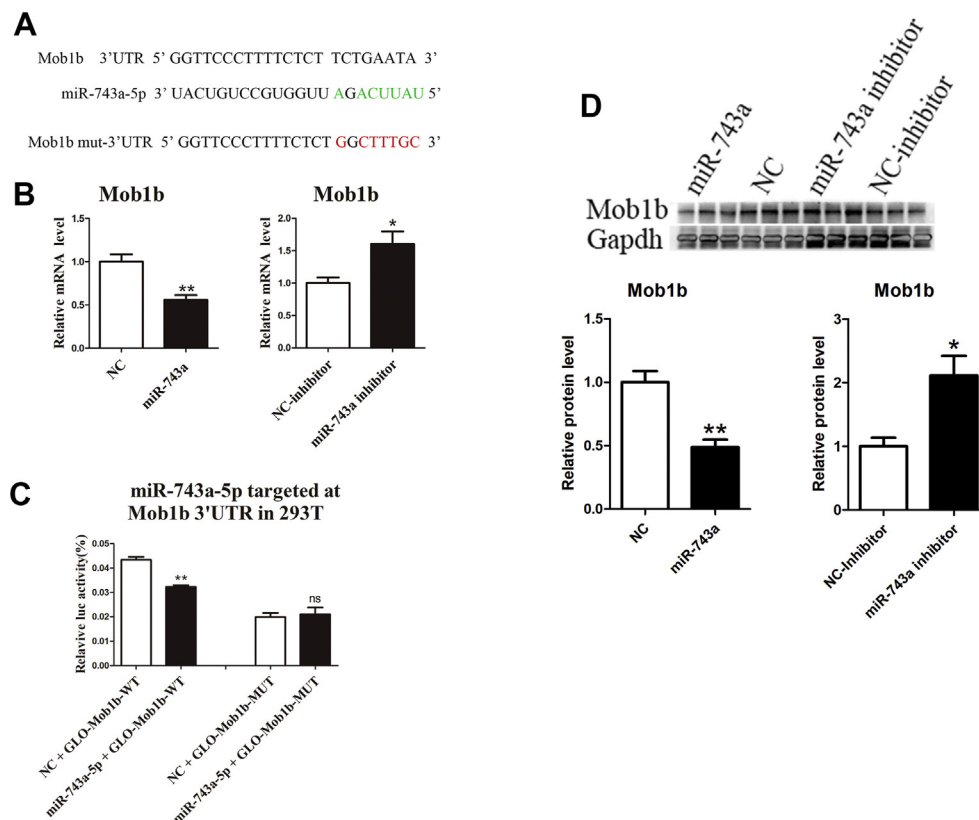
Figure 4   The validation of interaction network of miR-743a-5p-*Mob1b*. (**A**) The normal and mutant binding sites of *Mob1b* 3′UTR targeted by miR-743a-5p. (**B**) *Mob1b* was negatively regulated by miR-743a in C2C12 on mRNA expression level. (**C**) The Dual Luciferase Reporter Assay result showed that *Mob1b* target miR-743a-5p in 293T. (**D**) The protein expression level of Mob1b was influenced by miR-743a and inhibitor.

that miR-743a-5p promoted differentiation of myoblast cell.

Mob1b is a target regulated by mir-743a-5p

Bioinformatics analyses suggested that the miR-743a-5p were predicted to target the *Mob1b* gene at 3′UTR (Fig. 2A). Both microarray data and qPCR results showed that there was an opposite expression pattern between miR-743a-5p and *Mob1b* gene at mRNA level in C2C12 differentiation (Fig. S2B, C). These facts indicated that the *Mob1b* is a potential target for miR-743a-5p. In order to validate our conjecture, we transfected the miR-743a-5p mimics together with a re-constructed pmir-GLO vector containing normal and mutant binding sites of *Mob1b* 3′UTR (Fig. 4A) respectively. The result of dual-luciferase reporter assay indicated that *Mob1b* was a target gene for miR-743a-5p (Fig. 4C). Over-expression of miR-743a-5p effectively in-crease the expression of *Mob1b* in C2C12 while inhibition on the contrary (Fig. 4B). The Western blot analysis also showed that Mob1b expression at protein level was nega-tively regulated by miR-743a-5p (Fig. 4D).

