ORIGINAL RESEARCH

Electroacupuncture Promotes Skeletal Muscle Myogenic Differentiation and Protein Synthesis by Reducing Let-7c-5p Levels

Sisi Shen, MM; Yibei Lin, MM; Diyan Xu, MM; Yidan Zuo, MM; Liang Gu, MM; Xiaoping Wang, MM; Shan Zhu, MM; Zhen Su, MD

ABSTRACT

Objective • Acupuncture with low-frequency electrical stimulation (Acu-LFES) can attenuate muscle atrophy. Previous studies have found that Acu-LFES reduces the let-7 family in serum exosomes. This study explored the effects of let-7c-5p in chronic kidney disease (CKD) muscle atrophy.

Methods • A total of 24 mice were randomly divided into control group, Acu-LFES group, CKD group, and CKD/Acu-LFES group (n = 6/group). The 5/6 nephrectomy was performed to establish the CKD model in mice. After 20 weeks, the Acu-LFES group and CKD/Acu-LFES group were treated with electroacupuncture at the "Zu San Li" and "Yang Ling Quan" bilaterally points for 15 minutes once. Surface sensing of translation (SUnSET), Reverse Transcription-quantitative PCR(RT-qPCR), immunofluorescence staining, and Western blot were performed to examine each group's state of protein production and myogenic differentiation. we knocked down or exogenously expressed let-7c-5p in C2C12 myoblast, RT-qPCR, and Western blot were performed to examine protein synthesis and myogenic differentiation.

Results • The protein expressions of MyoD and Myogenin (MyoG) were decreased in the CKD group ($P = .029$ and $P = .026$) concomitant with a decrease in the muscle fiber cross-sectional area. Acu-LFES prevented muscle atrophy in CKD mice. The protein expressions of MyoD and MyoG were increased in the CKD/Acu-LFES group ($P = .006$ and $P = .001$). In muscle of CKD mice, IGF1, IGF1R, IRS1, phosphorylated mTOR and P70S6K proteins were decreased compared with control muscle ($P = .001$, $P = .007$, $P < .001$, $P < .001$ and $P < .001$), whereas atrogin-1/MAFbx and MuRF1 were dramatically increased ($P < .001$). Acu-LFES reversed these phenomena, indicating IGF1/mTOR signaling pathway was induced to promote muscle protein synthesis and myogenic differentiation. Meanwhile, Acu-LFES caused a decrease of let-7c-5p in skeletal muscle of CKD mice ($P = .034$). Inhibiting let-7c-5p promoted C2C12 myogenic differentiation ($P = .002$ and $P = .001$) and increased IGF1, IGF1R, IRS1 levels while upregulating mTOR and P70S6K phosphorylation ($P < .001$, $P = .002$, $P = .009$, $P < .001$ and $P = .007$). It is interesting to observe that the abundance of atrogin-1/MAFbx and MuRF-1 was unaffected by let-7c-5p ($P > .05$).

Conclusions • Acu-LFES-reduced expression of let-7c-5p can ameliorate CKD-induced skeletal muscle atrophy by upregulating the IGF1/mTOR signaling pathway, which enhances skeletal muscle protein synthesis and myogenic differentiation. Let-7c-5p may be a potential regulator for the treatment of muscle atrophy. (*Altern Ther Health Med.* 2024;30(1):472-480).

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INTRODUCTION

Skeletal muscle serves as the body's primary protein store. It is involved in exercise and energy metabolism, including the uptake, use, and storage of substrates like glucose, lipids, and amino acids. [1,2] In the physiological state, skeletal muscle protein synthesis and breakdown are in dynamic balance. When the balance is broken, skeletal muscle mass changes. Various stimuli can influence skeletal muscle.[3-5] Mechanical stress or anabolic hormones (such as testosterone or 2-adrenoceptor agonists) can cause hypertrophy, primarily controlled by the IGF1/AKT/mTOR pathway.[6,7] Atrophy occurs as a result of aging, metabolic diseases (such as cancer, diabetes, renal failure, respiratory insufficiency, or sepsis), neuromuscular disease, or stimulation by catabolic hormones(corticosteroids), and the mechanism underlying this process involves the activation of ubiquitin-mediated proteasomes (UPS).[8,9] In numerous studies, Acupuncture with low-frequency electrical stimulation (Acu-LFES), rather than high-frequency electroacupuncture (100 Hz), has been demonstrated to be effective in treating skeletal muscle atrophy, including

myasthenia caused by chronic renal disease, diabetes, sciatic nerve damage, spinal cord transection, and disuse-related muscular atrophy.[10-15] However, the mechanisms by which Acu-LFES medication affects muscle protein metabolism still need to be better understood.

MicroRNAs (miRNAs) are a class of highly conserved endogenous non-coding small RNAs that control the expression of genes by binding to the 3' untranslated region (UTR) of the target messenger RNA (mRNA). miRNAs play an important role in physiological and pathological processes, including cell proliferation, differentiation, and apoptosis. They are crucial for biological growth, development, and disease.[16-19] It has been reported that miRNAs are involved in muscle development, growth, regeneration, and atrophy.[20] According to research, miR-133 can increase myocyte proliferation by lowering serum response factor (SRF) protein levels[21]; miR-206 suppresses cell proliferation and participates in differentiation by regulating DNA synthesis.[22] Mice lacking the miR-206 gene take longer to recover from skeletal muscle denervation.[23] These miRNAs, enriched or specifically expressed in muscle, are called muscle-specific miRNAs (myomiRs).[24,25] Lethal-7 (let-7) was the second miRNA to be discovered, and there are 12 members of the let-7 miRNA family in humans.[26] Muscle biopsies showed that patients with oculo-pharyngeal muscular dystrophy, older adults, healthy men on bed rest for 21 days, and those with lower limb immobilization had significantly higher levels of let-7 expression in their skeletal muscles.[27-30] Another study found that healthy men on bed rest for 10 days had lower levels of let-7 expression.[31] In addition, let-7 levels were also lower in endomyocardial samples from patients with dilated cardiomyopathy.[32] However, cardiac hypertrophy correlated to let-7 overexpression.[33, 34] The let-7 family's effect on the muscle is currently debatable.

