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Role of miRNAs and lncRNAs in dexamethasone-induced myotube atrophy *in vitro*

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Abstract. Skeletal muscle atrophy is a well-known adverse effect of long-term glucocorticoid (GC) therapy. MicroRNAs (miRNAs or miRs) and long non-coding RNAs (lncRNAs) are important regulators in a number of physiological and pathological processes. However, the role of miRNAs and lncRNAs in the regulation of GC-treated muscle atrophy remains poorly understood. In the current study, muscular atrophy was induced and the results indicated that C2C12 myotubes were thinner than normal, while the expression of muscle ring finger protein 1 and Atrogin-1 was increased. The expression of nine miRNAs and seven lncRNAs associated with proliferation and differentiation were analyzed in a dexamethasone (DEX)-induced muscle atrophy cell model. In addition, the mRNA expression of the downstream targets of lncRNAs that were differentially expressed between DEX-treated and control cells were determined. The results indicated that the expression of miR-133a, miR-133b, miR-206 and five lncRNAs (increased Atrolnc-1, Dum, MAR1, linc-MD1 and decreased Myolinc) were significantly different between the DEX and the control group. Furthermore, the relative mRNA expression of Wnt5a and MyoD was significantly different between the two groups. The results of the current study indicated that some important miRNAs and lncRNAs are associated with DEX-induced muscle atrophy and have the potential to be further developed as a diagnostic tool for this condition.

Introduction

Glucocorticoids (GCs), which have anti-inflammatory and immune-suppressing properties, are commonly used in the treatment of a number of adult dyspnea syndromes (such as atypical pneumonia), asthma, autoimmune diseases and cardiopulmonary-related inflammation (1,2). However, high doses or prolonged use of GCs can cause many side effects (3-6). For muscle tissue, the main side effect caused by GCs is steroid myopathy, which is characterized by muscle weakness and atrophy (1,7,8). Glucocorticoid-induced skeletal muscle atrophy (GIMA) increases the disease risk for patients, leading to weakened immunity, increased infection rates, limited mobility, fracture and even paralysis (9,10). Severe systemic muscle atrophy can lead to swallowing disorders, breathing difficulty and other secondary life-threatening complications (5,11). The diseases induced by skeletal muscle atrophy seriously affect the quality of life and prognosis of patients. At present, the pathogenesis of GIMA is not fully understood, but some researchers believe that inhibition of protein anabolism or stimulation of protein catabolism is responsible for skeletal muscle atrophy (12,13), GCs can downregulate myogenic regulatory factors, thereby inhibiting proliferation and differentiation processes (1,10). GCs can activate skeletal muscle proteolysis through mechanisms such as the muscle-specific ubiquitin proteasome system, lysosomal system and calcium-dependent protease system (1). In addition, GCs induce muscular atrophy by affecting the production of growth factors such as insulin-like growth factor (10). Therefore, researchers are also aiming to explore the possibility of treating GIMA by blocking the mechanism of GC-induced muscular atrophy. For example, Bodine *et al* (14) demonstrated that the IGF1/PI3K/Akt pathway is sufficient to induce Myotube hypertrophy by activating the protein synthesis pathway. Stitt *et al* (15) indicated that inhibiting the activation of the muscular atrophy pathway can inhibit the upregulation of muscle RING finger protein 1 (MuRF1) and muscle atrophy F-box (MAFbx) induced by glucocorticoid. Despite recent significant advances in the understanding of the mechanism of GIMA pathogenesis, the mechanism by which GCs are expressed through atrophy-related genes, particularly long non-coding RNAs (lncRNAs), has not been fully described.

MicroRNAs (miRNAs) and lncRNAs are both non-coding RNAs; however, more attention has been paid to the role of miRNAs in skeletal muscle development (11,16-19). miRNAs are a class of small, non-coding RNAs that are ~22 nucleotides in length. The mechanism of miRNA regulation of

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muscle atrophy mainly involves muscle protein metabolism, muscle regeneration, angiogenesis and muscle cell apoptosis (20). miRNAs that are expressed specifically in muscles can alter the diseases that affect muscles (17,21-25). For example, in dexamethasone (DEX)-mediated muscular atrophy, muscle-specific miR-1 is induced and heat shock protein 70 (HSP70) levels are reduced (26). In addition, miR-23a is inhibited in diabetes and DEX-induced muscular atrophy (27). LncRNAs are a novel class of non-coding RNAs that are >200 nucleotides in length (28,29). Muscle-specific lncRNAs are important regulators of muscle proliferation, differentiation and atrophy (26,28-30). Long intergenic non-protein coding RNA muscle differentiation 1 (linc-MD1) regulates the expression of mastermind-like transcriptional coactivator 1 and myocyte enhancer factor 2C during muscle differentiation (31). The lncRNA muscle anabolic regulator 1 (MAR1) acts as a miR-487b sponge to regulate Wnt5a protein, resulting in muscle differentiation and regeneration (12). Given that aberrant gene expression underlies muscle atrophy, it is important to understand how lncRNAs regulate gene expression in response to diverse stresses or diseases that contribute to muscle atrophy (10,28,32).

A growing number of studies have demonstrated that the genetic hierarchies and transcriptional networks involved in myogenesis include lncRNA molecules (31,33-36). However, few studies have provided a comprehensive perspective on the regulation of miRNAs and lncRNAs in GC-induced skeletal muscle atrophy. Therefore, the aim of the current study was to investigate the expression of regulatory miRNAs and lncRNAs in GC-induced muscular atrophy within C2C12 cells. DEX is the most effective synthetic GC and conferred anti-inflammatory GC activity compared to natural cortisol and corticosterone (37). DEX has the potential to promote protein degradation and is considered an effective drug to induce muscle atrophy *in vivo* and *in vitro* (38-40). In the present study, the expression of selected miRNAs and lncRNAs that exhibited expression profiles similar to those previously reported (1 6,18,19,26,30,31,36), between control and DEX-treated C2C12 cells was investigated, and miRNAs and lncRNAs that were differentially expressed between normal (control) and atrophy conditions were identified. The results from the present study may provide candidate miRNAs and lncRNAs that may lead to a better understanding of the molecular pathways by which GCs regulate skeletal muscular atrophy.

Materials and methods

Cell culture. The mouse myoblast C2C12 cell line was purchased from the Stem Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences and cultured at 37˚C in 5% CO_2 and high glucose DMEM (cat. no. 12100046; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% (v/v) FBS (cat. no. SH30070.03; HyClone; Cytiva). For myotube differentiation, C2C12 myoblasts were incubated at 37˚C in 12-well plates with 2% horse serum (cat. no. SH30074.03; HyClone; Cytiva) for 72 h, according to previous studies (8,41).

DEX-induced muscle atrophy cell model. C2C12 cells were cultured to 70-80% confluence with high glucose DMEM (cat. no. 12100046; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% (v/v) FBS (cat. no. SH30070.03; HyClone; Cytiva), before being digested with trypsin. In total, $5x10^4$ cells were then seeded into the a 12-well plate, until they reached 80-90% confluence, following which they were differentiated by incubation in high glucose DMEM containing 2% horse serum (cat. no. SH30074.03; HyClone; Cytiva) (28). DEX-induced atrophy was performed by treating cells on the 3rd day of differentiation with 50, 100 and 200 μM DEX (Sigma-Aldrich; Merck KGaA) dissolved in ethanol for 12, 24 and 48 h. Control cells were incubated with 0.03% (v/v) ethanol (control) for 48 h. The cell medium was exchanged every 24 h (42). All incubations were performed at 37˚C.