To further the exploration whether miR-743a-5p func-tions in myogenesis by targeting *Mob1b* gene, we conducted the co-transfection of *Mob1b* over-expression vector together with miR-743a-5p mimics or *Mob1b* siRNA together with miR-743a-5p inhibitor, respectively. The result showed

that *Mob1b* inhibited the expression of marker genes (*MyoD*, *MyoG* and *MyHC*) for differentiation induced by miR-743a-5p mimics on mRNA level (Fig. 5A). The Western blot result of MyHC also displayed a negative influence (Fig. 5B) of *Mob1b* together with miR-743a-5p mimics on differentiation. The immunofluorescence result showed that *Mob1b* together with miR-743a-5p suppressed the myotube formation (Fig. 5C). While, *Mob1b* interruption rescued the inhibition of differentiation induced by miR-743a-5p inhibitor. *Mob1b* siRNA together with miR-743a-5p inhibitor promoted differentiation of myoblast C2C12 on mRNA (Fig. 5A) and protein expression level (Fig. 5B). The co-transfection of *Mob1b* siRNA together with miR-743a-5p inhibitor promoted the myotube formation (Fig. 5C). Therefore, these facts suggested that miR-743a-5p regu-lates myogenic differentiation via targeting *Mob1b*.

Differentiation is an essential process of muscle injury recovery during skeletal muscle development and regen-eration. To confirm the function of miR-743a-5p in myoblast differentiation *in vivo*, cardiotoxin (CTX, protein kinase C inhibitor) injection was conducted. The tissue slice after CTX injection displayed a clear process of myoblast dif-ferentiation accompany with injury regeneration (Fig. S2A). The expression of miR-743a-5p was up-regulated not only from D0 to D4 (Fig. S2C) *in vitro* but also from D0 to D7 after CTX injection (Fig. S2B) *in vivo*. It suggested that miR-743a-5p expression profile is consistent with muscle regeneration
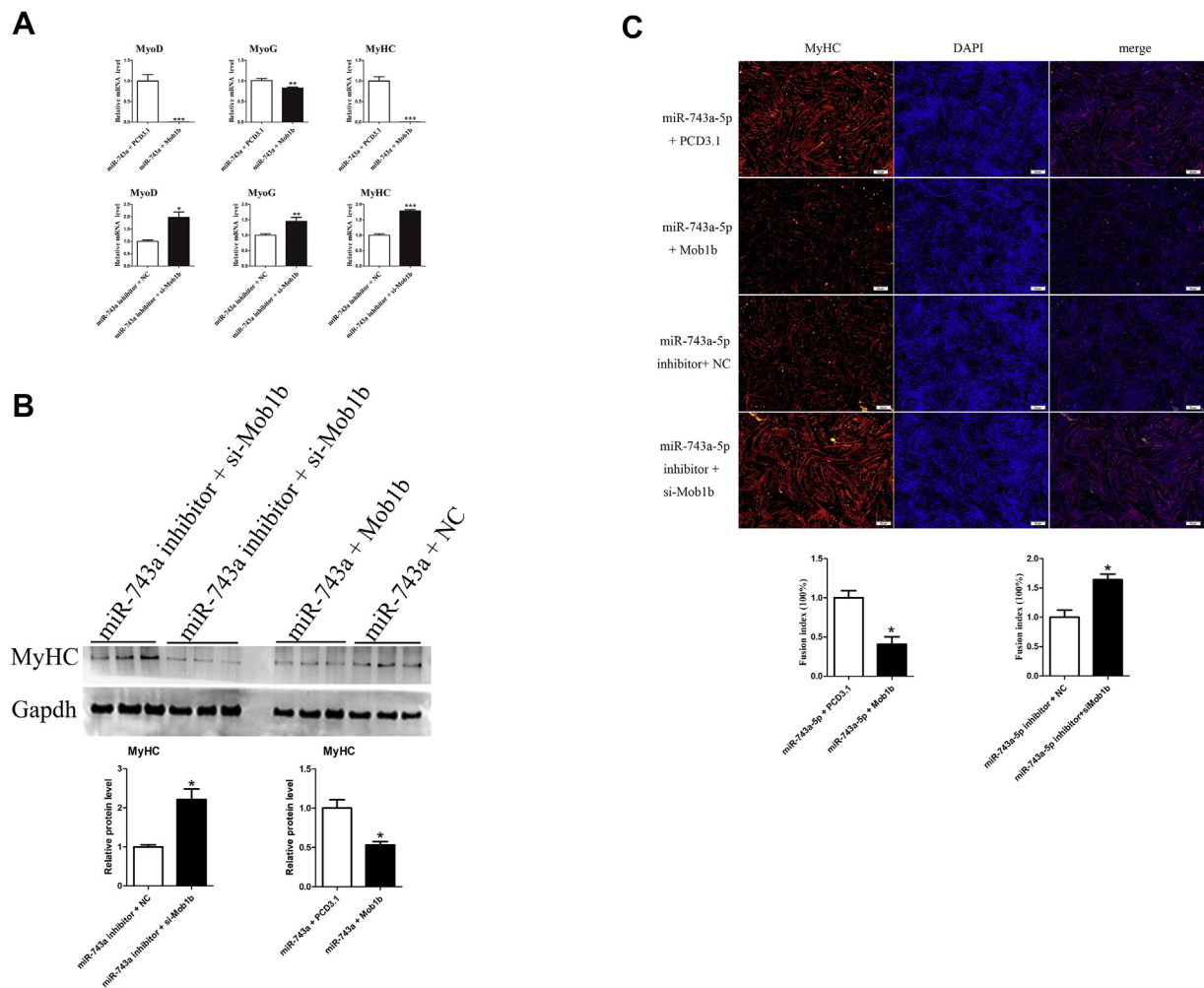
Figure 5   The influence of *Mob1b* together with miR-743a over-expression or inhibition on C2C12 differentiation. (**A**) The qPCR result showed that co-transfection of *Mob1b* over-expression vector together with miR-743a mimic could significantly reduce the expression of marker genes for differentiation; while co-transfection of siRNA-*Mob1b* together with miR-743a inhibitor on the contrary. (**B**) Western blot of MYHC showed a negative role of over-expression of *Mob1b* together with miR-743a in differentiation. (**C**) Immunofluorescence result showed that over-expression of *Mob1b* together with miR-743a could effectively inhibit differentiation while inhibition on the contrary.

