Long non-coding RNA Mir22hg-derived miR-22-3p promotes skeletal muscle differentiation and regeneration by inhibiting HDAC4

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Emerging studies have indicated that long non-coding RNAs (lncRNAs) play important roles in skeletal muscle growth and development. Nevertheless, it remains challenging to understand the function and regulatory mechanisms of these lncRNAs in muscle biology and associated diseases. Here, we identify a novel lncRNA, Mir22hg, that is significantly upregulated during myoblast differentiation and is highly expressed in skeletal muscle. We validated that Mir22hg promotes myoblast differentiation in vitro. Mechanistically, Mir22hg gives rise to mature microRNA (miR)-22-3p, which inhibits its target gene, histone deacetylase 4 (HDAC4), thereby increasing the downstream myocyte enhancer factor 2C (MEF2C) and ultimately promoting myoblast differentiation. Furthermore, in vivo, we documented that Mir22hg knockdown delays repair and regeneration following skeletal muscle injury and further causes a significant decrease in weight following repair of an injured tibialis anterior muscle. Additionally, Mir22hg gives rise to miR-22-3p to restrict HDAC4 expression, thereby promoting the differentiation and regeneration of skeletal muscle. Given the conservation of Mir22hg between mice and humans, Mir22hg might constitute a promising new therapeutic target for skeletal muscle injury, skeletal muscle atrophy, as well as other skeletal muscle diseases.

INTRODUCTION

Skeletal muscle is a highly dynamic and adaptive tissue that plays a pivotal role in regulating whole-body metabolism. However, disorders of skeletal muscle growth and development can lead to numerous diseases, such as muscular atrophy, dystrophy, hypertrophy, and myosarcoma.1 Thus, identification of the functional components in muscle biology, as well as elucidation of their regulatory mechanisms, will aid toward identifying potential therapeutic approaches for muscular diseases.

Long non-coding RNAs (lncRNAs), a type of non-coding RNA comprised of >200 nucleotides, are involved in diverse biological processes throughout an organism’s life span, including immunobiology,2 cell fate,3 cell differentiation and development,4 and diseases.5 lncRNAs act as regulators through multiple mechanisms, including chromosome modification, transcriptional activation, molecular sponge activity, competitive binding, mRNA translation, and protein stability.6 In particular, numerous studies indicate that lncRNAs play an important role in skeletal muscle biology and muscular diseases,7 as summarized in several reviews.8,9 For example, linc-MD1, the first lncRNA identified as specifically expressed in muscle tissue, reportedly controls the differentiation of muscle cells by interacting with microRNA (miR)-133 and miR-135 as a molecular sponge and is related to muscle atrophy.10 In addition, Inc-mg is highly expressed in skeletal muscle and is associated with muscle hypertrophy. It also serves as a competing endogenous RNA (ceRNA) against miR-125 to modulate insulin-like growth factor 2 (IGF2) levels, thereby regulating skeletal muscle differentiation.11

Currently, thousands of lncRNAs have been identified in skeletal muscles, and various skeletal muscle highly expressed lncRNAs and diverse regulatory mechanisms have been revealed.12,13,14 Further, the roles and regulatory mechanisms of some classically defined lncRNAs have also been verified.13,14,15 Nevertheless, it remains a tremendous challenge to understand the role of these molecules in the molecular mechanism in myogenesis and skeletal muscle growth as well as the development and molecular mechanisms of the thousands of lncRNAs that have been recently identified in skeletal muscles. Moreover, considering that skeletal muscle disease-related lncRNAs have been described, lncRNAs may represent a new potential therapeutic target for skeletal muscle diseases.7,14
In this study, we identified a new lncRNA, Mir22hg, that is characterized by high expression in skeletal muscle, for which we systematically defined its function in differentiation, repair, and regeneration. Our data indicated that Mir22hg promotes the differentiation of skeletal muscle cells in vitro and facilitates the repair and regeneration of injured skeletal muscle mass in vivo. Mechanistically, Mir22hg gives rise to miR-22-3p to inhibit the target gene histone deacetylase 4 (HDAC4), thereby promoting myocyte enhancer factor 2C (MEF2C) expression, myoblast differentiation, and the repair and regeneration of injured skeletal muscle.