RNA extraction and reverse-transcription quantitative (RT-q) PCR. DEX-treated C2C12 and control cell samples were washed with phosphate-buffered saline before lysis in TRIzol® reagent (cat. no. 15596018; Thermo Fisher Scientific, Inc.), and total RNA was extracted according to the manufacturer's instructions. Total RNA was reverse transcribed into complementary DNA (cDNA) at 37˚C for 15 min and 85˚C for 5 sec using PrimeScript™ RT Master mix (cat. no. RR 036A; Takara Bio, Inc.) for mRNA and lncRNA detection. At the same time, total RNA was reverse transcribed into cDNA using the Mir X™ miRNA First Strand Synthesis kit (cat. no. 638313; Takara Bio, Inc.) to detect miRNA. All the RNAs (mRNA, lncRNAs and miRNAs) were measured using TB Green™ Premix Ex Taq™ II (cat. no. RR820A; Takara Bio, Inc.). The PCR reaction was completed on a StepOnePlus Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the following program: Denaturation at 95˚C for 30 sec, followed by 40 cycles of denaturation at 95˚C for 5 sec, annealing at 55˚C for 30 sec and extension at 72˚C for 30 sec. The PCR for detecting miRNAs was performed as follows: Denaturation at 95˚C for 10 sec, followed by 40 cycles of denaturation at 95˚C for 5 sec, and annealing and extension at 60˚C for 30 sec. The $2^{-\Delta\Delta Cq}$ method was used to calculate the relative fold change among biological groups using 18S as an internal normalizer for mRNA and lncRNA, and U6 as an internal normalizer for miRNA (43). The list of primers and sequences is provided in Tables I and II.

Myotube area measurements. Myotube area was quantified by analyzing the area of myotubes covering the culture area. Images were acquired at a magnification of x400 using an optical electron microscope (Thermo Fisher Scientific, Inc.). The myotube area was measured using ImageJ (v1.44P; National Institutes of Health) software from randomly selected areas of the myotubes in the control group from three different wells and three different DEX treatments. A total of 40 myotubes were measured in each well.

Western blot analysis. Western blot analysis was performed to determine protein levels of MURF1 and Atrogin-1 in myotubes. The C2C12 cells were lysed in RIPA buffer containing protease inhibitor (Beyotime Institute of Biotechnology) and phenylmethylsulfonyl fluoride to extract total proteins. A total of 20 μg of protein, measured using the bicinchoninic acid Protein Assay Kit (cat. no. P0010S; Beyotime Institute of Biotechnology) was separated using

Table I. Primers for miRNAs.

miRNA	Sequence (5'-3')
miR-133a	TTTGGTCCCCTTCAACCAGC
miR-133b	GGTCCCCTTCAACCAGCTA
miR-206-3p	GGAATGTAAGGAAGTGTGTGG
miR-18a	GCCATCTAGTGCAGATAGAAAA
miR-186	GAATTCTCCTTTTGGGCTAAAA
miR-1a-3p	TGGAATGTAAAGAAGUATGTAT
miR-23a-3p	ATCACATTGCCAGGGATTTCC
miR-27b	TTCACAGTGGCTAAGTTCTGC
miR-29b-3p	TAGCACCATTTGAAATCAGTGTT
U6 forward	GGAACGATACAGAGAAGATTAGC
U6 reverse	TGGAACGCTTCACGAATTTGCG

miRNA/miR, microRNA.

10-12% SDS-PAGE. The protein was transferred to a PVDF membrane and blocked by 5% skimmed milk powder at room temperature for 1 h. MURF1 (1:1,000; cat. no. ab77577; Abcam), Atrogin-1 (1:2,000; cat. no. ab168372; Abcam) or tubulin-targeted primary antibody (1:5,000; cat. no. AC021; ABclonal Biotech Co., Ltd.) was added and incubated overnight at 4˚C. After washing the membrane five times, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody Goat Anti-Rabbit IgG (1:10,000; cat. no. as014; ABclonal Biotech, Co., Ltd.) at room temperature for 1 h. After washing the membrane five times, Immobilon western chemilum HRP substrate (cat. no. WBKlS0100; EMD Millipore) by 5% non-fat powdered milk was used for detection, and a Tanon series automatic chemiluminescence imaging analysis system (Tanon-V8 Pro; Tanon Sciences and Technology Co., Ltd.) was used for scanning the image. Tubulin was used as the internal control, and the relative expression of the target protein band was compared with the internal control.

Statistical analysis. GraphPad Prism 5.0 (GraphPad Software, Inc.) was used to perform statistical analyses. The experimental data were represented by the mean ± SD from triplicate data, and the Student's t-test was applied for the comparison of two groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Establishment of the DEX-induced muscle atrophy cell model. To establish an appropriate *in vitro* model, preliminary experiments were conducted by treating cells on day 3 of differentiation with 50, 100 and 200 μM DEX dissolved in ethanol for 12, 24 and 48 h. Under the treatment conditions aforementioned, compared with other doses and time points, the mean area of C2C12 myotubes and the protein expression levels of MURF1 and Atrogin-1 in C2C12 after treatment with 100 μM DEX for 48 h were significantly different, with the mean area of C2C12 myotubes reduced decreased and the expression of MURF1 and Atrogin-1 increased compared with those in control. Therefore, in the present study, the concentration of 100 μM DEX and the time point of 48 h were used to construct the muscular atrophy model (data not shown). Cells were treated on the 3rd day of differentiation with 100 μM DEX dissolved in ethanol for 48 h to construct the muscular atrophy model. The results of optical microscopy images indicated that the average area of C2C12 myotubes in control group was higher compared with the DEX group (Fig. 1A-C). RT-qPCR and western blot analysis revealed that the transcription and protein expression of MURF1 and Atrogin-1 in DEX-treated myotubes were increased (Fig. 1D and E), suggesting that GC-induced muscle atrophy of C2C12 cells was successfully established.

miRNA expression patterns in atrophic C2C12 cells. RT-qPCR was used to determine the expression of multiple miRNAs that have previously been revealed to be associated with muscle development in DEX-treated and control C2C12 cells. miRNAs that were previously reported in the literature were identified and nine miRNAs [miR-133a (19), miR-133b (18,44), miR-206 (45), miR-18a (46), miR-186 (47), miR-1a-3p (21,48), miR-23a-3p (27,49), miR-27b (50), miR-29b-3p (16)] were selected for examination in the present study, which exhibited expression similar to that previously reported. The results indicated that the expression of miR-133a, miR-133b and miR-206 was significantly increased in the DEX-treated group compared with the control group (Fig. 2A-C), while the abundance of the other six miRNAs (miR-1a-3p, miR-186, miR-18a, miR-23a-3p, miR-27b and miR-29b-3p) showed no significant change between DEX-treated group and the control group (Fig. 2C-I).

