

MicroRNA-23a-5p mediates the proliferation and differentiation of C2C12 myoblasts

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Abstract. Skeletal myogenesis is a highly ordered and complex biological process that is mediated by numerous regulatory factors. In previous studies, it has been demonstrated that microRNAs (miRs) and long non-coding RNAs (lncRNAs) serve key roles in skeletal myogenesis. The present study showed that the expression levels of *miR-23a-5p* showed a dynamic change from decrease to increase during C2C12 myoblast proliferation and differentiation. Functional analysis using 5-ethynyl-2'-deoxyuridine proliferation and Cell Counting Kit-8 detection assays indicated that overexpression of *miR-23a-5p* significantly promoted C2C12 myoblast proliferation compared with the negative control. In addition, in C2C12 myoblasts transfected with *miR-23a-5p* mimics, increased expression levels of regulators associated with cell proliferation (*Cyclin E*, *CCND1* and *Cyclin B*) were observed compared with the negative control. By contrast, overexpression of *miR-23a-5p* decreased the expression levels of specific-myogenesis factors (*MyoD*, *MyoG* and *Myf5*) and decreased C2C12 myoblast differentiation. Luciferase activity assays indicated that *miR-23a-5p* suppressed the luciferase activity of *lncDum*. Further analysis demonstrated that *miR-23a-5p* not only showed an opposite expression level pattern compared with *lncDum*, which was first increased and then decreased, but also had an opposite effect on the proliferation and differentiation of C2C12 myoblasts compared with *lncDum* which inhibited cell proliferation and promoted

cell differentiation. Taken together, these results indicated that *miR-23a-5p* may mediate the proliferation and differentiation of C2C12 myoblasts, which may be involved in *lncDum* regulation.

Introduction

The basic functions of the skeletal musculature include facilitating locomotor activity, postural behavior and breathing (1). In addition, the skeletal musculature is a key metabolic organ in the human body, which is involved in regulating metabolite homeostasis and maintaining metabolic health via processes such as mediating glucose uptake and insulin sensitivity (2). Skeletal muscle has been identified as a secretory organ, which may exert autocrine, paracrine or endocrine effects to affect the function of adipose tissue or the cardiovascular system (3). Therefore, impaired or loss of function of skeletal muscle can affect functional capacity and increase the risk of a number of diseases, such as diabetes mellitus (4), muscle atrophy (5) and cancer (6). Skeletal myogenesis is a complex biological process that involves myoblast proliferation, migration and differentiation, as well as fusion of multicellular myotubes into contractile skeletal muscle fibers (7). In addition, according to skeletal muscle contractility and expression levels of myosin heavy chain (MyHC) subtypes, skeletal muscle fibers are classified into two major types: Fast- (MyHC2b and MyHC2x) and slow-twitch fibers (MyHC1 and MyHC2a) (8-10). A number of transcription factors mediate skeletal myogenesis (11-15), including the myogenic regulatory family (MRF) and the myocyte enhancer 2 family (MEF2), which are among the most studied gene families involved in regulating skeletal myogenesis (14). The MRF family, also known as the MyoD family, consists of *MyoD*, *MyoG*, *Myf5* and *MRF4* (14), and serves a key role in regulating specific gene transcription in muscle cells, as well as cell growth cycle and differentiation (16). For instance, *MyoD* and *Myf5* induce expression levels of myogenin and other MEF2 family transcription factors (17). Subsequently, myogenin and MEF2 family members cooperate in the activation of muscle structural genes during differentiation and the formation of multinucleated myotubes (17). Megeney *et al* (18) demonstrated that mice lacking both *MyoD* and dystrophin displayed a marked increase in severity of

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myopathy leading to premature death, suggesting a role for *MyoD* in muscle regeneration.

Evidence has demonstrated that microRNAs (miRNAs/miRs) are a class of non-coding RNAs 17-24 nucleotides in length (19,20), which serve key roles in numerous physiological and pathological processes, such as cell proliferation, cell differentiation, or tumorigenesis (21-23). A number of studies have shown that miRNAs regulate the expression levels of key functional genes and transcription factors at the posttranscriptional level, and induce mRNA degradation or translation inhibition by interacting with the 3' untranslated regions (UTRs) of their target mRNAs (20,24). Involvement of miRNAs in skeletal muscle development and function has previously been reported (25,26). A number of studies have demonstrated that miRNAs function as key regulators of myogenesis via regulating myoblast proliferation and differentiation (25,27,28). *miR-1*, for example, promotes myogenesis via targeting histone deacetylase 4, a transcriptional repressor of the myogenesis gene (29). *miRNA-133* enhances myoblast proliferation by repressing serum response factor (29). *miRNA-206* regulates skeletal muscle satellite cell proliferation and differentiation by repressing *Pax7* (26), and *miRNA-139-5p* regulates C2C12 cell myogenesis by blocking the Wnt/ β -catenin signaling pathway (30). In addition, *miRNA-27b* regulates Pax3 protein levels, and downregulation of Pax3 causes rapid entry into the myogenic differentiation program (31). Changes in miRNA expression levels have been reported to be associated with skeletal myogenesis (32,33).

Long non-coding RNAs (lncRNAs), a class of non-protein-coding RNAs >200 nucleotides in length, have been identified as competing endogenous RNAs (ceRNAs) that sponge miRNAs via complementary base pairing to regulate biological process (34). To date, numerous lncRNAs have been identified in mice and humans, among which some serve important roles in muscle development and myogenesis (35,36). A number of studies have indicated that expression levels of *miR-23a* and *miR-23b* are increased in the early stage of C2C12 differentiation, and that these miRNAs are involved in the regulation of *TrxR1* expression levels via direct binding to the 3' UTR of *TrxR1* mRNA (37,38). Wang *et al.* (10) reported that *miR-23a-3p* downregulates the expression levels of *Myh1*, *2* and *4* via targeting the 3' UTR of mRNAs, thereby inhibiting myogenesis of C2C12 myoblasts and fast-twitch MyHC isoforms. However, the role of *miR-23a-5p* in myoblast proliferation and differentiation has not yet been fully elucidated. The present study demonstrated that *miR-23a-5p* affected myoblast proliferation and differentiation. It was hypothesized that *miR-23a-5p* promoted C2C12 myoblast proliferation and inhibited differentiation, which may be involved in *lncDum* regulation.