process. Besides, the expression profile of *MyoD, MyoG* and *MyHC* are consistent with miR-743a-5p, while *Mob1b* had a significant decrease after D1 (Fig. S2B). It even displayed an opposite trend in most periods of the process. These results indicated that the miR-743a-5p is closely related to muscle regeneration.

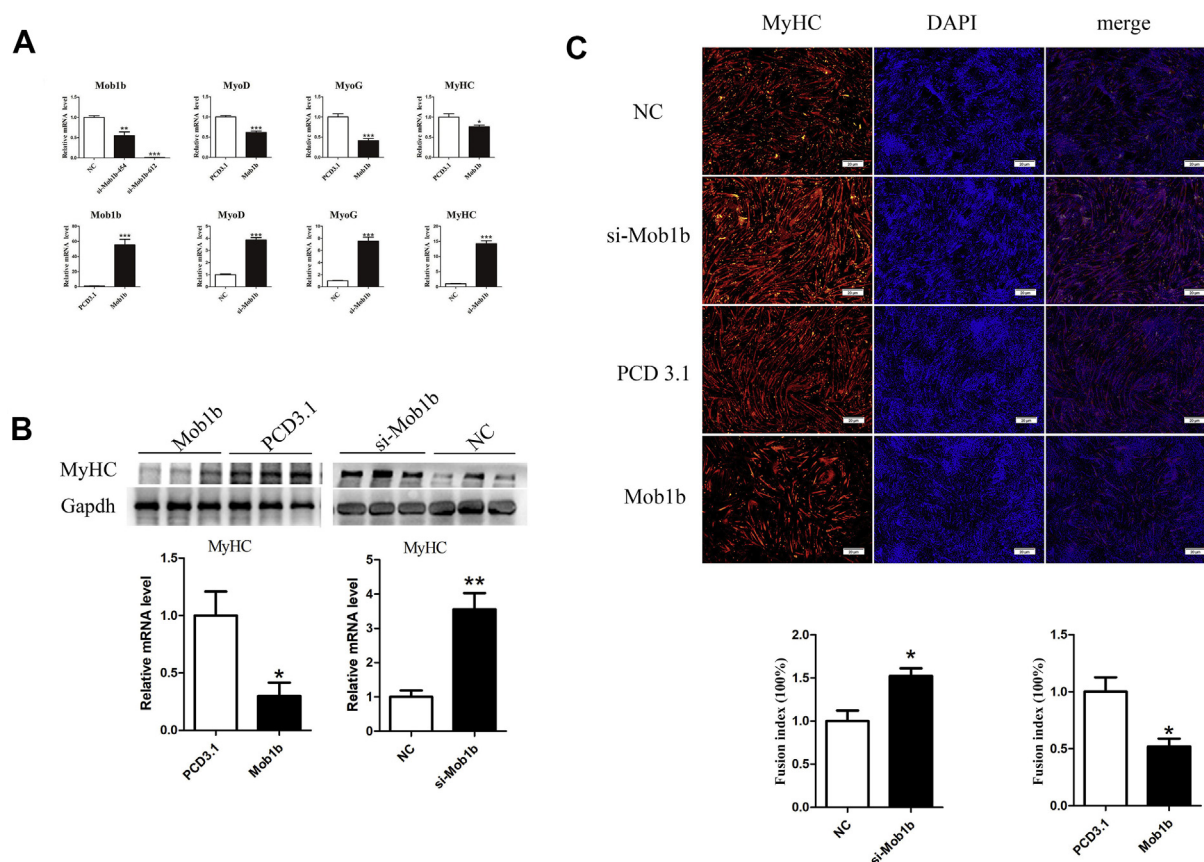## Mob1b inhibited differentiation of C2C12 myoblast