LncRNA expression patterns in atrophic C2C12 cells. RT-qPCR was used to detect the expression of multiple lncRNAs related to muscle development and determine if the expression differed between DEX-treated and control C2C12 cells. The results revealed that the expression of four lncRNAs (Atrolnc-1, Dum, MAR1 and linc-MD1) significantly increased in the DEX-treated group compared with the control group (Fig. 3A-D). However, the expression of Myolinc in the DEX group was significantly decreased (Fig. 3E). LncMyoD and lnc-mg showed no statistical difference between the two groups (Fig. 3F, G).

mRNA expression levels of the downstream targets of differentially expressed lncRNAs. To further determine whether the differentially expressed lncRNAs identified are involved in the regulation of DEX-induced muscular atrophy, PCR was performed to determine the mRNA expression of the downstream targets of Atrolnc-1, Dum, MAR1, linc-MD1 and Myolinc. The relative mRNA expression levels of Wnt5a (Fig. 4D) and MyoD (Fig. 4E), which are downstream targets of MAR1 and Myolinc, respectively, were significantly higher in the DEX group compared with the control group. However, the expression levels of Dppa2, MAML1, Mef2c and Acta1 were not significantly different between the two groups (Fig. 4A-C and F).

Discussion

In clinical practice, the cause of secondary muscular atrophy due to hormone use is often unclear to doctors and patients.

Table II. Primers for mRNAs and lncRNAs.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Atrogin-1	GAGTGGCATCGCCCAAAAGA	TCTGGAGAAGTTCCCGTATAAGT
MURF1	GTGTGAGGTGCCTACTTGCTC	GCTCAGTCTTCTGTCCTTGGA
Atrolnc-1	CAGCTGCCTACACCTGAAGA	AGGGCTCGCAGATTACACC
Dum	CACAAAGACAGGCAGACAGAC	TACCAAGCAGGTTCCTACGG
MAR1	CCAAAGGACTGTCTTGGAACA	AACAGCACTGAGCAGGGAC
linc-MD1	AGTGATTGAGGTGGACAGAAGG	CCCATTGAGGAGCATAGAACC
Myolinc	CGGTGCTATGGTTCTGATCG	TATGTGGGAAATACAGGGACA
lncMyoD	ACCCAAGGCAAGAAAAGTAGCA	ACTCACGAGTCAGCGGCAGAAC
lnc-mg	CTGCATCACGGAAGGAGATA	AACAATCCATCCTCATTGGC
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG

linc-MD1, long intergenic non-protein coding RNA muscle differentiation 1; lnc-mg, myogenesis-associated lncRNA; MAR1, muscle anabolic regulator 1; muscle RING finger protein 1.

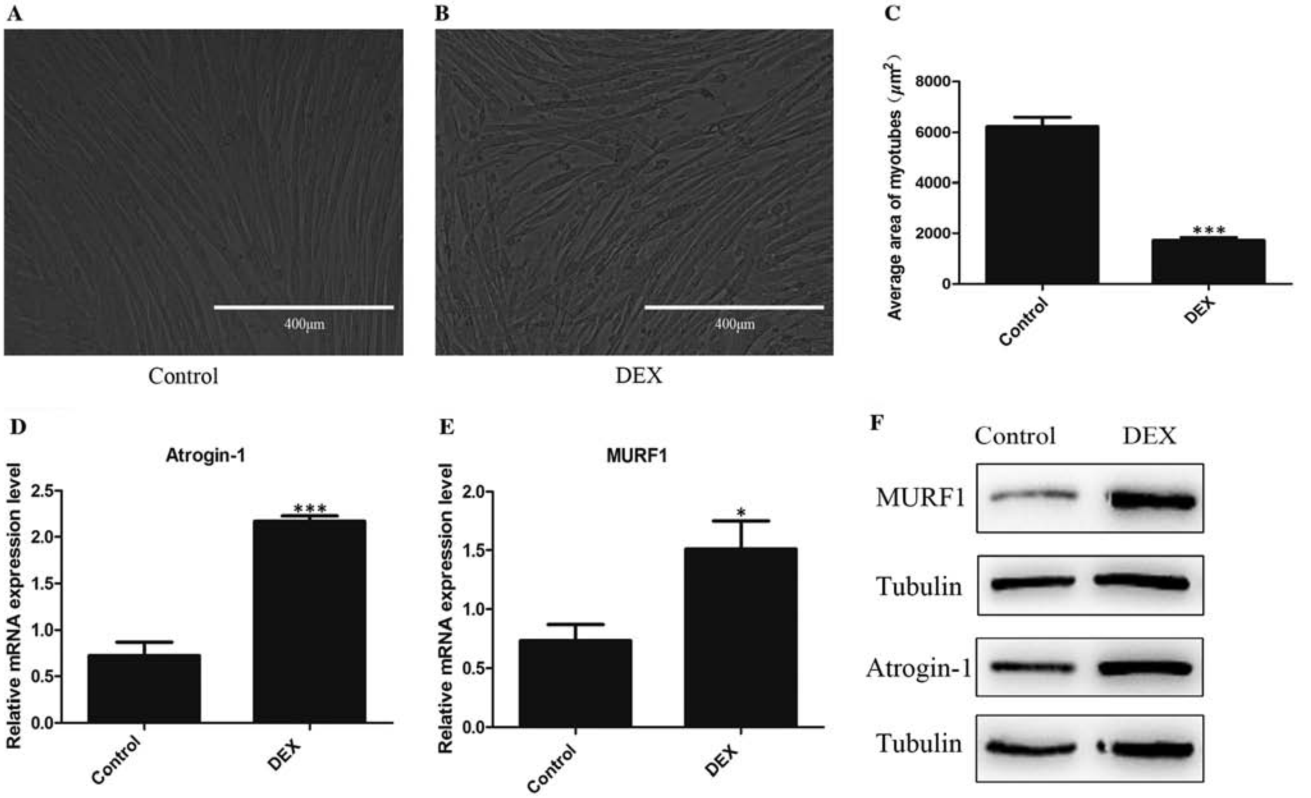

Figure 1. Establishment of the DEX-induced muscle atrophy cell model. (A) and (B) C2C12 myotubes in the DEX-induced muscle atrophy group were thinner than that of the control group under optical microscope. (C) The average area of C2C12 myotubes was lower in DEX-induced muscle atrophy C2C12 cells than in control cells. An increase in the mRNA levels of (D) MURF1 and (E) Atrogin-1 was observed in starved C2C12 cells. (F) An increase in the protein levels of MURF1 and Atrogin-1 was observed in starved C2C12 cells. *P<0.05 and ***P<0.001 vs. control. DEX, dexamethasone; MURF1, muscle RING finger protein 1.

Patients often refuse treatments that may cause side-effects such as muscular atrophy and muscle weakness, where development of these symptoms may reduce a patients' trust in their doctor and affect their clinical treatment outcome (51,52). Although GC mechanisms of action have been fully described, the mechanisms of non-coding RNAs in GIMA yet to be fully understood. The current study attempted to elucidate the regulatory mechanism of non-coding RNAs in GC-induced skeletal muscle atrophy to provide the groundwork for the development of drugs and treatment programs for the prevention and treatment of muscular atrophy in clinical practice. Non-coding RNAs were examined, which have been previously demonstrated to be involved in other muscle atrophy models. The current study aimed to identify whether non-coding RNAs were associated with the DEX model.