RESULTS
Mir22hg expression patterns
By integrating previously published data, we revealed that 74 overlapping lncRNAs were annotated in the UCSC database as showing upregulation with the progression of myogenesis (Figure S1). Notably, some of the upregulated lncRNAs have been reported to play important roles in skeletal myogenesis, such as H19 and 2310043L19Rik.12,18 Moreover, in the study by Zhou et al.,19 Gene Ontology (GO) function analysis showed that Mir22hg is involved in NADH oxidation in myoblasts and muscle contraction in myotubes (Figure S1); however, its function in skeletal myogenesis remained unclear.

Based on these results, we hypothesized that Mir22hg plays a crucial role in the differentiation of skeletal muscle cells. After 4 days of C2C12 differentiation, obviously fused myotubes were visible (Figure 1A), and the expression of differentiation markers myosin heavy chain (MyHC) (Figure 1B) and MyoD (Figure 1C) gradually increased during myogenesis. Consistent with our hypothesis, Mir22hg was also upregulated with increasing days of differentiation (Figure 1D), the trend for which was consistent with that of MyHC and MyoD, further confirming the previous RNA sequencing (RNA-seq) results.19 In addition, tissue expression profile showed that Mir22hg was highly expressed in skeletal muscle (Figure 1E). Furthermore, in the process of regeneration following skeletal muscle injury, MyHC expression first increased and subsequently decreased (Figure 1F). Notably, Mir22hg also displayed a similar expression pattern (Figure 1G), suggesting that Mir22hg might play an important role in the process of myogenesis and skeletal muscle regeneration.

Mir22hg promotes myoblast differentiation
To explore its functional roles, we constructed a vector that efficiently overexpressed Mir22hg (Figure 2A) and detected the effect of Mir22hg on the differentiation of skeletal muscle cells. Immunofluorescence revealed that Mir22hg overexpression induced differentiation on the third day, concomitant with significant increases in the number of MyHC (Figures 2B and 2C) and MyoD (Figures 2E and 2F)-positive cells, indicating that overexpression of Mir22hg promoted the formation of myotubes. Western blotting results confirmed that Mir22hg overexpression promoted the expression of MyHC protein (Figure 2D).

Moreover, we designed three short interfering (si)RNAs targeting Mir22hg and selected the one exhibiting the highest knockdown efficiency, siMir22hg-2, for subsequent experiments (Figure 2G). Conversely, the results showed that Mir22hg knockdown inhibited the expression of MyoD (Figure 2H) and MyHC (Figure 2I) at the mRNA level. Immunofluorescence results further demonstrated that knockdown of Mir22hg reduced MyHC expression (Figures 2J).
Figure 2. IncRNA Mir22hg enhances myoblast differentiation in vitro

(A) qRT-PCR analysis of Mir22hg expression in C2C12 cells transfected with control pcDNA3.1 or Mir22hg after 24 h in DM (n = 3). (B) Representative photographs of MyHC immunofluorescence staining in C2C12 cells differentiated for 4 days after overexpression of Mir22hg. (C) Quantitation of the MyHC-positive cells (n = 3). (D) Western blotting of MyHC in C2C12 cells differentiated for 3 days after overexpression of Mir22hg. (E) Representative photographs of MyoG immunofluorescence staining in C2C12 cells differentiated for 4 days after overexpression of Mir22hg. (F) Quantitation of the MyoG-positive cells (n = 3). (G) qRT-PCR analysis of Mir22hg knockdown in C2C12 cells transfected with control NC or three siMir22hg after 24 h (n = 3). (H and I) qRT-PCR analysis of mRNA expression of MyoD (H) and MyHC (I) in C2C12 cells transfected with negative control (NC) or siMir22hg and then cultured in DM for 2 days (n = 3). (J) Representative photographs of MyHC immunofluorescence staining in C2C12 cells differentiated for 4 days after transfection with control NC or siMir22hg. (K) Quantitation of the MyHC-positive cells (n = 3). (L) Western blotting of MyHC in C2C12 cells differentiated for 3 days after transfection with control NC or siMir22hg. Data are expressed as mean values ± SEM, and an unpaired two-tailed Student’s t test was used to analyze the statistical significance between two groups. *p < 0.05, **p < 0.01, ***p < 0.001.
and 2K), indicating that knockdown of Mir22hg inhibits the formation of myotubes. In addition, western blotting results revealed that Mir22hg knockdown significantly decreased MyHC protein levels (Figure 2L). Hence, Mir22hg serves as a critical positive regulator of myoblast differentiation.