Materials and methods

Animal experiments. A total of 21 female C57BL/6 mice (Dashuo Co., Ltd.) had free access to food and water in natural light cycle and 37°C temperature conditions. The muscle tissues of 2-6-week-old C57BL/6 mice were collected for RNA isolation. A total of three mice were included each group (2-week-old, 3-week-old, 4-week-old, 5-week-old, 6-week-old C57BL/6 mice). Tissues (liver, spleen, lung, heart, kidney,

muscle, inguinal fat, perirenal fat, brain, shoulder fat and gonads fat) of 6-week-old C57BL/6 mice were collected for RNA isolation. In order to establish a muscle atrophy model, muscle atrophy was induced by Dexamethasone (Dex; Beijing Solarbio Science & Technology Co., Ltd.)-mediated injury as described by Morimoto *et al.* (39). In brief, three 10-week-old mice were intramuscularly injected in the left hind leg with 100 μ l 10 μ M Dex. As a control, the right legs of the same mice were intramuscularly injected with PBS. The injection was performed every 7 days to ensure success. At 14 days after the first injection, muscle samples were collected for RNA isolation.

All animal procedures were approved by the Animal Care and Ethics Committee of Sichuan Agricultural University [approval no. DKY-(S20176903)] and performed in accordance with the institutional guidelines for the care and use of laboratory animals.

Cell culture. C2C12 myoblasts and HeLa cells (Beijing Stem Cell Bank; Chinese Academy of Science) were cultured in growth medium (GM) containing DMEM and 10% FBS (both Gibco; Thermo Fisher Scientific, Inc.). When C2C12 myoblasts reached 80% confluence, GM was replaced with differentiation medium (DM) containing DMEM supplemented with 2% horse serum (Gibco; Thermo Fisher Scientific, Inc.). Cells were cultured at 37°C with 5% CO₂.

Cell transfection. miR-23a-5p mimics (cat. no. miR10017019-1-5), negative control (NC) mimics (cat. no. miR01201-1-5) and small interfering (si)RNA molecules directed against *lncDum* (si-lncDum) were designed and synthesized by Guangzhou RiboBio Co., Ltd. Overexpression of *lncDum* (p-lncDum) was achieved by cloning *lncDum* complementary DNA into vector pcDNA3.1 (Beijing Tsingke New Industry TsingKe Biotechnology Co., Ltd.). Briefly, 5x10⁵ or 4x10⁴ C2C12 myoblasts were seeded in 12- or 96-well plates and grown to 80% confluency, then transfected with miRNA-23a-5p mimics, NC mimics, si-lncDum, p-lncDum, empty pcDNA3.1+ vector or siRNA control (si-NC) at a concentration of about 20 μ M using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The transfections were carried out every 48 h. Cells were harvested for 6 days, and bright-field microscope was used to observe the ability of C2C12 myoblasts to differentiate into myotubes 6 days post-transfection. The sequences of synthesized RNA oligonucleotides are presented in Table SI.

Reverse transcription-quantitative PCR (RT-qPCR). RNA from muscle tissue and C2C12 cells was obtained using TRIzol® (Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions. Reverse transcription was performed using a PrimeScript RT Master Mix kit for mRNA, or a PrimeScript™ miRNA RTPCR kit (both Takara Biotechnology Co., Ltd.) for miRNA, according to the manufacturer's instructions. qPCR was performed using the SYBR Premix Ex Taq kit (Takara Biotechnology Co., Ltd.) using a CFX96 system (Bio-Rad Laboratories, Inc.). *U6* and β -actin served as endogenous controls for miRNA and mRNA, respectively. The expression levels of miRNA and mRNA were quantified using the comparative threshold cycle (2^{- $\Delta\Delta$ C_t}) method (40). Primer sequences are presented in Table SII.

Cell proliferation analysis. In brief, C2C12 myoblasts were seeded in 96-well plates, and transfected with miR-23a-5p mimics, NC mimics, si-lncDum, p-lncDum, si-NC or pcDNA3.1⁺ empty vector, after which cell proliferation was determined at 0, 12, 24, 48, 72 and 96 h using a Cell Counting Kit (CKK)-8 (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. For 5-ethynyl-2'-deoxyuridine (EdU) proliferation analysis, C2C12 myoblasts were treated with 10 μ M EdU (Guangzhou RiboBio Co., Ltd.) after 24 h post-transfection and incubated for 3 h at 37°C. EdU staining was performed according to the manufacturer's protocol. Cell nuclei were stained with DAPI (Beyotime Institute of Biotechnology) for 10 min at room temperature. Images (magnification, x100) of the entire well were captured using an IX53 fluorescent inverted microscope (Olympus Corporation).

Western blotting. Total cellular proteins were extracted using RIPA lysis buffer (Wuhan Servicebio Technology Co., Ltd.) 96 h after transfection. The native protein lysate was collected, and the protein concentration was measured using the BCA protein concentration assay kit (Wuhan Servicebio Technology Co., Ltd.) according to the manufacturer's instructions. Proteins (30-50 μ g/per well) were resolved by SDS-PAGE with 10% gels, then transferred to PVDF membranes (EMD Millipore). The membranes were blocked with 5% non-fat milk in 0.5% TBS-Tween-20 (TBST) for 1 h at room temperature. The membranes were then incubated overnight at 4°C with primary antibodies against β -actin (dilution 1:1,000; cat. no. GB12001, Wuhan Servicebio Technology Co., Ltd.); MyoG (dilution ratio 1:1,000; cat. no. A6664, ABclonal), and MyHC1 (dilution ratio 1:1,000; cat. no. A7564, ABclonal). The membranes were washed with TBST and then incubated with the secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG; dilution 1:3,000; cat. no. GB23303; Wuhan Servicebio Technology Co., Ltd.) for 30 min at room temperature. The signals were visualized using enhanced chemiluminescent reagent (Wuhan Servicebio Technology Co., Ltd.), and the expression levels of β -actin were used for normalization. The AlphaEaseFC™ software processing system (version 3.2 β ; Alpha Innotech) was used to analyze the optical density value of the target bands.