In a recent study, we discovered that Acu-LFES enhances skeletal muscle exosome secretion and influences miRNA expression in distal organs. We collected exosomes from Acu-LFES-treated mice serum for microRNA deep sequencing, and four members of the let-7 family (let-7a-5p, let-7b-5p, let-7c-5p, and let-7e-5p) were observed to contain a significantly lower level than expected.[35] In this study, we hypothesized that Acu-LFES might facilitate skeletal muscle atrophy by inhibiting Let-7c-5p of exosome-derived. We investigated the effect of Acu/LFES on myogenesis and the IGF1 signaling pathway in CKD-associated muscle atrophy. We used C2C12 cells as an in vitro skeletal muscle model to further investigate the effect and mechanism of let-7c-5p on skeletal muscle protein metabolism.

MATERIALS AND METHODS

Animals and CKD model

The Wenzhou Medical University Animal Ethics Committee approved these experiments. All animal experiments complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The male C57BL/6J mice were housed in 12 hours of darkness and 12 hours of light while being fed adaptively for one week. The mice were randomly divided into four groups: control, Acu-LFES, CKD, and CKD/Acu-LFES groups (n = 6/group). 5/6 nephrectomy was performed to induce CKD.[36] After 20 weeks, the mice received Acu-LFES treatment. At the end of the experiment, the mice were euthanized, and we took out the gastrocnemius and tibialis anterior (TA) muscles and stored them at -80°C for a future experiment.

Acu-LFES treatment

The WHO Standard Acupuncture Guidelines were used to choose the acupuncture points.[37] The positive point (Yang Ling Quan, GB34) is located in a six mm-deep hollow on the exterior inferior to the caput fibulae. Both the superficial and deep fibular nerves are nearby at this position. The negative spot (Zu San li, ST36) is located on the lateral side of the knee joint, 5mm below the fibular head, close to the peroneal nerve. We immobilized the mice with special devices to remain recumbent during the Acu-LFES therapy process. A continuous wave with a frequency of 20 Hz and a current intensity of 1 mA (mice with a slight shaking of the limbs) was applied for 15 minutes every day for two weeks using a 0.25 mm × 25 mm sterile acupuncture needle. The needle was hooked to the SDZ-II Electronic Acupuncture Instrument (Hwato; Suzhou, China).

Cell culture and treatment

Mouse myoblast cell line C2C12 cells were kindly provided by Stem Cell Bank, Chinese Academy of Sciences (GNM26; Shanghai, China). The cell was cultured in growth medium (GM), which was made up of high glucose DMEM (Gibco, USA), 10% fetal bovine serum (Gibco, USA), and 1% penicillin-streptomycin antibiotic mixture (NCM Biotech, Jiangsu, China), and cultured in a constant temperature incubator with 5% CO_2 at 37°C, the culture medium was changed every two days. When the cell fusion reached about 70%-80%, it was changed into differentiation media (DM), which contains high glucose DMEM and 2% horse serum supplemented with penicillin and streptomycin, and the C2C12 myoblasts were induced to myotubes.

miRNA transfection

The specific let-7c-5p mimics, inhibitor, mimic negative control (NC), and inhibitor NC were purchased from RiboBio (Guangzhou, China). First, one day before transfection, logarithmic growth phase C2C12 cells were inoculated in 6-well plates at a density of 2×10^5 cells/well, and when the cell reached 50% fusion, we transfected miRNA mimics, inhibitor, mimics NC, and inhibitor NC to the C2C12 cells by using Lipofectamine 2000 reagent (Invitrogen, USA), respectively, according to the manufacturer's instruction. After 6 hours of transfection, the medium was replaced. Then, 48 hours after differentiation, cells were harvested for the following stage experiment.

Surface sensing of translation (SUnSET)

SUnSET uses puromycin to monitor protein synthesis rapidly. Puromycin contains a structure comparable to the ends of tRNA molecules and can be consolidated into

nascent polypeptide chains, disturbing protein synthesis.[38] Puromycin (P8230, Solarbio; Beijing, China) was included in the culture media of each well for 30 minutes before collecting myotube cells, with a final concentration of 1 μM. Cell proteins were extracted and then analyzed by Western blot to identify total protein synthesis. Anti-puromycin antibody was obtained from Millipore (MABE343, 1:25,000 dilution; United States).

Western blot

C2C12 myotubes or skeletal muscles were lysed in RIPA lysis buffer (Beyotime; Shanghai, China), which contained 1% protease inhibitor PMSF and 1% phosphatase inhibitor to extract total protein. Protein concentration was measured using a BCA protein assay kit (Beyotime; Shanghai, China). Equal amounts of proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), subsequently transferred to PVDF membranes, and then blocked with 5% skim milk powder for 1h at room temperature. The membranes were incubated with primary antibodies overnight at 4°C. Primary antibodies that we used included mTOR (Ab32028, 1:1000 dilution), phosphorylated mTOR (Ab109268, 1:1000 dilution) from Abcam (UK); P70S6K (#9202, 1:1000 dilution), phosphorylated P70S6K (Thr389; #97596, 1:1000 dilution), β-actin (#3700, 1:1000 dilution) were from Cell Signaling Technology (USA); IGF1 (28530, 1:500 dilution), IRS1 (17509, 1:1000 dilution) were from ProteinTech (USA), IFG1R (AF6123, 1:500 dilution) was from Affinity (USA); MyoD (WL04662, 1:1000 dilution), MyoG (WL01132, 1:1000 dilution) was from Wanleibio (Shenyang, China). In addition, goat Anti-Mouse IgG, Peroxidase - Conjugated, H+L (BL001A, 1: 10 000 dilution; Biosharp, Anhui, China) was used as a secondary antibody. The Protein bands were detected by ECL chemiluminescence reagent (Affinity; USA), and mean gray values of protein bands were measured by ImageJ.