**Mir22hg promotes myoblast differentiation via miR-22-3p**

To understand the role of Mir22hg in skeletal muscle cell differentiation, we performed bioinformatics analysis and found that Mir22hg was also highly conserved across many species, including humans and mice in addition to livestock animals (Figure S2), with the miR-22-3p-containing region of Mir22hg particularly highly conserved (Figure S3A). These results suggested that Mir22hg may exert its function by producing the conserved miR-22-3p. Furthermore, quantitative reverse transcription polymerase chain reaction (qRT-PCR) results showed that miR-22-3p levels gradually increased during differentiation (Figure S3B), and its expression was significantly positively correlated ($r = 0.732$, $p = 0.0019$) with that of Mir22hg during differentiation (Figure S3C). In turn, tissue expression profile indicated that miR-22-3p was also highly expressed in skeletal muscle and myocardium (Figure S3D), and its expression was significantly increased in C2C12 cells overexpressing Mir22hg (Figure S3E). Accordingly, we deduced that mature miR-22-3p might be derived from Mir22hg. To confirm this hypothesis, we investigated the effects of mutating the miR-22-3p seed sequence in the Mir22hg sequence on the expression of mature miR-22-3p. qRT-PCR results showed that miR-22-3p expression in the Mir22hg-mut group was significantly lower than that in the Mir22hg group, whereas no difference was observed compared with the control group (Figure S3F). Together, these results demonstrated that Mir22hg could yield mature miR-22-3p after shearing through post-transcriptional processing.

**HDAC4, a direct target of miR-22-3p, is crucial for the function of Mir22hg**

To explore the regulatory mechanism of Mir22hg, we first examined the cytoplasmic and nuclear distribution of Mir22hg in C2C12 cells. The results of the nucleocytoplasmic separation assay and RNA fluorescence in situ hybridization (RNA-FISH) showed that Mir22hg was highly expressed in the cytoplasm (Figures 3A and 3B). As Mir22hg produced miR-22-3p through processing in C2C12 cells, we proposed that Mir22hg might function by modulating miR-22-3p targets. Accordingly, we predicted the target genes of miR-22-3p, using three online tools: miRTarBase, TargetScan, and RNA22. As shown in the Venn diagram, seven overlapping target genes were predicted, including ERBB3, HDAC4, MECP2, DDIT4, KLF6, VSNL1, and SCAMP1 (Figure 3C). As HDAC4 is reported to inhibit myogenic differentiation and to promote C2C12 cell proliferation,20 we subsequently focused our attention on the HDAC4 gene. To verify whether HDAC4 was affected by miR-22-3p, we constructed wild-type (HDAC4-WT) and mutant (HDAC4-Mut) luciferase reporter vectors (Figure 3D) and performed dual-luciferase reporter assays. Results showed that the relative luciferase activity was significantly decreased after co-transfection of HDAC4-WT and miR-22-3p in HEK293T cells (Figure 3E). Furthermore, immunofluorescence results revealed that overexpression of miR-22-3p significantly inhibited the expression of HDAC4 (Figures 3F and 3G). These findings were supported by the results of western blotting (Figure 3H).

To further confirm the inhibitory effect of Mir22hg on HDAC4 by generating miR-22-3p, we performed a co-transfection experiment. Immunofluorescence results showed that the expression of HDAC4 was significantly decreased in the miR-22-3p overexpression group (pcDNA3.1 + mimics miR-22-3p) compared with that in the control group (pcDNA3.1 + mimics negative control (NC); Figures 4A and 4B). Similarly, the expression of HDAC4 was significantly decreased in the Mir22hg overexpression group (Mir22hg + mimics NC) compared with that in the control group (pcDNA3.1 + mimics NC), whereas the expression level of HDAC4 could be rescued in Mir22hg-overexpressing cells through the addition of a miR-22-3p inhibitor (Figures 4A and 4B). Moreover, western blotting results demonstrated that co-transfection of Mir22hg and the miR-22-3p inhibitor rescued the expression of HDAC4 (Figure 4C). Collectively, these results indicated that HDAC4 is a direct target of miR-22-3p and that Mir22hg inhibits HDAC4 expression through the generation of miR-22-3p.