Immunofluorescence analysis. For immunofluorescence analysis, C2C12 myoblasts were transfected with miR-23a-5p mimics, NC, si-lncDum, p-lncDum, si-NC or pcDNA3.1⁺ empty vector during differentiation, then washed three times with PBS, and fixed in 4% paraformaldehyde for 30 min at room temperature. Next, cells were washed three times with PBS, and blocked with 5% normal goat serum in PBS for 1 h at room temperature. Then, cells were incubated with an anti-myosin slow antibody (anti-MyHC1; dilution 1:300; cat. no. bs-9862R; BIOSS) overnight at 4°C, washed three times with PBS, and incubated with a fluorescent secondary antibody (dilution ratio 1:300; cat. no. bs-0295G-FITC; BIOSS) at 37°C for 1 h. Cell nuclei were stained with DAPI (Beyotime Institute of Biotechnology) for 20 min at room temperature. Images (magnification, x100) were captured using an IX53 fluorescent inverted microscope (Olympus Corporation). The

fusion index of myotubes as calculated by Image J software (Image J 1.50i; National Institutes of Health).

Luciferase reporter assay. HeLa cells were seeded in 96-well plates and cultured to 90% confluency prior to transfection. Wild-type (WT-lncDum) and mutant lncDum (Mut-lncDum) were amplified using primers containing *Xho*I and *Not*I restriction sites and cloned into a psi-CHECK™-2 promoter vector at the 3' end of the *Renilla* gene Beijing Tsingke New Industry TsingKe Biotechnology Co., Ltd.). For the luciferase reporter analysis, 4x10⁴ HeLa cells were seeded in 96-well plates and grown to 80% confluency, then co-transfected with either WT-lncDum or Mut-lncDum in conjunction with either miR-23a-5p mimics or mimics control (Guangzhou RiboBio Co., Ltd.) at a final concentration of 100 nM using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). Luciferase activity was measured using a Dual-Glo Luciferase Assay System (Promega Corporation) 24 h after transfection, according to the manufacturer's instructions. Luciferase activity levels were normalized to those of firefly luciferase.

Statistical analysis. Data are presented as the mean \pm SEM of three experiments. Statistical analysis was performed using SPSS software (version 22.0; IBM Corp.). Differences between groups were determined using an unpaired Student's t-test. Differences between 3 or more groups were determined by one-way ANOVA, followed by the Least Significant Difference method. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-23a-5p is associated with skeletal myogenesis. miR-23a-5p exhibited a similar expression profile to three myoblast-specific miRNAs [*miR-206-3p* (41), *miR-181a-5p* (42) and *miR-221* (43); Fig. 1A] in C2C12 myoblasts. The expression levels of *miR-23a-5p* in numerous types of tissue derived from 6-week-old WT mice were assessed, which demonstrated that *miR-23a-5p* was highly expressed in muscle, compared with adipose tissue and brain. However, *miR-23a-5p* was expressed at lower levels in muscle tissue compared with major organs, such as the liver, spleen, lung, heart and kidney (Fig. 1B). Furthermore, *miR-23a-5p* was highly expressed in the muscle tissue of 2-3-week-old mice, and notably decreased after 3 weeks and remained at low levels as mice aged (Fig. 1C). In order to confirm these results, a muscle atrophy model was established using Dex injection, as previously described (5,44,45). Compared with the control group, Dex treatment caused a significant decrease in *miR-23a-5p* expression levels in muscle tissue (Fig. 1D). Thus, the present findings indicated that *miR-23a-5p* may be associated with skeletal myogenesis.

miR-23a-5p promotes C2C12 myoblast proliferation. miR-23a-5p exhibited a notably decreased expression level during the proliferation and differentiation of C2C12 myoblasts compared with myogenesis-specific miRNAs, including *miR-206-3p*, *miR-181a-5p*, *miR-221/222* and *miR-29b-3p* (Fig. 2A). In order to identify the role of