Our microarray data showed that *Mob1b* was down-regulated with 2.6-fold changes in differentiated C2C12 (Table S2). The qPCR result also displayed a similar trend during C2C12 differentiation (Fig. S2B, C). We over-expressed and interrupted *Mob1b* with *Mob1b*-PCD3.1 and siRNAs, respectively. The result showed that *Mob1b* repressed differentiation of C2C12 myoblast (Fig. 6A). The Western blot result showed that over-expression of *Mob1b* decreased MyHC abundance at protein level while interruption of *Mob1b* increased MyHC expression (Fig. 6B).

Besides, immunofluorescence showed that interrupting *Mob1b* promoted differentiation and myotube formation while over-expressing *Mob1b* inhibited differentiation and myotube formation (Fig. 6C). Altogether, these results revealed a negative role of *Mob1b* in C2C12 differentiation.

Discussion

Our profiling of transcriptome provided the useful data for understanding the molecular regulation and miRNA—mRNA interaction in skeletal muscle development. There are already some mRNA—miRNA interactions found in C2C12 development.[21] However, a prediction with dry laboratory is far from enough for comprehensive validation. Here, not only a reliable network was constructed but also the regulation relationships in myoblast differentiation were confirmed by microarray analyses, qPCR and dual luciferase reporter assay. It was reported that miR-743a inhibited the proliferation of metanephric mesenchymal cell.[22] Our study

Figure 6 The function of *Mob1b* in C2C12 differentiation. (**A**) The qPCR result showed that *Mob1b* over-expression could significantly inhibit the expression of marker genes for differentiation; while siRNA-*Mob1b* interruption on the contrary. (**B**) Immunofluorescence result showed that over-expression of *Mob1b* could effectively inhibit differentiation while inhibition on the contrary. (**C**) Western blot of MYHC showed a negative role of *Mob1b* in differentiation.

suggested that miR-743a promoted the differentiation and inhibited the proliferation of C2C12 myoblast in myogenesis. The down-regulated *Mob1b* in our study is also consistent with another reported data.[23] Meanwhile other new discovered relationships conveying potential functions are consistent with published papers. The up-regulated miR-27a-5p can regulate insulin resistance in skeletal muscle through PPARγ.[24] The target of miR-27a-5p validated by us was *sln*. *Sln*, involved in Ca^{2+} transportation, ATP uptake and muscle relaxation,[25] is also a novel regulator of muscle metabolism and obesity.[26] The up-regulated miR-145a-5p was reported promoting myoblast differentiation.[27] Besides, its target gene *Fscn1* was reported participating in apoptosis regulated by miR-145.[28] The miR-200c-5p-*Tmigd1* relationship validated in our network also showed potential function in C2C12 migration. MiR-200c can inhibit muscle differentiation.[29] Meanwhile miR-200c-5p functions in proliferation and metastasis.[30] While *Tmigd1*, regarded as a novel adhesion molecule that protects epithelial cells from oxidative cell injury,[31] can also react as a novel cell adhesion molecule for human trophoblast cell migration.[32] All of these conveying potential profound functions in C2C12 indicated it a valuable network for exploration in C2C12 myoblast development. Meanwhile the mechanism of how miR-743a-5p regulates C2C12 differentiation was need to explore.

There had been no report about the function of miR-743a-5p or *Mob1b* in myogenesis. In our study, the expression profile of miR-743a-5p and *Mob1b* are closely related to differentiation. It was reported that miR-743a mediated up-regulation of the mitochondrial malate dehydrogenase under oxidative stress.[33] MiR-743a which is responsible for repressing metanephric mesenchymal (MM) cells proliferation[22] attracted our attention. MiRNAs which are negative in proliferation usually display a positive role in myoblast differentiation.[34] MiR-743a-5p actually enhanced differentiation and promoted myotubes formation. It displayed a potential positive role in differentiation. Generally, miRNAs can negatively regulate genes expression by binding to the 3′-untranslated region of their target genes.[17] Our study first validated the targeted relationship between miR-743a-5p and *Mob1b* during C2C12 differentiation.