Immunofluorescence staining

TA muscles were embedded under optimal cutting temperature (OCT) compound (SAKURA; USA) in cooled isopentane. Frozen tissues were cut into 7 mm sections and then fixed in 4% paraformaldehyde for 15 min and blocked in 5% bovine serum albumin for 1 h. Laminin (Ab133645, 1:250 dilutions; Abcam, USA) was used as a primary antibody and incubated at 4 °C overnight, followed by incubation with the corresponding secondary antibody for 1 h. In addition, 20 μL of anti-fluorescence attenuation DAPI staining solution was dropped and incubated at room temperature in the dark for 10 minutes. Images were captured under a fluorescence microscope (LV-150N; Nikon, Japan). 300 fibers were measured per muscle tissue.

Reverse Transcription-quantitative PCR(RT-qPCR)

The total RNA was extracted with TRIzol Reagent (Invitrogen, USA). For mRNA, RNA was reverse transcribed into cDNA using HiScript® II Q RT SuperMix for qPCR kit (Vazyme, Jiangsu, China). RT-qPCR was performed with Taq Pro Universal SYBR qPCR Master Mix Kit (Vazyme, Jiangsu, China), according to the manufacturer's instructions. For microRNA, we used the Bulge-Loop miRNA qPCR Starter Kit (RiboBio; Guangzhou, China) to synthesize reverse transcripts and quantify them. The relative gene expression level was calculated by using the $2^{-\Delta\Delta CT}$ method. Individual samples were run in triplicate. Bulge-loopTM miRNA qRT-PCR Primer Sets (one RT primer and a pair of qRT-PCR primers for each set) specific for let-7c-5p were designed by RiboBio (Guangzhou, China). The primer sequences are shown in Table 1.

Table 1. primer sequence

Gene	Prime (5'-3')	Sequence
IGF1	Forward	CGCTCTGCTTGCTCACCTTCAC
	Reverse	AAGCCCCTCGGTCCACACAC
IGF1R	Forward	GTGGGGGCTCGTGTTTCTC
	Reverse	GATCACCGTGCAGTTTTCCA
IRS1	Forward	TGCAGTAGTTCTCCAGCTGG
	Reverse	CCTGTTGGTGCACTTCCTAC
mTOR	Forward	ACCGGCACACATTTGAAGAAG
	Reverse	CTCGTTGAGGATCAGCAAGG
P70S6K	Forward	TTGACTTTCCAGTTCCCAAGGT
	Reverse	AAAAGGCGAATAGGAGGGCAG
MAFbx	Forward	CAGCTTCGTGAGCGACCTC
	Reverse	GGCAGTCGAGAAGTCCAGTC
MuRF1	Forward	GTGTGAGGTGCCTACTTGCTC
	Reverse	GCTCAGTCTTCTGTCCTTGGA
GAPDH	Forward	CAAGTTCAACGGCACAGTCAAG
	Reverse	ACATACTCAGCACCAGCATCAC

Statistical analysis

Data was performed on GraphPad Prism version 9 and expressed as Means ± SD. The Student's *t* test was used to compare the differences between the two groups; Multiple comparisons were established using the one-way ANOVA. Each experiment was repeated three times to ensure reproducibility. $P < .05$ was considered Statistically significant.

RESULTS

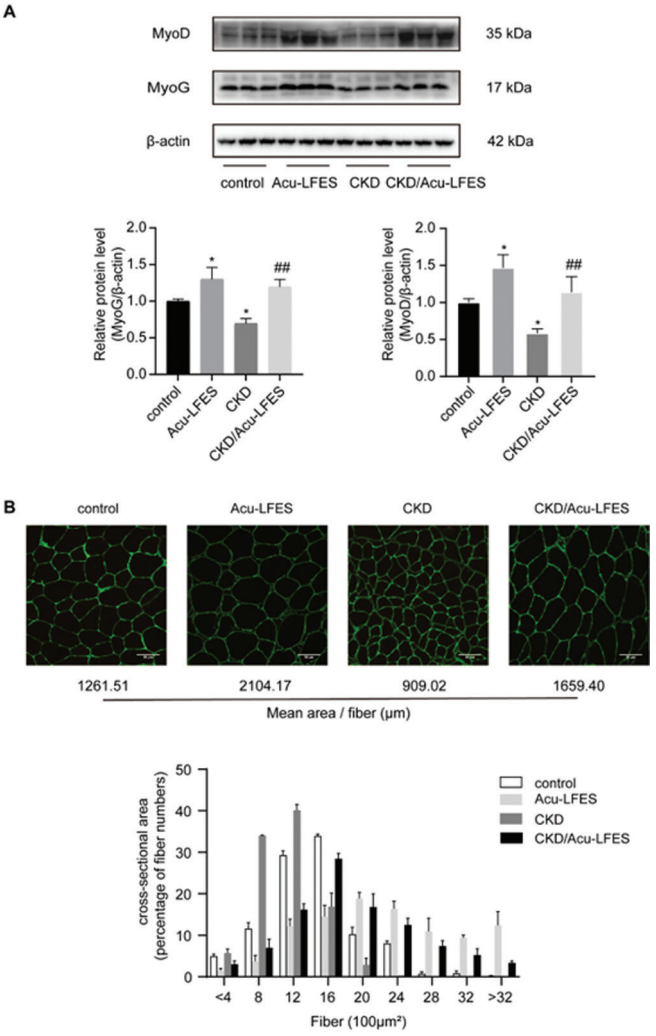
Acu-LFES improves muscle myogenic differentiation in control and CKD mice

We first measured protein amounts of myogenesis markers which indicate the myogenic differentiation capacity of the muscles. In the muscle of CKD mice, the protein expressions of MyoD and Myogenin (MyoG) were decreased by 42% and 30%, respectively, versus in control mice ($P = .029$ and $P = .026$). Interestingly, Acu-LFES reversed the CKD-induced myogenic protein suppression, which increased MyoD and MyoG expressions 2.0- and 1.7-fold, respectively ($P = .006$ and $P = .001$). Acu-LFES also stimulates muscle myogenic differentiation in healthy mice ($P = .016$ and $P = .027$) (Figure 1A). Meanwhile, the muscle fiber cross-sectional area was determined by Immunofluorescence. The fiber cross-sectional area frequency distribution shows that the percentage of larger fiber in CKD mice (move to the left) is reduced compared with the control. Furthermore, Acu-LFES suppressed the leftward shift in the fiber size distribution (Figure 1B). These results demonstrate that Acu-LFES counteracts CKD-induced muscle wasting by activating muscle myogenic differentiation.