**HDAC4 is a negative regulator of myoblast differentiation**

To confirm the role of HDAC4 in myoblast differentiation, we designed three siRNAs and selected siRNA-3, which exhibited the highest knockdown efficiency, for subsequent experiments (Figure S4A). Knocking down HDAC4 significantly increased expression of the downstream transcription factor MEF2C, which is known to promote myoblast differentiation.21 In addition, immunofluorescence results showed that knockdown of HDAC4 significantly increased the rate of MyHC (Figures S4B and S4C)- and MyoG (Figures S4D and S4E)-positive cells. These results indicated that HDAC4 serves as a negative regulator of myoblast differentiation.

**Mir22hg targets HDAC4 through miR-22-3p to regulate myoblast differentiation**

To demonstrate the promoting effect of Mir22hg on myoblast differentiation by generating miR-22-3p to inhibit HDAC4 expression, we carried out a co-transfection experiment. Overexpression of miR-22-3p or Mir22hg promoted myotube formation (Figure 5A). Moreover, Mir22hg overexpression significantly increased the proportion of MyHC (Figures 5B and 5C)- and MyoG (Figures 5D and 5E)-positive cells. Compared with the group transfected with Mir22hg alone, the rate of MyHC- and MyoG-positive cells was significantly decreased in the group co-transfected with Mir22hg and the miR-22-3p inhibitor (Figures 5B–5E). Similarly, western blotting results revealed that transfection with Mir22hg or miR-22-3p increased MyHC and MyoG protein levels (Figure 5F) compared with those of the control group. Meanwhile, the protein levels of MyHC and MyoG significantly decreased in the group co-transfected with Mir22hg and the miR-22-3p inhibitor compared with those in the group transfected with Mir22hg alone, which was consistent with the immunofluorescence results.

Furthermore, we induced mutations in the miR-22-3p seed sequence of Mir22hg and performed transfection experiments in C2C12 cells.
Western blotting results showed that, compared with the Mir22hg-WT group, the Mir22hg-mut group exhibited significantly increased HDAC4 protein expression, while decreasing that of MEF2C (Figure S5A). In addition, Mir22hg-mut failed to promote the expression of MyHC (Figures S5B and S5C) or MyoG (Figures S5D and S5E). In summary, our data showed that Mir22hg regulated C2C12 differentiation by producing miR-22-3p to inhibit HDAC4.

Knockdown of Mir22hg delays regeneration following skeletal muscle injury

After skeletal muscle injury, quiescent skeletal muscle satellite cells are activated and migrate to the injury sites to rapidly proliferate and differentiate to participate in skeletal muscle repair and regeneration. As our results confirmed that Mir22hg could promote myoblast differentiation, we speculated that Mir22hg might also be involved in the regeneration of injured skeletal muscle. To confirm this speculation, we generated a Mir22hg-knockdown short hairpin (sh)RNA lentivirus and performed a lentiviral injection experiment in vivo. The experimental design, including times of lentiviral and 1.2% BaCl2 (causing muscle injury) injection and sample collection time, are shown in Figure 7A. After 2 days, the evaluation of Mir22hg knockdown efficiency in the TA muscles revealed that its expression was reduced by >60% (Figures 6B and 6C). Next, we carried out regeneration experiments after skeletal muscle injury. Hematoxylin and eosin (H&E) staining results showed that the rate of skeletal muscle repair was slower in the Mir22hg knockdown group compared with the control group (Figure 6D). Moreover, after 9 days, many of the nuclei remained in the center of the muscle fibers in the Mir22hg knockdown group, whereas in the control group most of the muscle fiber nuclei had migrated to the edge of muscle fibers, as is characteristic of mature fibers. These results indicated that Mir22hg knockdown inhibited the regeneration of injured skeletal muscle. In addition, in the Mir22hg knockdown group tibialis anterior (TA) muscle weight was significantly lower than that of the control group at day 12 (Figures 6E and 6F).
and 6F). Together, our data demonstrated that Mir22hg knockdown caused muscle weight loss consequent to the delayed regeneration of injured skeletal muscle.