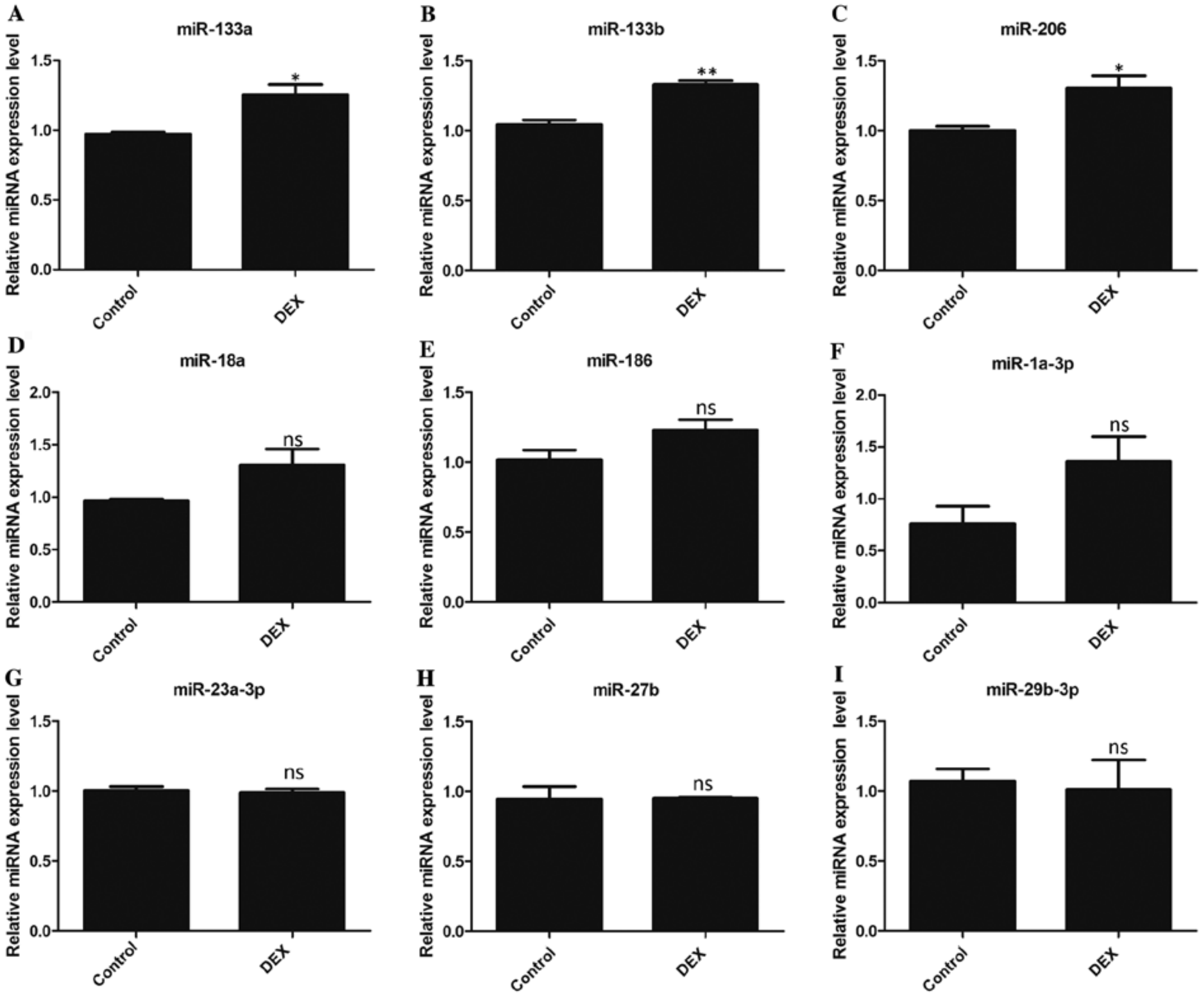

Figure 2. miRNA expression patterns in atrophic C2C12 cells. The expression levels of miRNAs (A) miR-133a, (B) miR-133b, (C) miR-206, (D) miR-18a, (E) miR-186, (F) miR-1a-3p, (G) miR-23a-3p, (H) miR-27b and (I) miR-29b-3p were detected in control C2C12 cells and DEX-induced muscle atrophy C2C12 cells. All data are presented as mean ± SD. *P<0.05 or **P<0.01 vs. control; n=3 per group. microRNA, miRNA or miR; DEX, dexamethasone; ns, not significant.

In skeletal muscle, non-coding RNAs serve multiple roles in muscle development and regeneration, such as in the regulation of genes involved in myogenesis, proliferation, and muscle fiber-type conversion (25,26,31-36). Among them, miRNA has been revealed to serve a role in regulating muscle atrophy by being associated with muscle protein metabolism, muscle regeneration, angiogenesis and muscle cell apoptosis (21). miR-206 inhibits the expression of Pax7 and Histone deacetylase 4to promote the differentiation of muscle satellite cells for the purpose of muscle regeneration (46). miR-27b promotes the differentiation of myogenic satellite cells and promotes muscle regeneration by targeting myogenic regulatory inhibitory proteins (47). The current study indicated that the expression of miR-133a, miR-133b and miR-206 were upregulated in the DEX group. These results were consistent with previous studies that demonstrated increased expression of miR-133a and miR-206 in animal models of muscular dystrophy and in the serum of effected patients (30,48,53). Myogenic regulatory factors include MyoD, MyoG, Myf5 and MRF4, which servean important role in the regulation of myogenic differentiation after proliferation of minisatellite cells (54). In the present study, C2C12 cells treated with DEX were indicated to exhibit increased levels of miR-133a, miR-133b and miR-206 and of MyoD and Wnt5. This may be the result of DEX activating a compensatory mechanism for muscle atrophy, or MyoD may servea role in this mechanism as it may contribute toother functional mechanisms that are not yet fully understood. For example, it may be suggested that MyoD promotes muscle atrophy under the action or involvement of an unknown molecule, such as c-Myc. It is well known that c-Myc is a representative lncRNA that promotes the development and function of muscles (55,56). However, Eischen *et al* (57) revealedthat c-Myc inhibits Beclin 1 and Bax by inhibiting the anti-apoptotic protein Bcl-2, whichenhances autophagy and apoptosis, respectively, leading to musclewastage (58). In addition, Amirouche *et al* (45) indicated that overexpression of miR-206 can promote the expression of MyoD, and miR-206 can act as a multi-effect modulator to regulate muscle atrophy that is caused by Duchenne muscular dystrophy by targeting a variety of key miRNAs. It may be speculated that miR-206 may participate in the compensatory effect of muscle atrophy under DEX-induced conditions via unknown mechanisms, and this should be examined in future studies. The expression of miR-18a, miR-186, miR-1a-3p, miR-23a-3p, miR-27b and miR-29b-3p exhibited no significant change, suggesting that their role in DEX-induced skeletal muscle atrophy may be