Acu-LFES improves muscle protein metabolism

The expression of IGF1, IGF1R, and IRS1 were evaluated to understand better how Acu-LFES enhances muscle mass.

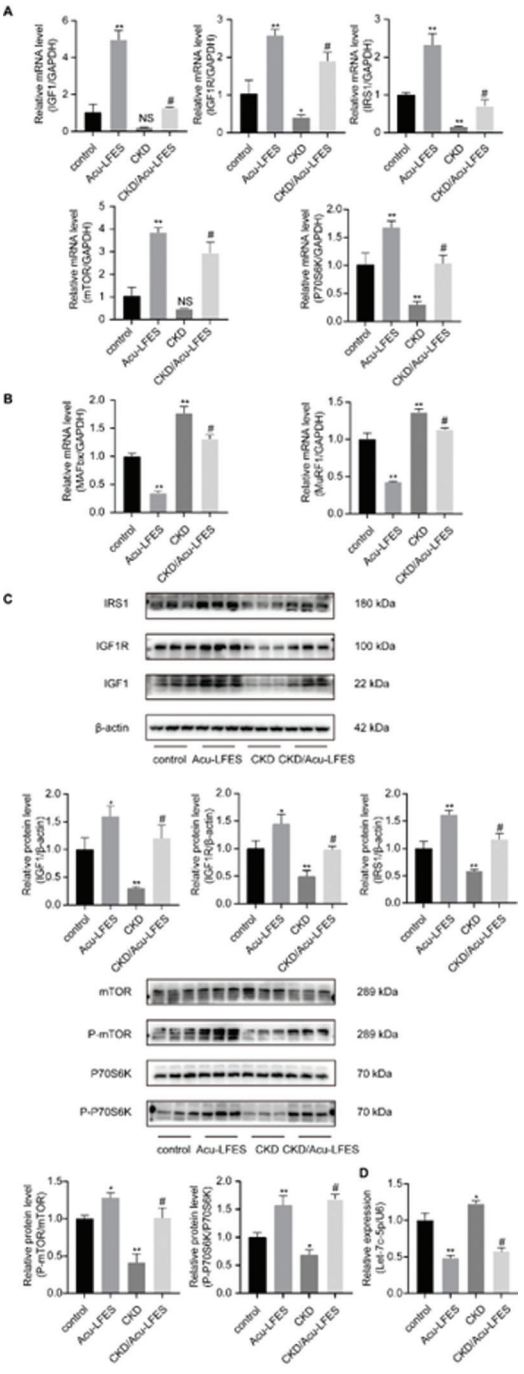
Figure 1. Acu-LFES improves muscle myogenic differentiation in control and CKD mice**.** (A) Western blot of MyoD and MyoG in hindlimb (gastrocnemius and tibialis anterior) muscle lysates from control, Acu-LFES, CKD, and CKD/Acu-LFES mice. (B) Tibialis anterior (TA) muscle sections were immunostained with anti-laminin antibody (green, cell perimeter). representative fiber cross-sections are shown in control, Acu-LFES, CKD, and CKD/Acu-LFES mice. Scale bar, 50 μm. The frequency distribution of fiber cross-sectional area in control, Acu-LFES, CKD, and CKD/Acu-LFES mice was presented as a percent cross-section area of fibers. n = 6/group; Each column represents the Means ± SD.



*$P < .05$ vs control
#$P < .01$ vs CKD.

In CKD mice, the mRNA level of IGF1 was decreased compared with the control group, but the difference was not statistically significant ($P > .05$), while the mRNA level of IGF1R and IRS1 were reduced ($P = .037$ and $P = .001$) (Figure 2A). In the meantime, we examined genes in all mice involved in protein synthesis and degradation. The RT-qPCR analysis results revealed, compared to the control group, atrogin-1/MAFbx and MuRF1 were dramatically increased in CKD mice ($P < .001$); in contrast, P70S6K was significantly

Figure 2. Acu-LFES improves muscle protein metabolism. A and B: Total RNA isolated from hindlimb muscle of control, Acu-LFES, CKD, and CKD/Acu-LFES mice were assayed for (A) IGF1, IGF1R, IRS1, mTOR, P70S6K, (B) protein degradation-related markers (atrogin-1/MAFbx, MuRF1), and (D) let-7c-5p expression by RT-qPCR. (C) The protein metabolism-related markers IGF1, IGF1R, IRS1, mTOR, P-mTOR, P70S6K, and P-P70S6K were measured by Western blot in hindlimb muscle lysates from control, Acu-LFES, CKD, and CKD/Acu-LFES mice. n = 6/group; Each column represents the Means ± SD.



** $P < .01$, and * $P < .05$ vs control, # $P < .05$ vs CKD.

decreased (P = .001), while the difference in mTOR was not statistically significant (P > .05) (Figure 2B). This phenomenon was reversed in CKD and control mice models by Acu-LFES. Furthermore, the proteins of IGF1, IGF1R, IRS1, phosphorylated mTOR, and P70S6K were dramatically elevated in CKD mice after Acu-LFES (P = .001, P = .007, P < .001, P < .001 and P < .001) (Figure 2C). These results indicate that Acu-LFES increased muscle protein synthesis while decreasing protein breakdown.