Additionally, qRT-PCR results showed that the mRNA expression of MyHC and skeletal muscle α-actin was significantly lower in the Mir22hg knockdown group compared with the control group on days 1 and 3 during the repair process of injured TA muscle (Figures 7A and 7B). Similarly, MyHC and MyoG protein levels were significantly lower in the Mir22hg knockdown group than those in the control group on days 3, 5, 7, and 9 (Figure 7C). Conversely, HDAC4 protein in the Mir22hg knockdown group exhibited higher expression than that in the control group on days 7 and 9. In addition, we observed that Mir22hg expression was significantly positively correlated with the ratio of TA muscle to body weight ($r = 0.5055, p = 0.0084$; Figure 7D). Moreover, H&E staining results showed that the cross-sectional area of muscle fibers of the injured TA muscle was significantly lower in the Mir22hg group than in the control group (Figures 7E and 7F). These results indicated that Mir22hg knockdown in the TA muscle decreased the regenerative capacity of skeletal muscle fiber, thereby delaying the repair of injured muscle and ultimately leading to skeletal muscle weight loss and reduction of total skeletal muscle fiber area.

**DISCUSSION**

lncRNAs are characterized by fewer exons, are less evolutionarily conserved, and have lower expressed abundance but higher cell and tissue specificity than mRNA. In this study, we screened Mir22hg as highly expressed in skeletal muscle based on previously published data sets and demonstrated its ability to promote skeletal muscle differentiation and regeneration. Notably, the functional roles of Mir22hg were achieved through the generation of mature miR-22-3p to inhibit HDAC4, thereby enhancing the expression of MEF2C, a critical transcription factor for promoting myoblast differentiation.

Among the diverse regulatory mechanisms, transcriptional activation and molecular sponges represent the most common regulatory mechanisms in skeletal myogenesis. For example, some lncRNAs that are highly expressed in the cytoplasm primarily function as ceRNAs by acting as a molecular sponge to absorb miRNA, such as Inc-mg1 and LncIRS1. Other lncRNAs that are highly expressed in the nucleus, such as SYISL and Linc-YY1, execute their functions by interacting with transcription factors to activate or inhibit downstream target genes, thereby regulating the proliferation and differentiation of skeletal muscle. In the present study, we found that Mir22hg functions through the production of miR-22-3p, which then activates downstream pathways to promote skeletal muscle differentiation and regeneration. Indeed, a similar mechanism has been discovered in the skeletal muscle regulation of lncRNA H19, whose exon1 produces two conserved miRNAs, miR-675-3p and miR-675-5p. We therefore further explored the precise regulatory mechanisms of Mir22hg in skeletal myogenesis.

We initially observed that the expression of Mir22hg was significantly increased during myoblast differentiation, whereas Mir22hg expression first increased and then decreased during the repair and regeneration of injured skeletal muscle, which is consistent with the change in MyHC expression in this process. These results suggest that Mir22hg may play an important role in regulating skeletal myogenesis in vivo and in vitro. Subsequently, we verified the functional roles of Mir22hg in promoting the differentiation of skeletal muscle cells through both overexpression and knockdown technologies. Notably, we observed that the mature miR-22-3p contained in the Mir22hg region is highly evolutionarily conserved across species. Moreover, miR-22-3p expression was markedly upregulated and significantly positively correlated with the expression of Mir22hg during myoblast differentiation and was also highly expressed in skeletal muscle. Our results further revealed that overexpression of Mir22hg significantly increased the expression of miR-22-3p, whereas mutating the seed
sequence of miR-22-3p in Mir22hg completely abolished miR-22-3p expression. Thus, these results indicate that Mir22hg executes its function of promoting skeletal muscle differentiation by generating mature miR-22-3p.