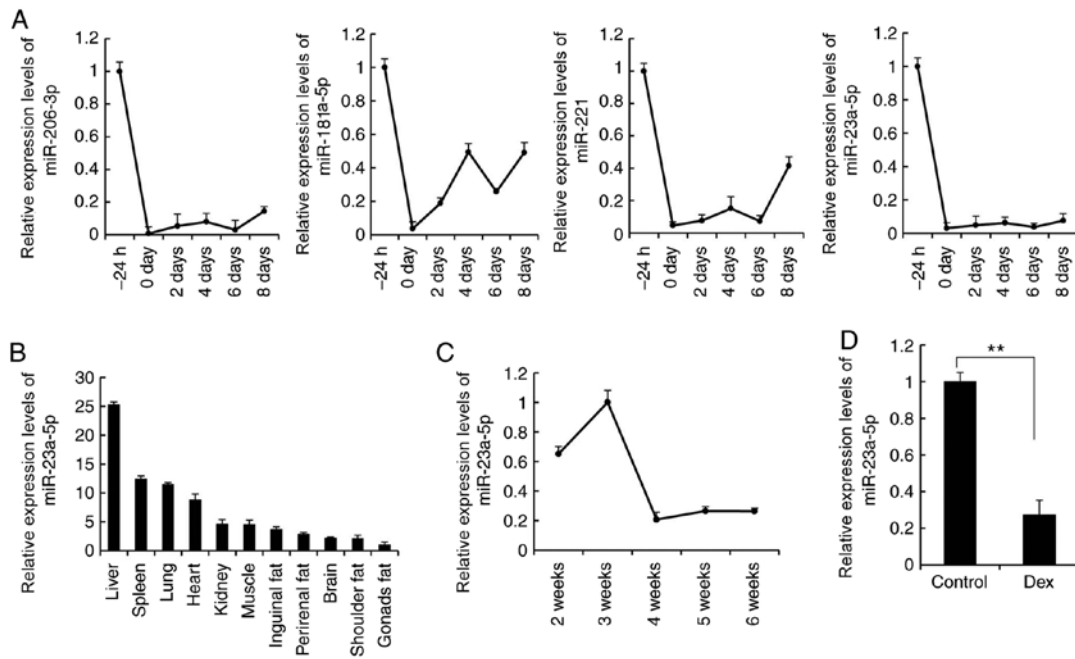


Figure 1. miR-23a-5p is associated with myogenesis. Reverse transcription-quantitative PCR was used to measure the expression levels of (A) miRs during myoblast proliferation and differentiation, and miR-23a-5p in (B) different types of tissue in 6-week-old mice, (C) muscle tissue of mice of different ages and (D) muscle tissue following treatment with Dex. Data are presented as the mean ± SEM (n=3). **P<0.01. miR, microRNA; Dex, dexamethasone.

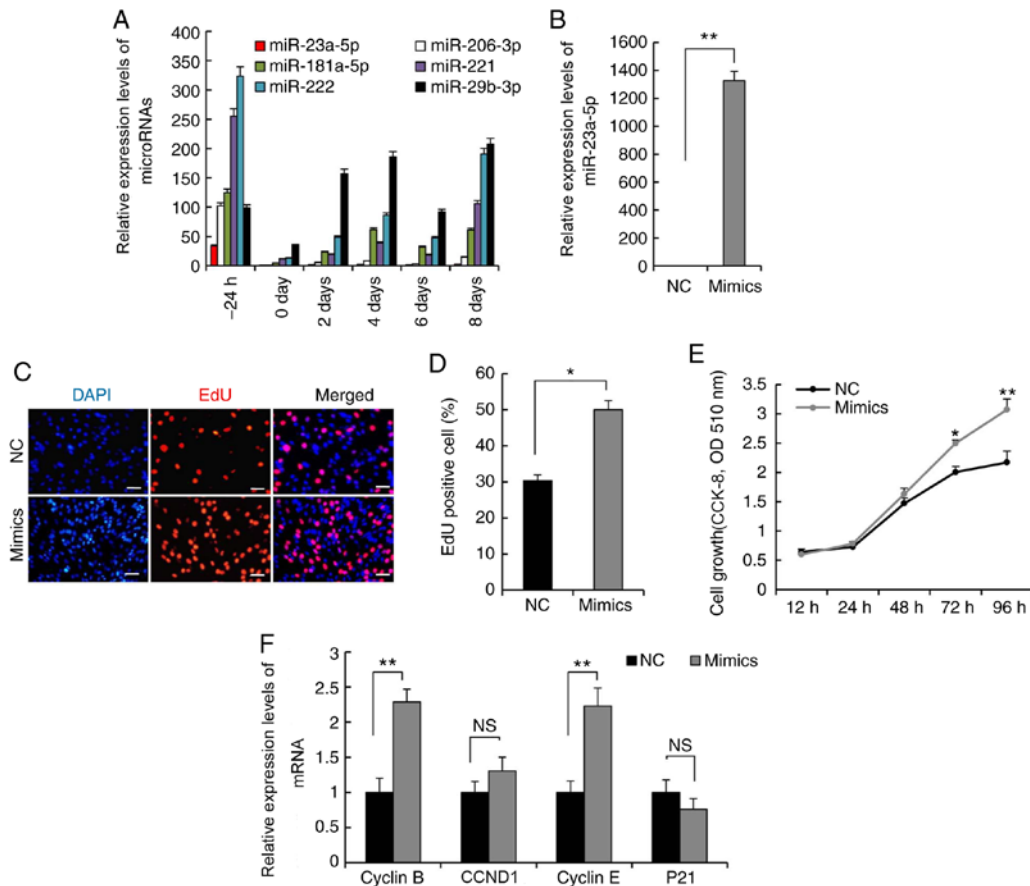


Figure 2. miR-23a-5p positively modulates C2C12 myoblast proliferation. RT-qPCR was used to measure expression levels of (A) miRNAs during C2C12 myoblast proliferation and differentiation, and (B) miR-23a-5p in C2C12 myoblasts transfected with miR-23a-5p mimics or NC during proliferation. (C) EdU assay was performed following transfection for 24 h. Cells undergoing DNA replication were stained with EdU (red) and cell nuclei were stained with DAPI (blue). Scale bar, 100 μm. (D) Percentage of EdU-positive cells/DAPI-positive cells was quantified. (E) Cell count was measured by an CCK-8 assay. (F) RT-qPCR analysis of the expression levels of *Cyclin E*, *Cyclin B*, *CCND1* and *P21*. Data are presented as the mean ± SEM (n=3). *P<0.05, **P<0.01 vs. NC or as indicated. miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR; NC, negative control; CCK-8, Cell Counting Kit-8; EdU, 5-ethynyl-2'-deoxyuridine; OD, optical density; NS, not significant.