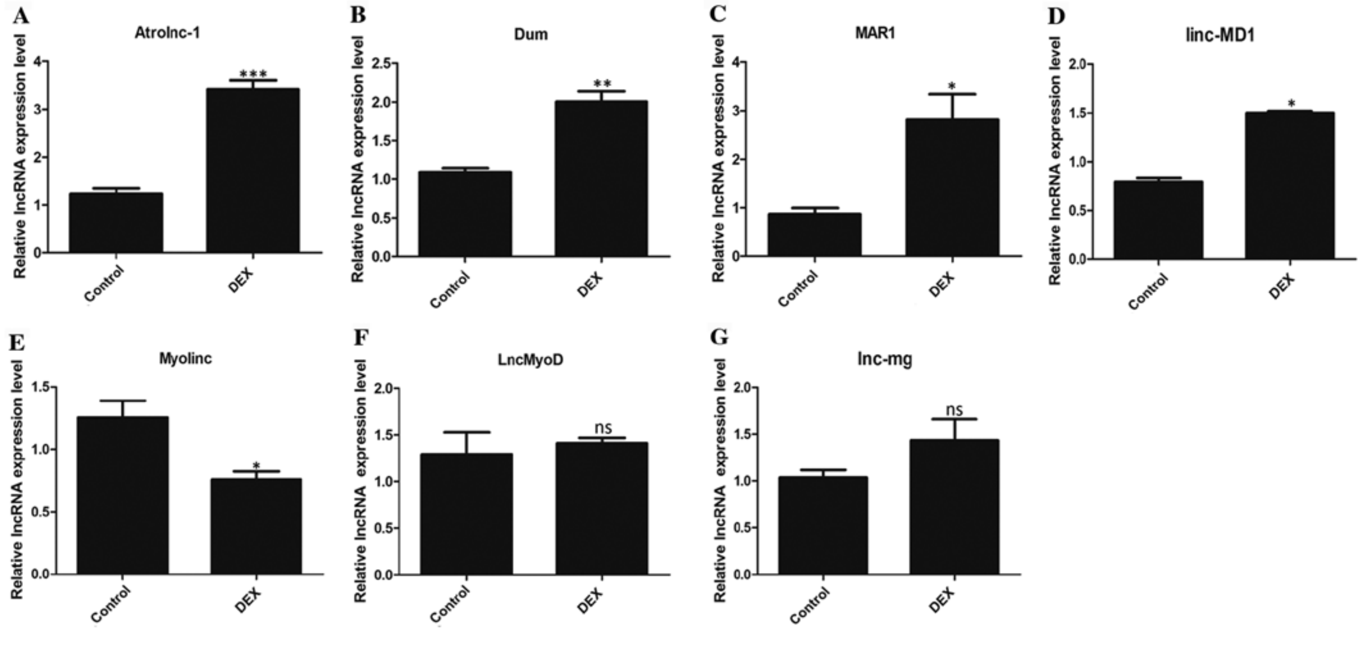

Figure 3. LncRNA expression patterns in atrophic C2C12 cells. The expression levels of lncRNAs (A) Atrolnc-1, (B) Dum, (C) MAR1, (D) linc-MD1, (E) Myolinc, (F) LncMyoD and (G) lnc-mg were detected in control C2C12 cells and DEX-induced muscle atrophy C2C12 cells. All data are presented as mean ± SD. *P<0.05, **P<0.01 or ***P<0.001 vs. control; n=3 per group. LncRNA, long non-coding RNA; DEX, dexamethasone; ns, not significant; linc-MD1, long intergenic non-protein coding RNA muscle differentiation 1.

minimal. In muscle atrophy under pathological conditions, the factors that cause miRNA changes are more complicated (59). Studies have demonstrated that changes in the activity of the hypothalamus-pituitary-adrenal axis in patients receiving glucocorticoid therapy may also affect muscle and miRNA expression (60,61). The current study only investigated the changes in miRNA expression at the cellular level *in vitro*; therefore, it is necessary to further verify the mechanism of the aforementioned miRNAs in hormone-induced muscular atrophy *in vivo*.

In a previous study, a total of 2,922 lncRNAs and 581 circular RNAs exhibited differential regulation during C2C12 differentiation, suggesting that they may be involved in muscle development (41). Among the lncRNAs (nine miRNAs and seven lncRNAs) detected in the current study, only some may be associated with the DEX-induced muscular atrophy. In the current study, a number of lncRNAs, including Atrolnc-1, Dum, MAR1, linc-MD1 and Myolinc, were revealed to be differentially expressed in control and atrophic myotubes. A large number of studies have demonstrated that lncRNAs can inhibit or activate gene expression by regulating gene transcription, mRNA stability, pre-mRNA splicing, protein translation and protein stability (62,63). LncRNA MAR1 acts as a miR-487b sponge to regulate Wnt5a protein expression, resulting in the promotion ofmuscle differentiation and regeneration (36). Myolinc recruits TDP-43 to the promoters of Filamin-A-interacting protein 1 and muscle marker genes (such as MyoD) to regulate myogenic regulatory networks (26). Additionally, lncRNAs can act as ‘sponges’ for miRNAs by pairing with and titrating them off their mRNA targets (64). In C2C12 cells, MAR1 has been indicated to promote myogenic differentiation by acting as a sponge for miR-487b, thereby regulating the expression of Wnt5a, which serves an important role in muscle regeneration (36). The present study also demonstrated that MAR1 expression in C2C12 cells treated with DEX was higher compared with the control group, and the expression of the downstream target gene Wnt5a was also increased, indicating a compensatory increase in MAR1 expression. Zhang *et al* (36) reported that the lncRNA MAR1 was significantly downregulated in mouse gastrocnemius muscle under senescence and mechanical unloading conditions during muscular atrophy. LncRNAs may change dynamically during muscular atrophy, or the same lncRNAs involved in muscular development may respond in different ways to different stimuli (65,66). linc-MD1 and Dum have been indicated to regulate the expression of MYHC and servean important role in the differentiation and development of skeletal muscle cells (30,31). Therefore, increased expression of linc-MD1 and Dum may be a compensatory response to atrophy. Inhibition of MURF1 and Atrogin-1 expression has been indicated to inhibit muscle loss and reduce muscle atrophy (36). In the current study, the expression of Atrolnc-1 in the DEX-induced atrophy model was significantly increased compared with the control cells. These results are consistent with studies that revealed that Atrolnc-1 significantly enhanced atrophic muscles in mouse models of chronic kidney disease, starvation and cancer (30,49). Atrolnc-1 interacts with A20 binding inhibitors of NF-κB-1 to promote its activation, leading to increased expression of MURF1 (30). The high expression of Atrolnc-1 and MURF1 observed in the present study further indicates that muscular atrophy occurred in DEX-treated C2C12 cells.

It has been suggested that lncRNAs and miRNAs may mutually restrict muscle development in muscular atrophy model (33,67). The results of the present study demonstrated that the expression of miR-133a and miR-133b in DEX-treated C2C12 cells increased compared with the control cells, which is consistent with the ability of linc-MD1 to modulate expression.