Subsequently, we predicted seven potential co-regulated target genes of miR-22-3p, using multiple bioinformatics tools. Among the encoded proteins, we focused our attention on HDAC4, a member of the HDAC family, because HDAC4 is critical for skeletal muscle differentiation and regeneration and regulates muscle-related diseases.\(^29,30\) Next, we used a dual-luciferase reporter vector assay to demonstrate the targeting relationship between miR-22-3p and HDAC4. Moreover, overexpression of miR-22-3p or Mir22hg significantly inhibited the expression of HDAC4 \textit{in vitro}. Further, knockdown of HDAC4 promoted the differentiation of myoblasts by upregulating the expression of MEF2C, which is consistent with previous studies.\(^20,31\) Conversely, myogenic differentiation was markedly inhibited when a miR-22-3p inhibitor was added to Mir22hg-overexpressing skeletal muscle cells. Recently, lncMGPF has been reported to regulate myogenesis by the MEF2C pathway.\(^32\) Interestingly, lncMGPF also functions as a miRNA sponge of miR-135a-5p, weakening the inhibitory effects of miR-135a-5p on MEF2C and thereby increasing expression of the MEF2C. Both lncMGPF and Mir22hg are highly expressed in skeletal muscle and regulate myogenesis through the MEF2C pathway. Therefore, it will be interesting to explore the potential synergistic regulation of lncMGPF and Mir22hg in myogenesis in future study.

To further validate the function of Mir22hg during myogenesis, we constructed a murine skeletal muscle injury model and performed a lentiviral injection experiment to inhibit Mir22hg expression to observe the effect on injured skeletal muscle repair and regeneration. As expected, during the repair period following skeletal muscle injury, Mir22hg knockdown delayed skeletal muscle regeneration and significantly reduced TA muscle weight. Moreover, we observed that the gene expressions of differentiation-related factors (MyHC, skeletal muscle \(\alpha\)-Actin) were significantly inhibited. In addition, the mean cross-sectional area of muscle fibers after regeneration was reduced.
and the expression of Mir22hg in the TA muscle was significantly positively correlated with the ratio of TA muscle to body weight. We speculated that satellite cell differentiation was partially inhibited after Mir22hg knockdown, leading to the slower formation of muscle fibers, thereby affecting regeneration after skeletal muscle injury. These results were similar to the regulatory mechanism by which IncRNAs H19 and MAR1 regulate skeletal muscle regeneration. Therefore, our findings suggest that Mir22hg may constitute a new target for the treatment of skeletal muscle injury.

In summary, we identified a new IncRNA, Mir22hg, that is highly expressed in skeletal muscle and could give rise to mature miR-22-3p to inhibit HDAC4 expression, thereby activating the downstream MEF2C pathway to promote skeletal muscle differentiation and regeneration (Figure 8). Our findings suggest that Mir22hg may be useful as a new therapeutic target for the treatment of skeletal muscle injury, skeletal muscle atrophy, and other muscular diseases.

MATERIALS AND METHODS

Pipeline for the discovery of candidate IncRNAs affecting skeletal muscle differentiation

To screen the critical candidate IncRNAs affecting skeletal muscle cell differentiation, we first downloaded IncRNA microarray data from various differentiation stages of mouse C2C12 from the study by
Jin et al.\textsuperscript{25} and conducted a comparative analysis with the lncRNAs identified in the C2C12 (mouse myoblast) differentiation process annotated in the UCSC database (https://genome.ucsc.edu/). Among the overlapping lncRNAs, 74 upregulated lncRNAs were annotated in the UCSC database. As the functions of one of these lncRNAs, Mir22hg, had also been predicted in myoblasts and myotubes in the study by Zhou et al.,\textsuperscript{19} it was selected as a promising candidate lncRNA in skeletal myogenesis.

Animals

BALB/c mice were purchased from Qinglongshan Laboratory Animal Company (Nanjing, China). Mice were housed in specific pathogen-free rooms and kept under controlled conditions of temperature (20\textdegree C–23\textdegree C) and illumination (12 h light-dark cycle) and had free access to food and water throughout the study. All mice were handled following the Animal Research Institute Committee guidelines of Nanjing Agricultural University, China.

Cell culture and differentiation

Mouse C2C12 cells and human embryonic kidney HEK293T cells were cultured in high-glucose Dulbecco’s modified Eagle’s medium (DMEM; HyClone, Logan, UT, USA) supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin-streptomycin (Gibco, Grand Island, NY, USA) at a constant temperature of 37\textdegree C in 5% (v/v) CO\textsubscript{2}. For the induction of C2C12 cell differentiation, C2C12 cells were induced with high-glucose DMEM supplemented with 2% horse serum and 1% penicillin-streptomycin.