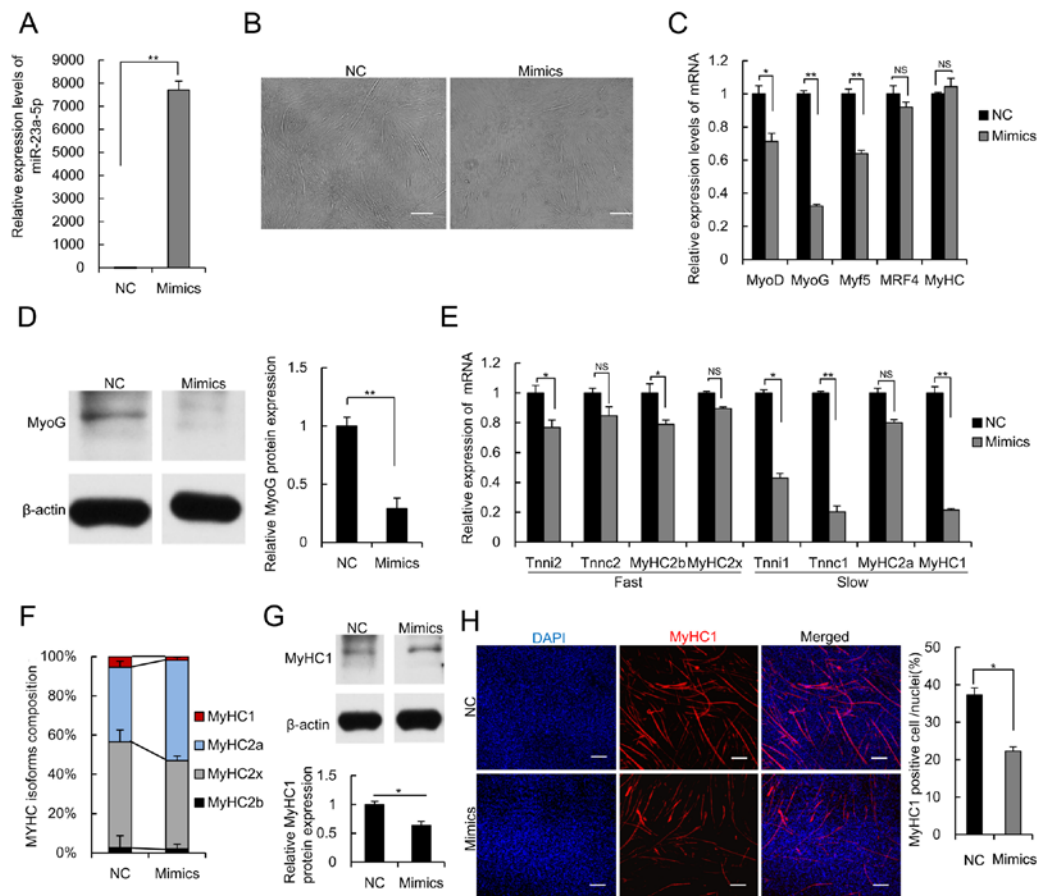


Figure 3. miR-23a-5p negatively modulates C2C12 myoblast differentiation and formation of muscle fiber type. (A) RT-qPCR analysis of the expression levels of *miR-23a-5p* in C2C12 myoblasts transfected with miR-23a-5p mimics or NC during differentiation. (B) Bright-field microscopy was used to observe the ability of C2C12 myoblasts to differentiate into myotubes at 6 days post-transfection. Scale bar, 100 μ m. (C) RT-qPCR analysis of the expression levels of *MyoG*, *MyoD*, *MRF4*, *Myf5* and *MyHC*. (D) Western blotting analysis of MyoG protein levels in C2C12 myoblasts transfected with miR-23a-5p mimics or NC during differentiation. RT-qPCR analysis of (E) expression levels of genes associated with fast- and slow-twitch fibers, and (F) The composition of MyHC (MyHC1, MyHC2a, MyHC2b and MyHC2x) in myoblasts transfected with miR-23a-5p mimics or NC. (G) Western blotting analysis of MyHC1 protein levels in C2C12 myoblasts transfected with miR-23a-5p mimics or NC during differentiation. (H) Immunofluorescence staining of MyHC1 was used to analyze myosin-slow-positive myotubes. The fusion index was calculated as MyHC-positive cells to total nuclei (total DAPI-positive cells). Scale bar, 100 μ m. Data are presented as the mean \pm SEM of three independent repeats. * P <0.05, ** P <0.01. miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR; NC, negative control; MyHC, myosin heavy chain; NS, not significant.

miR-23a-5p in regulating skeletal myogenesis, *miR-23a-5p* mimics were transfected into C2C12 myoblasts using GM to evaluate whether *miR-23a-5p* mediates proliferation of these cells. Transfection of *miR-23a-5p* mimics increased the expression levels of *miR-23a-5p* by 1,327-fold compared with the NC group, indicating that *miR-23a-5p* was successfully overexpressed in proliferating C2C12 myoblasts (Fig. 2B). Subsequently, an EdU proliferation assay was performed to evaluate the effect of miR-23a-5p on C2C12 myoblast proliferation; the results demonstrated that miR-23a-5p significantly increased the proliferation of EdU-positive cells compared with NC cells (Fig. 2C and D). These findings were also validated by an CCK-8 assay: Following transfection for 72 h, a greater number of C2C12 myoblasts were observed in the miR-23a-5p mimics group compared with the NC group (Fig. 2E). Furthermore, RT-qPCR analysis demonstrated that the expression levels of *Cyclin E* and *Cyclin B* were significantly upregulated in C2C12 myoblasts transfected with miR-23a-5p mimics, compared with the NC group (Fig. 2F). Although increasing *miR-23a-5p* tended to cause a decrease in *P21* expression levels compared with the NC group, the

difference was not significant. Taken together, these data indicated that *miR-23a-5p* positively regulated C2C12 myoblast proliferation.

miR-23a-5p inhibits C2C12 myoblast differentiation. In order to investigate the effect of *miR-23a-5p* on myoblast differentiation, miR-23a-5p mimics were transfected into C2C12 myoblasts using DM. At 6 days after transfection of miR-23a-5p mimics (Fig. 3A), bright-field microscopy was performed, which demonstrated that *miR-23a-5p* overexpression markedly inhibited the fusion of myotubes compared with the NC group (Fig. 3B). In order to confirm these results, the expression levels of various myogenic transcription factors, such as *MyoD*, *Myf5*, *MyoG*, *MRF4* and *MyHC*, were evaluated. Transfection with miR-23a-5p mimics resulted in a significant decrease in the expression levels of *MyoD*, *MyoG* and *Myf5* compared with the NC group (Fig. 3C). Furthermore, MyoG protein expression levels in *miR-23a-5p*-overexpressing C2C12 cells were assessed. It was revealed that MyoG was downregulated following *miR-23a-5p* overexpression, as determined via western blotting analysis (Fig. 3D).