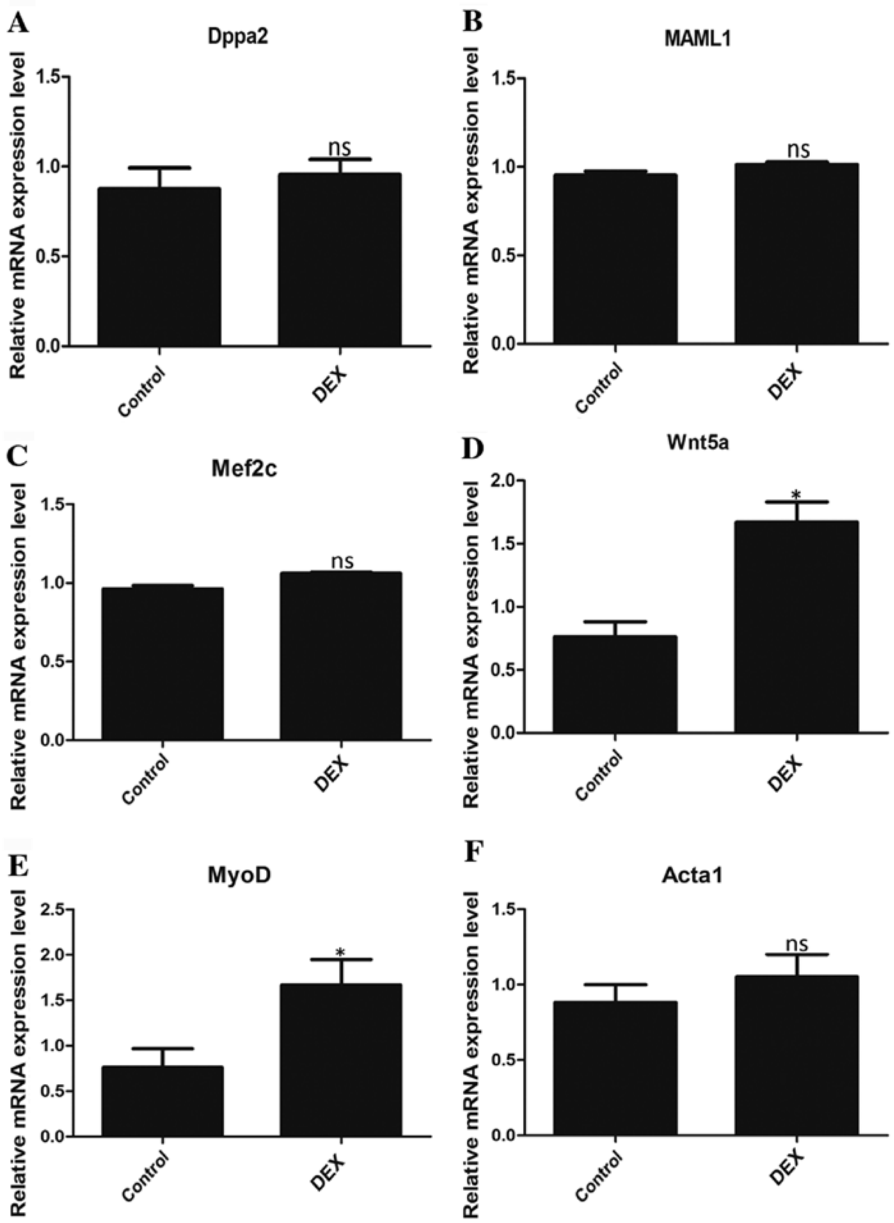

Figure 4. mRNA expression patterns of downstream targets in atrophic C2C12 cells. The expression levels of mRNAs (A) Dppa2, (B) MAML1, (C) Mef2c, (D) Wnt5a, (E) MyoD and (F) Acta1 were detected in control C2C12 cells and DEX-induced muscle atrophy C2C12 cells. All data are presented as mean ± SD. *P<0.05 vs. control; n=3 per group. DEX, dexamethasone; ns, not significant.

Legnini *et al* (34) indicated that in normal skeletal muscle cells, increased expression of miR-133a led to the cleavage of linc-MD1 to form miR-133b. Therefore, it can be speculated that, under the action of DEX, linc-MD1 may be upregulated and exhibit a compensatory effect via increased expression of miR-133a and miR-133b. However, the expression of the downstream mRNAs MAML1 and Mef2c were not indicated to be significantly different between the DEX-treated C2C12 and control cells, so may serve a role in this mechanism.