Extraction of total RNA and qRT-PCR

Total RNA was extracted from mouse tissues and C2C12 cells with TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). The concentration and integrity of RNA were assessed with NanoDrop 2000 (Thermo Fisher Scientific). cDNA for mRNA and miRNA was synthesized with the PrimeScript RT Master Mix (Perfect Real Time, Takara) and Mix-X miRNA First-Strand Synthesis Kit (Takara), respectively. mRNA and miRNA detection was performed by qRT-PCR with AceQ qPCR SYBR Green Master Mix (Vazyme, Nanjing, China) on a Step-One Plus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The primers for quantitative analyses are shown in Table S1. The relative expression levels were calculated with the 2\textsuperscript{−ΔΔCT} method,\textsuperscript{36} and mouse GAPDH, U6 snRNA, and Hprt were used for normalization of gene expression levels as endogenous reference genes.

Cytoplasmic and nuclear lncRNA Mir22hg

Cytoplasmic and nuclear RNAs were extracted from C2C12 cells with protocols described previously.\textsuperscript{37} In brief, the cells were washed with cold phosphate-buffered saline (PBS), lysed in cell lysis buffer from a Magna ChIP A/G Chromatin Immunoprecipitation Kit (Sigma-Aldrich), and centrifuged twice at 4\textdegree C. The supernatant was taken as the cytoplasmic fraction, and the bottom of the precipitate was collected as the nuclear fraction. RNA from the cytoplasmic and nuclear fractions were extracted with TRIzol reagent. The relative expression levels of Mir22hg, Metastasis associated lung adenocarcinoma transcript 1 (Malat1), and Gapdh in both cytoplasmic and nuclear fractions were determined using qRT-PCR.

Figure 7. Knockdown of lncRNA Mir22hg decreases the expression of post-injury skeletal muscle regeneration-related genes

(A and B) qRT-PCR analysis of the mRNA expression level of (A) MyHC and (B) skeletal muscle α-Actin after Mir22hg knockdown in the TA muscle (n = 3). (C) Western blotting of HDAC4, MyHC, and MyoG protein expression after Mir22hg knockdown in the TA muscle. (D) Correlation analysis between the weight of TA muscle (right leg) and Mir22hg expression (n = 28). (E) H&E staining of the muscle fiber area of TA muscle. Control lentivirus was injected into the left leg of mice and Mir22hg knockdown lentivirus was injected into the right leg, and the regenerated TA muscles at 12 days after skeletal muscle injury were collected for H&E staining. (F) Quantitation of cross-sectional area of muscle fiber (n = 3). Quantification was conducted with ImageJ Pro Plus. Data are expressed as mean values ± SEM, and a paired two-tailed Student’s t test was used to analyze the statistical significance between two groups. *p < 0.05.
nuclear fractions were determined by qRT-PCR. Malat1\(^{28}\) was used as a reference for high expression of lncRNA in the nucleus, and GAPDH was used as a reference for high expression in the cytoplasm.

**RNA fluorescence in situ hybridization**

FISH was performed with the lncRNA FISH Kit (Guangzhou RiboBio, Guangzhou, China) according to the manufacturer’s instructions. Briefly, cells were fixed with 4% formaldehyde for 10 min at room temperature. After being washed three times with PBS, cells were permeabilized with 0.5% Triton X-100 for 10 min at 4°C. Then, cells were incubated with RNA probes in hybridization buffer overnight at 37°C. The RNA probes were directly conjugated with a fluorophore. The cells were then washed three times with saline sodium citrate buffer and stained with DAPI. Images were captured by confocal microscopy (LSM700META; Zeiss, Oberkochen, Germany).

**Mut seed sequences of miR-22-3p in the Mir22hg**

WT and Mut seed sequences of miR-22-3p in the Mir22hg gene were ligated into pcDNA3.1. qRT-PCR analysis of miR-22-3p expression levels in C2C12 cells was performed 24 h after transfection with Mir22hg-mut and Mir22hg-WT overexpressing vectors in growth medium.
(dilution 1:2,000; Cell Signaling Technology, Danvers, MA, USA). Horseradish peroxidase-labeled anti-rabbit/goat IgG secondary antibody (dilution 1:5,000; Cell Signaling Technology) was used to detect protein expression. Targeted proteins were imaged on a Tanon 5200 instrument (Tanon, Shanghai, China) and analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA). Western blot bands were quantified with ImageJ.29