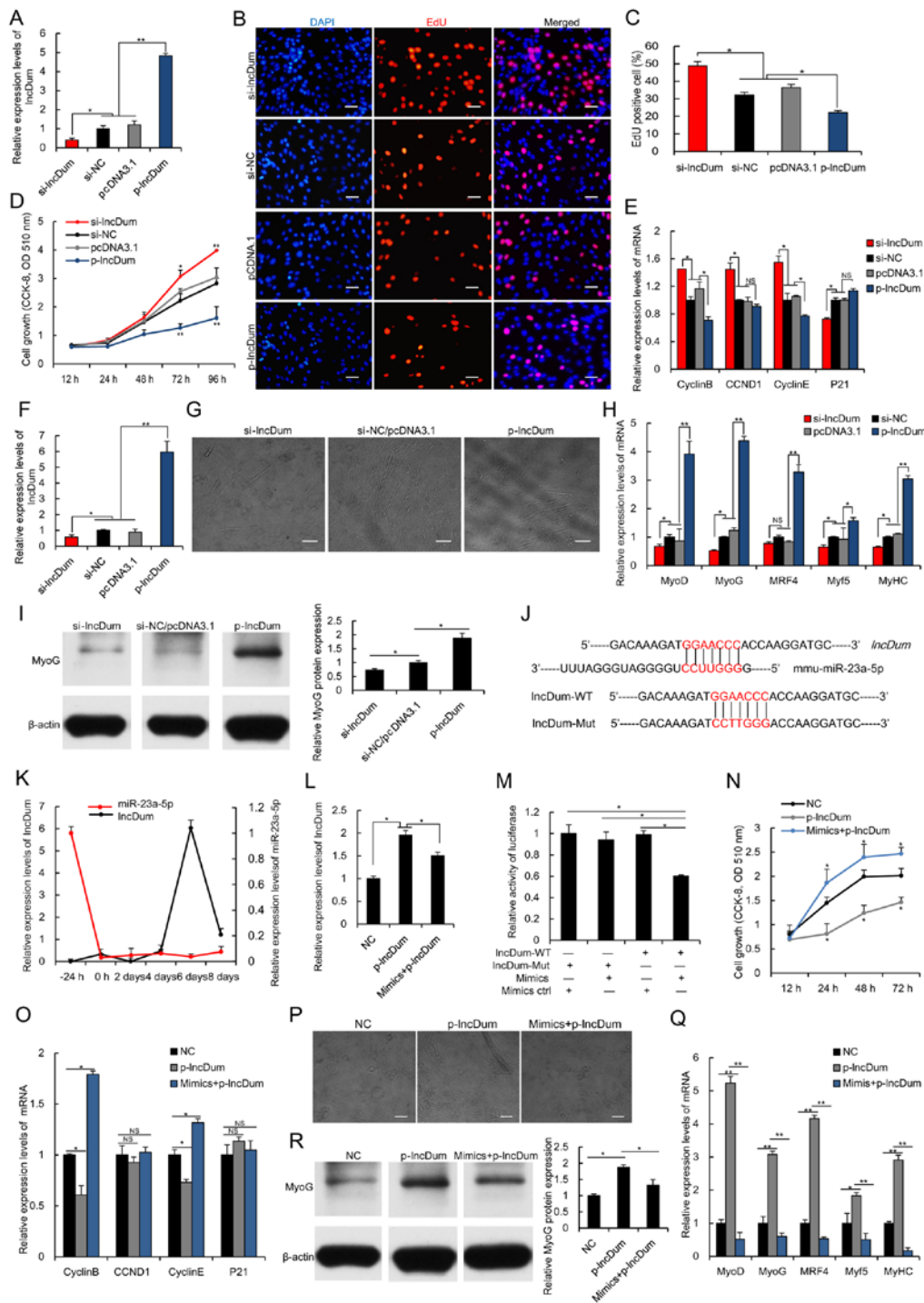


Figure 4. miR-23a-5p regulates C2C12 myoblast proliferation and differentiation by interacting with *lncDum*. (A) RT-qPCR analysis of the expression levels of *lncDum* in myoblasts transfected with si-*lncDum*, si-NC, p-*lncDum* or pcDNA3.1⁺ empty vector during proliferation. (B) EdU assay was performed following 24 h of transfection. Scale bar, 100 μ m. (C) Percentage of EdU-positive/DAPI-positive cells was quantified. (D) Cell count was measured via a CCK-8 assay in C2C12 myoblasts transfected with si-*lncDum*, si-NC, pcDNA3.1⁺ empty vector or p-*lncDum*. RT-qPCR analysis of the expression levels of (E) *Cyclin E*, *Cyclin B*, *CCND1* and *P21*, and (F) *lncDum* in myoblasts transfected with si-*lncDum*, si-NC, pcDNA3.1⁺ empty vector or p-*lncDum* during differentiation. (G) Bright-field microscopy was used to observe the ability of C2C12 myoblasts to differentiate into myotubes at 6 days after transfecting with si-*lncDum*, si-NC, pcDNA3.1⁺ empty vector or p-*lncDum*. Scale bar, 100 μ m. (H) RT-qPCR measured the expression levels of *MyoG*, *MyoD*, *MRF4*, *Myf5* and *MyHC*. (I) Western blotting analysis of MyoG protein levels in C2C12 myoblasts transfected with si-*lncDum*, si-NC, p-*lncDum* or pcDNA3.1⁺ empty vector during differentiation. (J) *lncDum* contained a binding site for miR-23a-5p. RT-qPCR analysis of the expression levels of (K) *lncDum* and miR-23a-5p during myoblast proliferation and differentiation, and (L) *lncDum* in myoblasts transfected with miR-23a-5p mimics and p-*lncDum*, NC or p-*lncDum* during proliferation. (M) Luciferase assays assessed the effect of miR-23a-5p on the activity of *lncDum*. (N) Cell count was measured via a CCK-8 assay in C2C12 myoblasts transfected with p-*lncDum*, NC or p-*lncDum* and miR-23a-5p mimics. (O) RT-qPCR analysis of the expression levels of *Cyclin E*, *Cyclin B*, *CCND1* and *P21*. (P) Bright-field was used to microscopy observe the ability of C2C12 myoblasts to differentiate into myotubes at 6 days after transfecting with p-*lncDum*, NC or p-*lncDum* and miR-23a-5p mimics. Scale bar, 100 μ m. (Q) RT-qPCR analysis of the expression levels of *MyoG*, *MyoD*, *MRF4*, *Myf5* and *MyHC*. (R) Western blotting analysis of MyoG protein levels in C2C12 myoblasts transfected with miR-23a-5p mimics and p-*lncDum*, NC or p-*lncDum* during differentiation. Data are presented as the mean \pm SEM of three independent repeats. * $P < 0.05$, ** $P < 0.01$. miR, microRNA; lnc, long non-coding RNA; RT-qPCR, reverse transcription-quantitative PCR; si, small interfering; NC, negative control; CCK-8, Cell Counting Kit-8; MyHC, myosin heavy chain; OD, optical density; NS, not significant.