In conclusion, DEX increased catabolism in skeletal muscle cells and elevated the expression of key genes for muscular atrophy, which suggested the successful establishment of the muscular atrophy model in C2C12 cells. Recent studies have demonstrated that some important miRNAs and lncRNAs may be involved in regulating the mechanism of action behind muscular atrophy (16,33,44,67); however, this remains to be further explored. The results of the present study provide a novel perspective for studies on miRNAs and lncRNAs in GC-induced muscular atrophy, and suggest that they may be used as potential diagnostic tools. Further studies are required to improve the understanding ofthe role of non-coding RNAs in GC-induced muscle atrophy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RC conceived and designed the experiments. YL, HS, SZ, SL and YS performed the experiments analyzed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Schakman O, Kalista S, Barbé C, Loumaye A and Thissen JP: Glucocorticoid-induced skeletal muscle atrophy. Int J Biochem Cell Biol 45: 2163-2172, 2013.
2. Rhen T and Cidlowski JA: Antiinflammatory action of glucocorticoids-new mechanisms for old drugs. N Engl J Med 353: 1711-1723, 2005.
3. Rauch A, Seitz S, Baschant U, Schilling AF, Illing A, Stride B, Kirilov M, Mandic V, Takacz A, Schmidt-Ullrich R, *et al*: Glucocorticoids suppress bone formation by attenuating osteoblast differentiation via the monomeric glucocorticoid receptor. Cell Metab 11: 517-531, 2010.
4. Ma K, Mallidis C, Bhasin S, Mahabadi V, Artaza J, Gonzalez-Cadavid N, Arias J and Salehian B: Glucocorticoid-induced skeletal muscle atrophy is associated with upregulation of myostatin gene expression. Am J Physiol Endocrinol Metab 285: E363-E371, 2003.
5. Waddell DS, Baehr LM, van den Brandt J, Johnsen SA, Reichardt HM, Furlow JD and Bodine SC: The glucocorticoid receptor and FOXO1 synergistically activate the skeletal muscle atrophy-associated MuRF1 gene. Am J Physiol Endocrinol Metab 295: E785-E797, 2008.
6. Braun TP, Szumowski M, Levasseur PR, Grossberg AJ, Zhu X, Agarwal A and Marks DL: Muscle atrophy in response to cytotoxic chemotherapy is dependent on intact glucocorticoid signaling in skeletal muscle. PLoS One 9: e106489, 2014.
7. Braun TP and Marks DL: The regulation of muscle mass by endogenous glucocorticoids. Front Physiol 6: 12, 2015.
8. Shimizu N, Yoshikawa N, Ito N, Maruyama T, Suzuki Y, Takeda S, Nakae J, Tagata Y, Nishitani S, Takehana K, *et al*: Crosstalk between glucocorticoid receptor and nutritional sensor mTOR in skeletal muscle. Cell Metab 13: 170-182, 2011.
9. Bodine SC and Furlow JD: Glucocorticoids and skeletal muscle. Adv Exp Med Biol 872: 145-176, 2015.
10. Schakman O, Gilson H, Kalista S and Thissen JP: Mechanisms of muscle atrophy induced by glucocorticoids. Horm Res 72 (Suppl 1): S36-S41, 2009.
11. Zhao SQ, Xu SQ, Cheng J, Cao XL, Zhang Y, Zhou WP, Huang YJ, Wang J and Hu XM: Anti-inflammatory effect of external use of escin on cutaneous inflammation: Possible involvement of glucocorticoids receptor. Chin J Nat Med 16: 105-112, 2018.
12. Zheng B, Ohkawa S, Li H, Roberts-Wilson TK and Price SR: FOXO3a mediates signaling crosstalk that coordinates ubiquitin and atrogin-1/MAFbx expression during glucocorticoid-induced skeletal muscle atrophy. FASEB J 24: 2660-2669, 2010.
13. Watson ML, Baehr LM, Reichardt HM, Tuckermann JP, Bodine SC and Furlow JD: A cell-autonomous role for the glucocorticoid receptor in skeletal muscle atrophy induced by systemic glucocorticoid exposure. Am J Physiol Endocrinol Metab 302: E1210-E1220, 2012.
14. Bodine SC, Latres E, Baumhueter S, Lai VK, Nunez L, Clarke BA, Poueymirou WT, Panaro FJ, Na E, Dharmarajan K, *et al*: Identification of ubiquitin ligases required for skeletal muscle atrophy. Science 294: 1704-1708, 2001.
15. Stitt TN, Drujan D, Clarke BA, Panaro F, Timofeyva Y, Kline WO, Gonzalez M, Yancopoulos GD and Glass DJ: The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. Mol Cell 14: 395-403, 2004.
16. Li J, Chan MC, Yu Y, Bei Y, Chen P, Zhou Q, Cheng L, Chen L, Ziegler O, Rowe GC, *et al*: miR-29b contributes to multiple types of muscle atrophy. Nat Commun 8: 15201, 2017.
17. Horak M, Novak J and Bienertova-Vasku J: Muscle-specific microRNAs in skeletal muscle development. Dev Biol 410: 1-13, 2016.
18. Chen JF, Mandel EM, Thomson JM, Wu Q, Callis TE, Hammond SM, Conlon FL and Wang DZ: The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. Nat Genet 38: 228-233, 2006.
19. McCarthy JJ and Esser KA: MicroRNA-1 and microRNA-133a expression are decreased during skeletal muscle hypertrophy. J Appl Physiol (1985) 102: 306-313, 2007.
20. Soares RJ, Cagnin S, Chemello F, Silvestrin M, Musaro A, De Pitta C, Lanfranchi G and Sandri M: Involvement of microRNAs in the regulation of muscle wasting during catabolic conditions. J Biol Chem 289: 21909-21925, 2014.
21. Walden TB, Timmons JA, Keller P, Nedergaard J and Cannon B: Distinct expression of muscle-specific microRNAs (myomirs) in brown adipocytes. J Cell Physiol 218: 444-449, 2009.
22. Bartel DP: MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 116: 281-297, 2004.
23. Ivey KN and Srivastava D: microRNAs as developmental regulators. Cold Spring Harb Perspect Biol 7: a008144, 2015.
24. Lei Z, Sluijter JP and van Mil A: MicroRNA therapeutics for cardiac regeneration. Mini Rev Med Chem 15: 441-451, 2015.
25. Shen H, Liu T, Fu L, Zhao S, Fan B, Cao J and Li X: Identification of microRNAs involved in dexamethasone-induced muscle atrophy. Mol Cell Biochem 381: 105-113, 2013.
26. Militello G, Hosen MR, Ponomareva Y, Gellert P, Weirick T, John D, Hindi SM, Mamchaoui K, Mouly V, Döring C, *et al*: A novel long non-coding RNA myolinc regulates myogenesis through TDP-43 and Filip1. J Mol Cell Biol 10: 102-117, 2018.
27. Xiong W, Jiang YX, Ai YQ, Liu S, Wu XR, Cui JG, Qin JY, Liu Y, Xia YX, Ju YH, *et al*: Microarray analysis of long non-coding RNA expression profile associated with 5-fluorouracil-based chemoradiation resistance in colorectal cancer cells. Asian Pac J Cancer Prev 16: 3395-3402, 2015.
28. Chen R, Jiang T, She Y, Xie S, Zhou S, Li C, Ou J and Liu Y: Comprehensive analysis of lncRNAs and mRNAs with associated co-expression and ceRNA networks in C2C12 myoblasts and myotubes. Gene 647: 164-173, 2018.
29. Boltaña S, Valenzuela-Miranda D, Aguilar A, Mackenzie S and Gallardo-Escárate C: Long noncoding RNAs (lncRNAs) dynamics evidence immunomodulation during ISAV-Infected Atlantic salmon (Salmo salar). Sci Rep 6: 22698, 2016.
30. Sun L, Si M, Liu X, Choi JM, Wang Y, Thomas SS, Peng H and Hu Z: Long-noncoding RNA Atrolnc-1 promotes muscle wasting in mice with chronic kidney disease. J Cachexia Sarcopenia Muscle 9: 962-974, 2018.
31. Cesana M, Cacchiarelli D, Legnini I, Santini T, Sthandier O, Chinappi M, Tramontano A and Bozzoni I: A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. Cell 147: 358-369, 2011.
32. Li Y, MengX, Li G, Zhou Q and Xiao J: Noncoding RNAs in muscle atrophy. Adv Exp Med Biol 1088: 249-266, 2018.