**Immunofluorescence staining**

After transfection, C2C12 cells were cultured in a 12-well plate with differentiation medium (DM) for 3–4 days. The cells were then washed three times with PBS and fixed for 30 min in 4% paraformaldehyde, followed by three washes with PBS. The cells were subsequently incubated in ice-cold 0.5% Triton X-100 at 4°C for 15 min and washed a further three times. Next, the cells were incubated in blocking solution (1% bovine serum albumin) at room temperature (25°C) for 1 h. The cells were then incubated with anti-MyHC antibody (dilution 1:50; Developmental Studies Hybridoma Bank) and anti-MyoG antibody (1:100; ABClonal, Wuhan, China) at 4°C for 16 h. The cells were then washed three times and incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) antibody (dilution 1:100; ZSGB-BIO, Beijing, China) and rhodamine [tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG (H+L) antibody (dilution 1:100; ZSGB-BIO, Beijing, China) in the dark. After 1 h of incubation at room temperature, the cells were washed three times. The cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) in the dark. After being washed three times, the glass slides were sealed with fluorescence anti-quenching agents (Biosharp, Hefei, China). Images were captured by confocal microscopy (LSM700META; Zeiss, Oberkochen, Germany). Quantification of immunofluorescence intensities was conducted with ImageJ.40

**Lentiviral vector construction and lentivirus production**

To generate Mir22hg-knockdown lentiviral vector, shRNA targeting Mir22hg or NC scramble sequences were subcloned into the GV112 vector (Shanghai GeneChem). The shRNA sequence (shMir22hg: 5′-GGGATAGTACAAATTGTGAA-3′) was synthesized by Shanghai GeneChem. For production of the lentivirus, the expression vectors were co-transfected with packaging plasmid pHelper 1.0 vector (Shanghai GeneChem) and envelope plasmid pHelper 2.0 vector (Shanghai GeneChem) into HEK293T cells with the GeneChem transfection reagent (Shanghai GeneChem). The supernatant was collected 48 and 72 h after transfection, concentrated by ultracentrifugation at 25,000 rpm for 90 min, and resuspended in an appropriate volume of Opti-MEM. The lentivirus was concentrated by ultracentrifugation at 25,000 rpm for 90 min and stored at −80°C.

**In vivo lentiviral particle administration**

For the lentiviral particle administration experiment, we used sixty 8-week-old male mice. Mice were sacrificed by cervical dislocation, and all efforts were made to minimize animals’ discomfort. Lentiviral particles for Mir22hg knockdown were injected into the mid-portion of one side of the TA muscles of each mouse at a dosage of 1.0 × 10⁸ TU/mL. A total of 10 μL of viral preparation was injected into each muscle. Control lentivirus was injected into the left leg, and Mir22hg-knockdown lentivirus was injected into the right leg. At 2 days after lentiviral injection 1.2% BaCl₂ was injected, and the TA muscles were collected on days 0, 1, 3, 5, 7, 9, and 12 for H&E staining, qRT-PCR, and western blotting analysis. Moreover, the TA muscles collected at 12 days were weighed. H&E staining of TA muscles was performed according to a previous report.31 The mean muscle fiber cross-sectional area was quantified with ImageJ Pro Plus according to a previous study protocol.42,43 Mir22hg levels and mRNA levels of myogenic markers (MyHC, α-Actin) were detected by qRT-PCR. The protein levels of HDAC4 and myogenic markers (MyHC, MyoG) were evaluated by western blotting analysis.

**Statistical analysis**

Statistical analysis was performed with Prism 7 software (GraphPad Software, San Diego, CA, USA). Pearson correlation coefficients between the expression levels of Mir22hg and miR-22-3p and between the ratio of the weight of the right leg TA muscle of 28 adult male mice (8 weeks old) and the expression of Mir22hg in the TA muscle were calculated. A two-tailed Student’s t test was used to analyze the statistical significance between two groups. Violin plots were drawn with R version 3.5.3. Data are expressed as mean values ± SEM unless otherwise noted, and the level of significance was set at p < 0.05.

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2021.02.025.

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**AUTHOR CONTRIBUTIONS**

H.L., W.W., and R.L. conceived and designed the experiments; R.L. and W.W. drafted the manuscript, and H.L., W.W., and R.L. revised the manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing interest.

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