Acu-LFES decreased let-7c-5p in skeletal muscles in control and CKD mice

In our previous study, four members of the let-7 family in serum exosomes were significantly decreased by Acu-LFES, with let-7c-5p having the most alteration. In mice skeletal muscle, let-7c-5p was detected. The let-7c-5p level was increased 1.2-fold in CKD mice relative to the control (P = .01), indicating there may be a link between CKD and elevated let-7c-5p. After using Acu-LFES, we discovered that the expression of let-7c-5p was significantly decreased by 53% in CKD/ Acu-LFES mice versus the CKD group (P = .034) (Figure 2D). These results demonstrated that Acu-LFES-treatment improved muscle atrophy in CKD accompanied by the downregulation of let-7c-5p. Based on the above, let-7c-5p may affect muscle metabolism.

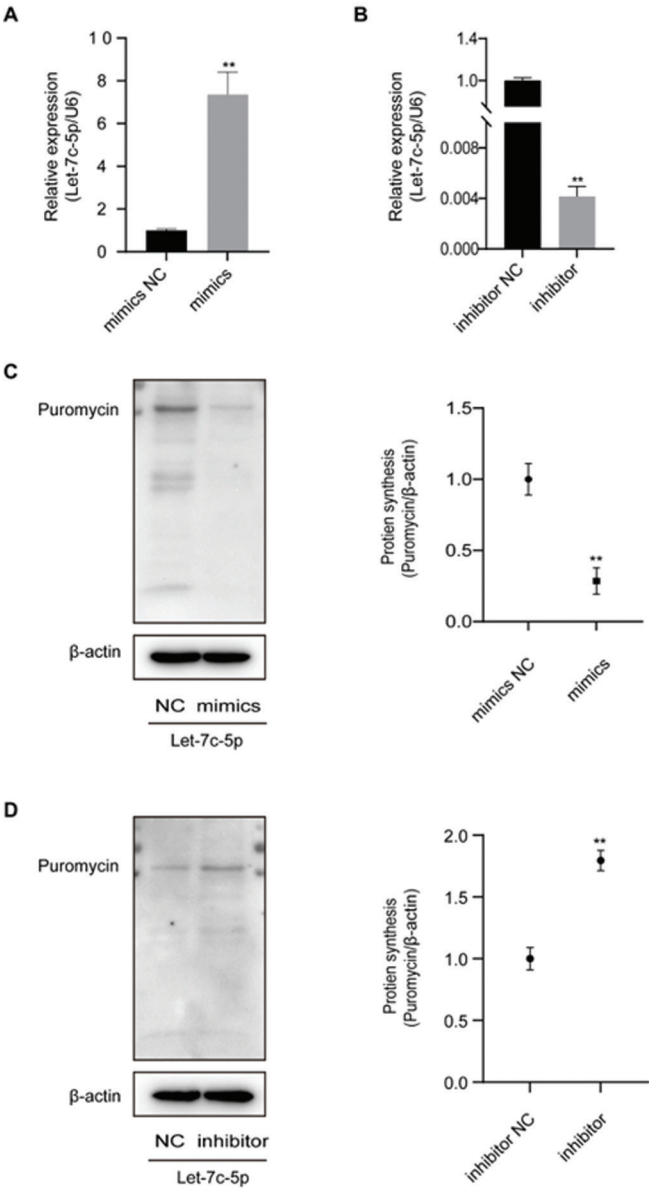
Let-7c-5p Inhibitor protein synthesis in C2C12

C2C12 cells were transfected with let-7c-5p mimics and its inhibitor to examine the effects of let-7c-5p on muscle protein metabolism. By using RT-qPCR, we first assessed the transfection efficiency. In the mimics group, let-7c-5p expression was 7.4-fold higher than the mimics NC group (P = .008) (Figure 3A). However, after transfection with the miRNA inhibitor, the expression of let-7c-5p was significantly decreased compared with the NC group (P = .001) (Figure 3B). We subsequently assessed the total protein synthesis in transfected cells using a puromycin incorporation assay. After let-7c-5p was overexpressed, the total amount of protein was dramatically decreased by 71%, compared to the mimics NC group P = .001) (Figure 3C). In contrast, protein synthesis in the let-7c-5p inhibitor group was 1.8-fold higher than in the inhibitor NC group (P < .001) (Figure 3D).