As muscle fiber type composition is key for muscle biological function and body metabolism, the present study aimed to determine whether *miR-23a-5p* mediates muscle fiber type composition. RT-qPCR demonstrated that overexpression of *miR-23a-5p* notably inhibited the expression levels of several genes associated with fast- (*Tnni2*, *Tnnc2*, *MyHC2b* and *MyHC2x*) and slow-twitch fibers (*Tnni1*, *Tnnc1*, *MyHC2a* and *MyHC1*) (46) compared with the NC group, indicating that *miR-23a-5p* may be involved in regulating the composition of skeletal muscle fiber type (Fig. 3E). The expression levels of *MyHC1*, *-2a*, *-2b* and *-2x*, following transfection of C2C12 myoblasts with *miR-23a-5p* mimics or NC were further analyzed during differentiation. Overexpression of *miR-23a-5p* in C2C12 myotubes decreased the percentage of *MyHC1* and increased the percentage of *MyHC2a* by RT-qPCR (Fig. 3F). In addition, overexpression of *miR-23a-5p* decreased *MyHC1* protein level by western blotting analysis (Fig. 3G), as demonstrated by immunofluorescence analysis of *MyHC1* (Fig. 3H). Taken together, these results indicated that *miR-23a-5p* may affect C2C12 myoblast differentiation and mediate skeletal muscle fiber type composition.

miR-23a-5p regulates C2C12 myoblast proliferation and differentiation by interacting with *lncDum*. A previous study have demonstrated that *lncDum* serves a key role in regulating cell differentiation and muscle regeneration (47). In order to confirm the function of *lncDum* on C2C12 myoblast proliferation and differentiation, si-*lncDum*, si-NC, p-*lncDum* or empty pcDNA3.1⁺ vector were transfected into C2C12 myoblasts during proliferation (Fig. 4A). CCK-8 and EdU assays revealed that inhibition of *lncDum* significantly promoted C2C12 myoblast proliferation compared with the control (Fig. 4B-D). Consistent with these findings, *Cyclin E*, *CCND1* and *Cyclin B* were upregulated, and *P21* was downregulated in C2C12 myoblasts that were transfected with si-*lncDum* (Fig. 4E). Consistent with previous findings (47) that *lncDum* enhances C2C12 myoblast differentiation, overexpression of *lncDum* promoted the fusion of myotubes and increased the expression levels of *MyoD*, *MRF4*, *MyoG*, *Myf5* and *MyHC*, whereas *lncDum* inhibition decreased the fusion of myotubes and downregulated these genes (Fig. 4F-H). In addition, western blotting analysis showed that the expression levels of *MyoG* were also upregulated by *lncDum* overexpression and downregulated by *lncDum* inhibition compared with the control (Fig. 4I), thereby indicating that *lncDum* promoted myoblast differentiation.

A number of lncRNAs that function in skeletal myogenesis have previously been identified as ceRNAs that sponge miRNAs via complementary base pairing (48,49). In the present study, it was observed that *lncDum* contained a complementary sequence to that of *miR-23a-5p*, and that *lncDum* and *miR-23a-5p* exhibited opposite mRNA expression level patterns during C2C12 myoblast proliferation and differentiation (Fig. 4J and K). Moreover, RT-qPCR analysis demonstrated that overexpression of *miR-23a-5p* significantly inhibited the increase of *lncDum* induced by transfection of p-*lncDum* (Fig. 4L). A luciferase reporter assay demonstrated that, compared with the control, overexpression of *miR-23a-5p* suppressed the luciferase activity of WT-*lncDum*, but had little effect on *miR-23a-5p* binding sites in Mut-*lncDum*

(Fig. 4M), demonstrating that *miR-23a-5p* interacted with *lncDum* via complementary base pairing. Moreover, rescue experiments were performed to evaluate whether the effects of *miR-23a-5p* on the proliferation and differentiation of C2C12 myoblasts were regulated by *lncDum*. The results indicated that *miR-23a-5p* significantly attenuated the effect of *lncDum* overexpression on C2C12 myoblast proliferation and differentiation (Fig. 4N-R). Taken together, these data suggested that *miR-23a-5p* mediated myogenic proliferation and differentiation via interactions with *lncDum*.