As a core-component of Hippo signaling, Mps One Binder Kinase Activator-Like 1B (*Mob1b*) is predominant in skeletal muscle development. Functions as activator of LATS1/2 in the Hippo signaling pathway, accompany with *Mob1a*, *Mob1b* plays a pivotal role in organ size control and tumor suppression.[35,36] MOB1, mediating phospho-recognition,[37] is a multifunctional protein best characterized as its integrative role in regulating mammalian Hippo (MST1/2), Warts (LATS1/2), and Tricornered (NDR1/2).[38] The indirect evidence is that *Mob1b* together with *Mob1a* is essential for

embryonic development. *Mob1b* double knockout mouse showed growth failure during E5-E8 accused of susceptibility to tumorigenesis and hperproliferation.[18] Coincidentally, the specification of myotomal cell and differentiation of myocytes also start at around embryonic stage (E9).[39] The muscle-specific genes *Six 1/4*, *Pax 3*, *Pax 7*, *Myf*5 and *MyoD* involved in muscle satellite stem cells specification are all highly expressed during this stage around embryonic stage (E9).[40] There are six classified biological processes of myoblast cell development in order: I stemness for embryonic progenitors, II specification for satellite stem cells, III commitment for committed satellite cells, IV activation and proliferation for myoblasts, V early differentiation for myocytes, and VI late differentiation for myotubes and myofibers. According to current evidence, *Mob1b* plays predominant role in muscle satellite cell specification at the first stage. As an early regulator in myogenesis, *Mob1b* start to function earlier than *Myod*, *Myog* and *Mrf4*. It indicated a more potential role of *Mob1b* in the early embryonic stage of myogenesis other than late differentiation in mature myocytes. Further studies on the mechanisms correlated with embryonic expression profile containing both *Mob1b* and miR-743a should be made in the near future. In our study, the up-regulated *Mob1b*, responsible of inhibiting differentiation, was negatively regulated by miR-743a-5p. The expression profile of *Mob1b* is consistent with the recent report that many hippo components were closely related to chromosome accessibility in differentiated C2C12.[23] These indicated an essential role of *Mob1b* in cell differentiation. Besides, *Mob1b* also showed an important role in differentiation in *vivo* after CTX injection. All of these suggested that miR-743a-5p promote C2C12 differentiation by targeting *Mob1b*.

In total, we combined expression profiling mRNA and miRNA to identify candidate mRNA, miRNAs and their interaction involved in C2C12 differentiation. A validated mRNA—miRNA network involved in myogenesis was constructed. A comprehensive transcriptome profiling reveals that miR-743a-5p plays a potential role in myogenesis. The core-component of Hippo signaling pathway *Mob1b* was regulated by miR-743a-5p in myoblast differentiation. It made a solid foundation for mechanism exploration in skeletal muscle development. It also brings us new inspiration in exploring Hippo signal pathway in myogenesis. A potential miRNA-based target therapies for skeletal muscle recovery and regeneration might by enhanced.

Author contributions

ZhongLin Tang designed, managed project and wrote manuscript. YongSheng Zhang carried out experiments and wrote the paper. YuWen Liu and GuoQiang Yi provided help in analysis of microarray, network construction and validation. YiLong Yao contributed to the experiments in cell differentiation and tissue slice. ZiShuai Wang and AdeyinkaAbiola Adetula made writing review and editing. DanLu and SiYuan Liu provide help for vector construction. YuanYuan Zhang prepared for the C2C12 differentiation and sample collection. Min Zhu and YaLan Yang contributed to the bioinformatics for mRNA-miRNA interaction network. XinHao Fan and MuYa Chen analyzed the microarray data.

YiJie Tang and Yun Chen assist in animal experiments and RNA extraction. All authors contributed to the article and approved the submitted version.

Data availability statement

We confirm that all data are contained within the manuscript. All primers in Table S3 used for experiments are from NCBI. The microarray source data underlying Table S1 and S2, Figure S1—S6 and Figure S1 and S2 are provided as a source data file. The original microarray data was submitted to GEO with a number GSE154440.

Ethics statement

This research was approved by the Ethical Committee of Agricultural Genomics Institute at Shenzhen, Chinese Academy of Agricultural Sciences. All mice care and use were conducted in strict accordance with the Animal Research Committee guidelines. All surgery was performed under cervical dislocation method anesthesia, and all efforts were made to minimize suffering.

Conflict of interests

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2020.11.018.

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