Let-7c-5p negatively modulates IGF1/mTOR signaling pathway in C2C12 myotubes

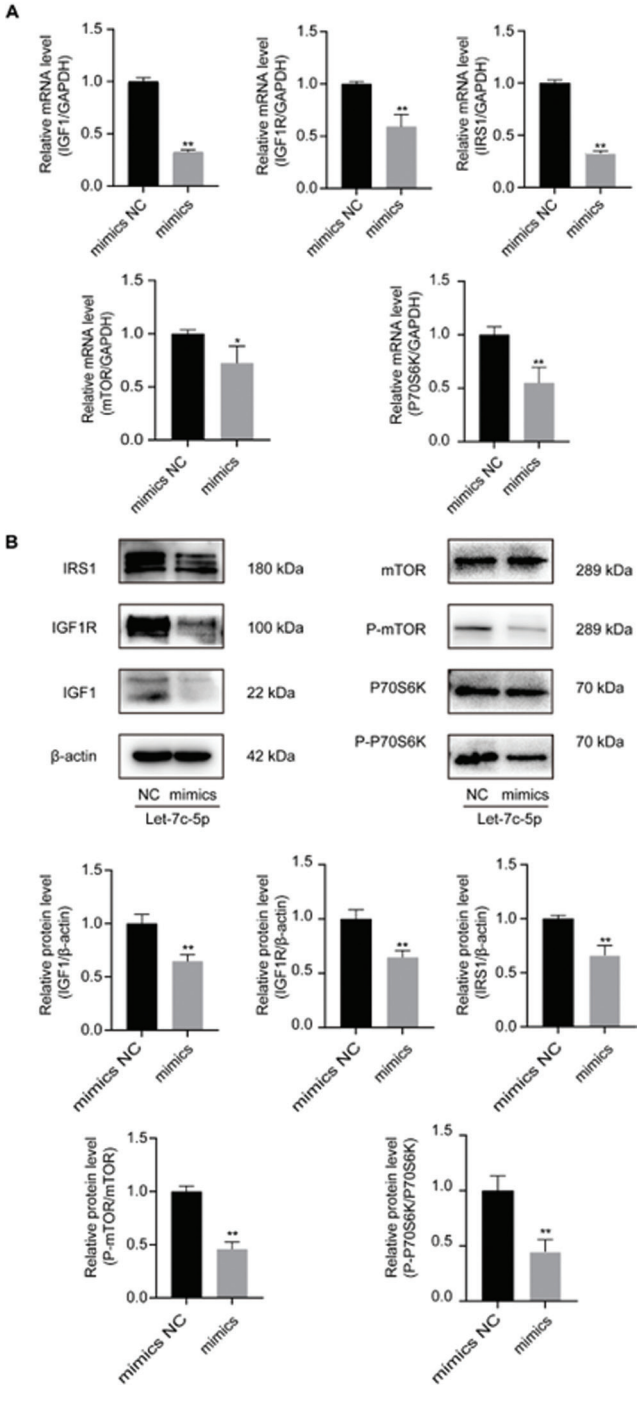
The mRNA expression level of muscle protein synthesis-related genes, such as IGF1, IGF1R, IRS1, mTOR, and P70S6K, were considerably lower in the group with overexpression let-7c-5p compared to those in the control group (P < .001, P = .004, P < .001, P = .042 and P = .008), according to the results of the RT-qPCR analysis (Figure 4A). In addition, western blot results confirmed that the protein level of IGF1, IGF1R, IRS1, phosphorylated mTOR, and p70S6K were significant decreased upon transfection of miRNA mimics (P = .004, P = .002, P = .003, P < .001 and P = .005) (Figure 4B).

Figure 3. Let-7c-5p Inhibitor protein synthesis in C2C12 myotubes. A and B: Total RNA was isolated from C2C12 myotubes transfected with mimics negative control (NC), let-7c-5p mimics, inhibitor NC, and let-7c-5p inhibitor. qRT-PCR was used to assay the let-7c-5p expression after transfection with let-7c-5p (A) mimics and (B) inhibitors. C and D: Puromycin was added into the cell culture medium (1 μM final concentration) 30 min before harvesting the cells. Protein was isolated from the C2C12 myotubes transfected with mimic NC, let-7c-5p mimics, inhibitor NC, and let-7c-5p inhibitor. Puromycin in cell lysates was measured by Western blot after transfection with let-7c-5p (C) mimics and (D) inhibitors. n = 6/group; Each column represents the Means ± SD.



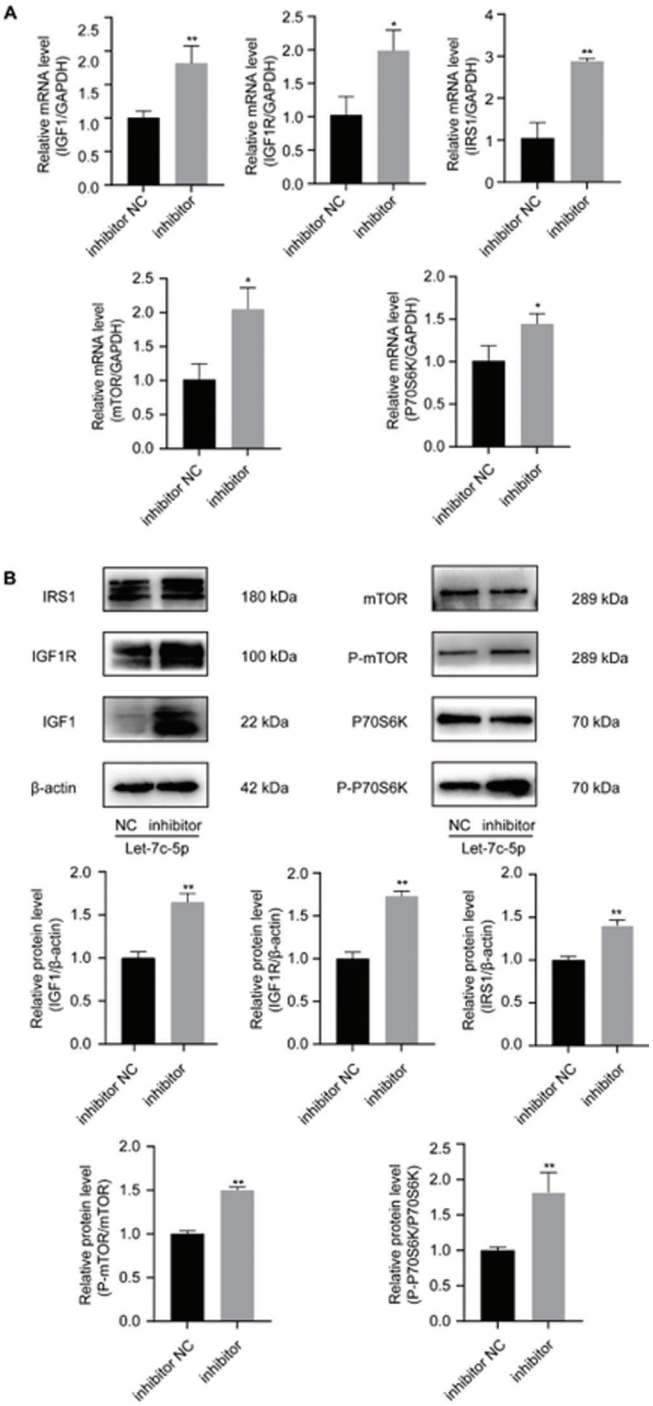
** P < .01 vs mimics NC or inhibitor NC.