33. Zhang ZK, Li J, Guan D, Liang C, Zhuo Z, Liu J, Lu A, Zhang G and Zhang BT: Long noncoding RNA lncMUMA reverses established skeletal muscle atrophy following mechanical unloading. Mol Ther 26: 2669-2680, 2018.
34. Legnini I, Morlando M, Mangiavacchi A, Fatica A and Bozzoni I: A feedforward regulatory loop between HuR and the long noncoding RNA linc-MD1 controls early phases of myogenesis. Mol Cell 53: 506-514, 2014.
35. Wang L, Zhao Y, Bao X, Zhu X, Kwok YK, Sun K, Chen X, Huang Y, Jauch R, Esteban MA, *et al*: LncRNA Dum interacts with Dnmts to regulate Dppa2 expression during myogenic differentiation and muscle regeneration. Cell Res 25: 335-350, 2015.
36. Zhang ZK, Li J, Guan D, Liang C, Zhuo Z, Liu J, Lu A, Zhang G and Zhang BT: A newly identified lncRNA MAR1 acts as a miR-487b sponge to promote skeletal muscle differentiation and regeneration. J Cachexia Sarcopenia Muscle 9: 613-626, 2018.
37. Fappi A, Neves JC, Sanches LN, Massaroto E Silva PV, Sikusawa GY, Brandão TPC, Chadi G and Zanoteli E: Skeletal muscle response to deflazacort, dexamethasone and methylprednisolone. Cells 8: 406, 2019.
38. Troncoso R, Paredes F, Parra V, Gatica D, Vásquez-Trincado C, Quiroga C, Bravo-Sagua R, López-Crisosto C, Rodriguez AE, Oyarzún AP, *et al*: Dexamethasone-induced autophagy mediates muscle atrophy through mitochondrial clearance. Cell Cycle 13: 2281-2295, 2014.
39. Becker DE: Basic and clinical pharmacology of glucocorticosteroids. Anesth Prog 60: 25-32, 2013.
40. Son YH, Jang EJ, Kim YW and Lee JH: Sulforaphane prevents dexamethasone-induced muscle atrophy via regulation of the Akt/Foxo1 axis in C2C12 myotubes. Biomed Pharmacother 95: 1486-1492, 2017.
41. Chen R, Jiang T, Lei S, She Y, Shi H, Zhou S, Ou J and Liu Y: Expression of circular RNAs during C2C12 myoblast differentiation and prediction of coding potential based on the number of open reading frames and N6-methyladenosine motifs. Cell Cycle 17: 1832-1845, 2018.
42. Massaccesi L, Goi G, Tringali C, Barassi A, Venerando B and Papini N: Dexamethasone-induced skeletal muscle atrophy increases O-GlcNAcylation in C2C12 cells. J Cell Biochem 117: 1833-1842, 2016.
43. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
44. Townley-Tilson WD, Callis TE and Wang D: MicroRNAs 1, 133, and 206: Critical factors of skeletal and cardiac muscle development, function, and disease. Int J Biochem Cell Biol 42: 1252-1255, 2010.
45. Amirouche A, Jahnke VE, Lunde JA, Koulmann N, Freyssenet DG and Jasmin BJ: Muscle-specific microRNA-206 targets multiple components in dystrophic skeletal muscle representing beneficial adaptations. Am J Physiol Cell Physiol 312: C209-C221, 2017.
46. Liu C, Wang M, Chen M, Zhang K, Gu L, Li Q, Yu Z, Li N and Meng Q: miR-18a induces myotubes atrophy by down-regulating IgfI. Int J Biochem Cell Biol 90: 145-154, 2017.
47. Antoniou A, Mastroyiannopoulos NP, Uney JB and Phylactou LA: miR-186 inhibits muscle cell differentiation through myogenin regulation. J Biol Chem 289: 3923-3935, 2014.
48. Lei S, She Y, Zeng J, Chen R, Zhou S and Shi H: Expression patterns of regulatory lncRNAs and miRNAs in muscular atrophy models induced by starvation in vitro and in vivo. Mol Med Rep 20: 4175-4185, 2019.
49. Mercatelli N, Fittipaldi S, De Paola E, Dimauro I, Paronetto MP, Jackson MJ and Caporossi D: MiR-23-TrxR1 as a novel molecular axis in skeletal muscle differentiation. Sci Rep 7: 7219, 2017.
50. Hou L, Xu J, Jiao Y, Li H, Pan Z, Duan J, Gu T, Hu C and Wang C: MiR-27b promotes muscle development by inhibiting MDFI expression. Cell Physiol Biochem 46: 2271-2283, 2018.
51. Oray M, Abu Samra K, Ebrahimiadib N, Meese H and Foster CS: Long-term side effects of glucocorticoids. Expert Opin Drug Saf 15: 457-465, 2016.
52. Stout A, Friedly J and Standaert CJ: Systemic absorption and side effects of locally injected glucocorticoids. PM R 11: 409-419, 2019.
53. Matsuzaka Y, Kishi S, Aoki Y, Komaki H, Oya Y, Takeda S and Hashido K: Three novel serum biomarkers, miR-1, miR-133a, and miR-206 for Limb-girdle muscular dystrophy, Facioscapulohumeral muscular dystrophy, and becker muscular dystrophy. Environ Health Prev Med 19: 452-458, 2014.
54. Li G, Li QS, Li WB, Wei J, Chang WK, Chen Z, Qiao HY, Jia YW, Tian JH and Liang BS: miRNA targeted signaling pathway in the early stage of denervated fast and slow muscle atrophy. Neural Regen Res 11: 1293-1303, 2016.
55. Luo W, Chen J, Li L, Ren X, Cheng T, Lu S, Lawal RA, Nie Q, Zhang X and Hanotte O: c-Myc inhibits myoblast differentiation and promotes myoblast proliferation and muscle fibre hypertrophy by regulating the expression of its target genes, miRNAs and lincRNAs. Cell Death Differ 26: 426-442, 2019.
56. Lin CH, Jackson AL, Guo J, Linsley PS and Eisenman RN: Myc-regulated microRNAs attenuate embryonic stem cell differentiation. EMBO J 28: 3157-3170, 2009.
57. Eischen CM, Packham G, Nip J, Fee BE, Hiebert SW, Zambetti GP and Cleveland JL: Bcl-2 is an apoptotic target suppressed by both c-Myc and E2F-1. Oncogene 20: 6983-6993, 2001.
58. Alessio E, Buson L, Chemello F, Peggion C, Grespi F, Martini P, Massimino ML, Pacchioni B, Millino C, Romualdi C, *et al*: Single cell analysis reveals the involvement of the long non-coding RNA Pvt1 in the modulation of muscle atrophy and mitochondrial network. Nucleic Acids Res 47: 1653-1670, 2019.
59. van de Worp WR, Theys J, van Helvoort A and Langen RC: Regulation of muscle atrophy by microRNAs: 'AtromiRs' as potential target in cachexia. Curr Opin Clin Nutr Metab Care 21: 423-429, 2018.
60. Hildebrandt T, Shope S, Varangis E, Klein D, Pfaff DW and Yehuda R: Exercise reinforcement, stress, and β-endorphins: An initial examination of exercise in anabolic-androgenic steroid dependence. Drug Alcohol Depend 139: 86-92, 2014.
61. Ng TP, Lu Y, Choo RW, Tan CT, Nyunt MS, Gao Q, Mok EW and Larbi A: Dysregulated homeostatic pathways in sarcopenia among frail older adults. Aging Cell 17: e12842, 2018.
62. Zhu M, Liu J, Xiao J, Yang L, Cai M, Shen H, Chen X, Ma Y, Hu S, Wang Z, *et al*: Lnc-mg is a long non-coding RNA that promotes myogenesis. Nat Commun 8: 14718, 2017.
63. Devaux Y, Zangrando J, Schroen B, Creemers EE, Pedrazzini T, Chang CP, Dorn GW II, Thum T and Heymans S; Cardiolinc network: Long noncoding RNAs in cardiac development and ageing. Nat Rev Cardiol 12: 415-425, 2015.
64. Ebert MS, Neilson JR and Sharp PA: MicroRNA sponges: Competitive inhibitors of small RNAs in mammalian cells. Nat Methods 4: 721-726, 2007.
65. Cichewicz MA, Kiran M, Przanowska RK, Sobierajska E, Shibata Y and Dutta A: MUNC, an enhancer RNA upstream from the MYOD gene, induces a subgroup of myogenic transcripts in trans independently of MyoD. Mol Cell Biol 38: e00655-17, 2018.
66. Mueller AC, Cichewicz MA, Dey BK, Layer R, Reon BJ, Gagan JR and Dutta A: MUNC, a long noncoding RNA that facilitates the function of MyoD in skeletal myogenesis. Mol Cell Biol 35: 498-513, 2015.
67. Li Z, Cai B, Abdalla BA, Zhu X, Zheng M, Han P, Nie Q and Zhang X: LncIRS1 controls muscle atrophy via sponging miR-15 family to activate IGF1-PI3K/AKT pathway. J Cachexia Sarcopenia Muscle 10: 391-410, 2019.