Discussion

Skeletal myogenesis is a complex biological process (7). In addition to the involvement of myoblast proliferation and differentiation, differentiated myoblasts fuse into multinucleate myotubes, which give rise to diverse types of muscle fiber that build the complex skeletal muscle architecture essential for body movement, postural behavior and breathing (1,50). Previous studies have also demonstrated the roles of miRNAs in skeletal myogenesis. For example, Naguibneva *et al* (42) reported that miR-181 regulates mammalian myoblast differentiation via targeting homeobox protein Hox-A11. Mi *et al* (30) reported that *miR-139-5p* regulates C2C12 cell myogenesis via blocking the Wnt/ β -catenin signaling pathway. Wang *et al* (10) reported that *miR-23a-3p* regulates myogenic differentiation by inhibiting the expression levels of fast MyHC isoforms. To the best of our knowledge, however, the role and molecular mechanism of *miR-23a-5p* in myoblast proliferation and differentiation has not previously been fully elucidated. The present study aimed to determine whether *miR-23a-5p* was involved in skeletal myogenesis. Previous studies have indicated that *miR-206-3p* (41), *miR-181a-5p* (42) and *miR-221* (43) serve key roles in the regulation of skeletal myogenesis. The results of the present study demonstrated that *miR-23a-5p* exhibited a similar expression level pattern to these three myoblast-specific miRNAs. The present study also revealed that *miR-23a-5p* was ubiquitously expressed in different types of tissue and moderately expressed in muscle tissue. Furthermore, *miR-23a-5p* expression levels in muscle tissue were significantly decreased following Dex treatment. Dex, a potent synthetic glucocorticoid, has been widely used to induce muscle atrophy due to its ability to stimulate protein degradation (51). Muscle atrophy induced by muscular dysfunction can affect functional capacity. The data obtained in the present study supported the hypothesis that *miR-23a-5p* is associated with skeletal myogenesis. As *miR-23a-5p* exhibited low expression during the proliferation and differentiation of C2C12 myoblasts compared with myogenesis-specific miRNAs, including *miR-206-3p*, *miR-181a-5p*, *miR-221/222* and *miR-29b-3p*, it was hypothesized that overexpression of *miR-23a-5p* could affect skeletal myogenesis.

Myoblast proliferation and differentiation are key processes in skeletal myogenesis (7). *Cyclin E*, *Cyclin B* and *CCND1*, which bind cyclin-dependent protein kinases (Cdk) to control cell cycle progression, such as G1-S and G2-M transition, are expressed during the course of the cell cycle (52-54). By contrast, as a Cdk inhibitor, *p21* can bind and inactivate Cdk-cyclin complexes to repress specific steps of cell cycle progression (55-57). The results of the present study demon-

strated that *miR-23a-5p* positively regulate the expression levels of these genes during C2C12 myoblast proliferation. EdU and CCK-8 assays also showed that *miR-23a-5p* significantly promoted C2C12 myoblast proliferation.

Previous studies have shown that myogenic differentiation is controlled by complex myogenic transcription factors, such as *MyoD*, *Myf5*, *MyoG*, *MRF4* and *MyHC* (58,59). *MyoD* and *Myf5* participate in controlling early differentiation, and *MyoG* and *MRF4* induce myoblast differentiation at later stages (30,42). *MyoG* and *MyoD* are key transcription factors in myogenesis and regulate transcription of the majority of muscle-specific genes (60). *MyoG* and *MyoD* serve an important role in the regulation of myoblast differentiation. *MyoD* is considered to act as a myogenic determination gene (61,62), whereas *MyoG* is essential for terminal differentiation of committed myoblasts (59). The present results indicated that *miR-23a-5p* decreased the expression levels of myogenesis-specific factors and that *miR-23a-5p* inhibits C2C12 myoblast differentiation. Previous studies have demonstrated that regulators could modulate muscle fiber type transition via altering the percentage of MyHC isoforms associated with slow- or fast-twitch fibers (63-65). Fibers expressing MyHC2a and 2x have intermediate characteristics between MyHC types 1 and 2b (66). In addition, muscle fiber type composition is key for muscle biological function and body metabolism; therefore, the present study aimed to determine whether *miR-23a-5p* mediates muscle fiber type composition. The results suggested that *miR-23a-5p* is involved in regulating the composition of skeletal muscle fiber type in a more complex manner. Taken together, these results indicated that *miR-23a-5p* may affect C2C12 myoblast differentiation and mediate skeletal muscle fiber-type composition.

A number of lncRNAs that are key regulators of skeletal muscle physiology and are involved in skeletal myogenesis have been identified as ceRNAs that sponge miRNAs via complementary base pairing (34,67). Wang *et al* (47) indicated that *lncDum* serves an important role in regulating cell differentiation and muscle regeneration. These results suggest that *miR-23a-5p* interacted with *lncDum* by complementary base pairing, and that *lncDum* promotes C2C12 myoblast proliferation and differentiation. Taken together, these data indicated that *miR-23a-5p* mediates myogenic proliferation and differentiation via interacting with *lncDum*.

In conclusion, the present study demonstrated that the expression levels of *miR-23a-5p* were showed a dynamic change, from decrease to increase, during myogenesis in mice. Analysis revealed that *miR-23a-5p* overexpression promoted C2C12 myoblast proliferation, inhibited C2C12 myoblast differentiation and regulated muscle fiber type composition. The present study indicated that *lncDum* affected C2C12 myoblast proliferation and differentiation, which was contrary to the effects of *miR-23a-5p*. Taken together, the present results suggested that *lncDum* may be a target of *miR-23a-5p* in the regulation of skeletal myogenesis. However, the association between *miR-23a-5p* inhibition and the regulation of skeletal myogenesis was not determined in the present study, and further investigation is required to elucidate the regulatory mechanisms underlying *miR-23a-5p* in skeletal muscle development.

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Availability of data and material

All data generated or analyzed during this study are included in this published article.

Authors' contributions

SZ, LZ and ML conceptualized and designed the experiments. XZ, HG, LW and PZ performed the experiments. JD, LS and DJ collected the animal samples. JW and XL analyzed the data. XZ drafted the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All animal procedures were conducted in accordance with institutional guidelines for the care and use of laboratory animals was approved by the Animal Care and Ethics Committee of Sichuan Agricultural University, Sichuan, China [approval no. DKY-(S20176903)].

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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