Figure 4. Overexpression of let-7c-5p inhibited the IGF1/mTOR signaling pathway in C2C12 myotubes. (A) Total RNA was isolated from C2C12 myotubes transfected with mimics NC, let-7c-5p mimics, inhibitor NC, and let-7c-5p inhibitor. The expression of IGF1, IGF1R, IRS1, mTOR, and P70S6K was assayed by RT-qPCR. (B) The protein synthesis-related markers IGF1, IGF1R, IRS1, mTOR, P-mTOR, P70S6K, and P-P70S6K were measured by Western blot from C2C12 myotubes transfected with mimics NC, let-7c-5p mimics, inhibitor NC, and let-7c-5p inhibitor. n = 6/group; Each column represents the Means ± SD.



**$P < .01$, and *$P < .05$ vs mimics NC.

Figure 5. Downregulation of let-7c-5p promoted IGF1/mTOR signaling pathway in C2C12 myotubes. (A) Total RNA was isolated from C2C12 myotubes transfected with mimics NC, let-7c-5p mimics, inhibitor NC, and let-7c-5p inhibitor. IGF1, IGF1R, IRS1, mTOR, and P70S6K were assayed by RT-qPCR. (B) IGF1, IGF1R, IRS1, mTOR, phosphorylated mTOR, P70S6K, and phosphorylated P70S6K were measured by Western blot from C2C12 myotubes transfected with mimics NC, let-7c-5p mimics, inhibitor NC, and let-7c-5p inhibitor. n = 6/group; Each column represents the Means ± SD.



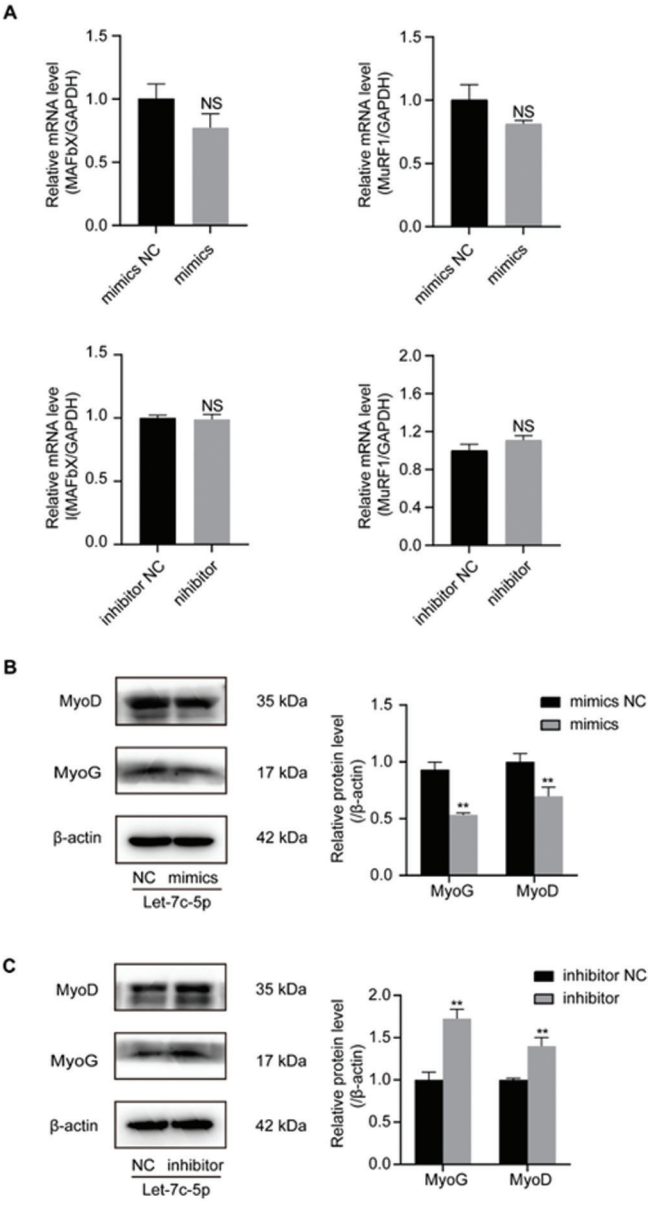
**$P < .01$, and *$P < .05$ vs inhibitor NC.

However, myotubes transfected with miRNA inhibitor showed a considerable rise in the mRNA expression level of IGF1, IGF1R, IRS1, mTOR, and P70S6K ($P = .007$, $P = .015$, $P = .001$, $P = .010$ and $P = .024$) (Figure 5A). The Western blot results showed that the expression level of IGF1, IGF1R, IRS1, phosphorylated mTOR, and p70S6K was declined in the group transfected with the let-7c-5p inhibitor ($P<0.001$, $P = .002$, $P = .009$, $P < .001$ and $P = .007$) (Figure 5B). Let-7c-5p does not change the protein degradation markers atrogin-1/MAFbx and MuRF1 in C2C12 myotubes ($P > .05$) (Figure 6A). These results demonstrate that the downregulation of let-7c-5p promotes muscle protein synthesis by activating IGF1/mTOR/P70S6K signaling pathway.

Let-7c-5p negatively modulates C2C12 myotubes differentiation

The Western blot results showed that the overexpressing of let-7c-5p was associated with lower levels of myogenic differentiation-related markers. We found that the protein expression level of MyoD and MyoG in the let-7c-5p mimics transfected group were significantly decreased than in the control group after differentiation for two days ($P = .008$ and $P = .005$) (Figure 6B). Inhibition of endogenous let-7c-5p increased protein expression level of MyoD and MyoG ($P = .002$ and $P = .001$) (Figure 6C).

Figure 6. Let-7c-5p negatively modulates C2C12 myotubes differentiation. (A) Total RNA was isolated from C2C12 myotubes transfected with mimics NC, let-7c-5p mimics, inhibitor NC, and let-7c-5p inhibitor. RT-qPCR was used to assay for atrogin-1/MAFbx and MuRF1. B and C: The myogenic markers MyoD and MyoG were measured by Western blot from C2C12 myotubes after transfection with let-7c-5p (B) mimics and (C) inhibitor. n = 6/group; Each column represents the Means ± SD.



**$P < .01$, and *$P < .05$ vs mimics NC or inhibitor NC.

DISCUSSION

The skeletal muscle is the largest organ in the human body, which plays a vital role in maintaining posture, body temperature, and exercise. It is also the largest endocrine organ.[39,40] Various diseases can cause skeletal muscle atrophy, seriously affecting daily activities and quality of life. Acu-LFES can improve skeletal muscle atrophy caused by various diseases and has been used in clinical.[12,41,42] Our results indicate that Acu-LFES could promote myogenic differentiation and increase skeletal muscle cross-sectional area by upregulating MyoD and MyoG (Figure 1). Skeletal muscle myogenic differentiation and regeneration depend on the modulation of myogenic regulatory factors (MRFs). The MRF family includes MyoD, Myf5, MRF4, and MyoG.[43,44] MyoD primarily affects the myogenic differentiation process, prompting pre-myogenic cells to differentiate into myogenic cells. Davis et al.,[45] convert a variety of non-myogenic cells into myoblasts by overexpressing MyoD. Moreover, a lack of MyoD has been demonstrated to delay muscle regeneration.[46] MyoG is crucial for the maturation and maintenance of muscle fibers as well as the terminal differentiation of myocytes. It also regulates the fusion of myoblasts. [47]

IGF1 signaling is an essential pathway for protein synthesis and degradation in skeletal muscle, and the liver is the primary source of IGF1. However, autocrine or paracrine IGF1, rather than serum IGF1, primarily controls the metabolism of skeletal muscle proteins. [48-50] IGF1 binds and activates IGF1R, which subsequently recruits IRS1, leading to activation of the PI3K/Akt signaling pathway,[51] and further activation of downstream mTOR, P70S6K, and 4E-BP1 proteins. Phosphorylated P70S6K activates ribosomal s6 protein, which regulates the initiation of mRNA translation proteins. However, when 4EBP1 is phosphorylated, it will be released from the EIF4E-4EBP1 inhibitory complex, thereby allowing the translation initiation factor eIF4E to bind to eIF4G, accelerating translation initiation and promoting protein synthesis.[6,52] Several studies have shown that

upregulation of IGF1 signaling improves muscle atrophy by stimulating cell proliferation, differentiation, and inhibition of apoptosis.[53-56] In line with previous studies, we found that the expression of IGF1 was downregulated in CKD-related muscle atrophy. Acu-LFES upregulates the mRNA and protein expression of IGF1, followed by increased mTOR and P70S6K phosphorylation (Figure 2A, C). In addition, activation of the IGF1 signaling pathway can also lead to FOXO3a activation, and phosphorylated FOXO3a is excluded from the nucleus, and the expression of the E3 ubiquitin ligases atrogin-1/MAFbx and MuRF1 is regulated.[8] The UPS pathway is essential for protein degradation. It consists of ubiquitin, E1 ubiquitin-activating enzyme, E2 ubiquitin-binding enzyme, E3 ubiquitin ligase, proteasome, and deubiquitinating enzymes, which regulate protein by forming polyubiquitin chains.[57]

In our study, we also observed Acu-LFES reverse the increase in MAFbx and MuRF1 induced by CKD (Figure 2B). We confirm that Acu-LFES can regulate skeletal muscle protein synthesis and degradation through the IGF1 signaling pathway.

We also found that Acu-LFES downregulated let-7 expression in skeletal muscle (Fig. 2D). The let-7 family is highly conserved among different animal species and is an essential regulator of individuals' normal development and tumor.[26,58] Studies have shown that let-7 inhibits multiple components of the insulin/PI3K/mTOR pathway, leading to insulin resistance and impaired glucose tolerance,[59] let-7 transgenic mice have impaired glucose tolerance. Let-7 blocks glucose-induced pancreatic insulin secretion through the insulin/IGF1 pathway, and the knockout of let-7 improves pancreatic β cell function and insulin resistance.[60] There has been a report that inhibition of let-7 improves glucose uptake and insulin resistance in diabetic myocardium and protects the myocardium against ischemia-reperfusion injury through the Akt/mTOR pathway.[61] Let-7 affects cell proliferation or differentiation by targeting IGF1 or IGF1R in endometrial stromal cells and spermatogonia.[62,63] Furthermore, let-7 is related to muscle development: Jiang et al.[64] found that let-7 reduced skeletal muscle glucose uptake and glycogen synthesis by inhibiting IL-3 secretion, affecting skeletal muscle mass. In addition, let-7 can improve cardiac hypertrophy by negatively regulating cyclin D2.[34] Therefore, we hypothesized that let-7 is a critical factor in IGF1 upregulation induced by Acu-LFES. We explored the effect of let-7c-5p on myogenesis and protein metabolism of C2C12 cells. By inhibitor IGF1, let-7c-5p suppresses the phosphorylation levels of mTOR and P70S6K. Both the MyoD and MyoG proteins were reduced at the same time. Contrary to expectations, this study did not find a significant difference between atrogin-1/MAFbx and MuRF1. These data suggested that let-7c-5p may not affect protein degradation in skeletal muscle. Nuclear factor-κB (NF-κB) is expressed in skeletal muscle and activated by inflammatory factors, especially tumor necrosis factor-alpha (TNFα). It has been reported that NF-κB is sufficient to cause skeletal muscle atrophy.[65] NF-κB inhibited the differentiation of C2C12 cells and upregulated MuRF1 expression.[66,67] In addition, TNFα promoted MAFbx expression through p38.[68] We tentatively put forward that Acu-LFES inhibitor UPS by NF-κB pathway rather than IGF1 signal, and let-7c-5p do not influence the NF-κB pathway.

In summary, our study suggests that the downregulation of let-7c-5p by electroacupuncture can enhance myogenic differentiation and protein synthesis in skeletal muscle through IGF1 signaling.

CONFLICTS OF INTEREST

The authors report no conflict of interest.

AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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