Electrical acupuncture increases protein synthesis through downregulation of let-7

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**Running title:** inhibition of Let-7 increase protein synthesis

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Abstract (350 words)

**Background:** Our previous study found that acupuncture with low frequency electrical stimulation (Acu/LFES) prevents muscle atrophy by attenuation of protein degradation in chronic kidney disease. However, it is not clear whether Acu/LFES can increase protein synthesis in skeletal muscle.

**Methods:** Normal C57/BL6 mice were given Acu/LFES treatment, applied for 30 minutes once. Gastrocnemius and triceps brachii muscles were harvested at 0, 6, 24, 48 and 72 hours after treatment. Protein synthesis was measured by the surface sensing of translation (SUnSET) assay. Exosomes were harvested using serial centrifugations and subjected to microRNA deep sequencing. The mature microRNA library was validated using a High Sensitivity DNA chip. The Student’s t-test and ANOVA were used for statistics.

**Results:** Protein synthesis was enhanced in the both hindlimb (gastrocnemius) and forelimb (triceps brachii) muscles. To identify how exosomes regulate protein synthesis, we performed microRNA deep sequencing in serum exosomes isolated from treated and untreated mice and found that the 34 microRNAs were altered by Acu/LFES. Specifically, five members of the let-7 miRNA family were significantly decreased in the Acu/LFES treated mice. Blocking exosome secretion using GW4869 before treatment decreased the Acu/LFES-induced increases in protein synthesis. This provided evidence that the increased protein synthesis in response to Acu/LFES is exosome mediated. In cultured C2C12 myotubes, overexpressing let-7c-5p resulted in decreased protein synthesis. Conversely, inhibition of let-7c not only increased protein synthesis, but also increased the expression of Igf1, Igf1 receptor, insulin receptor and Igf2 receptor mRNA. In addition, inhibiting let-7c resulted in enhanced protein abundance of Igf1, Igf1 receptor, mTOR and p70S6. In silico searching suggested that let-7 could target Igf1. Using a luciferase reporter assay, we demonstrated that let-7 directly inhibited Igf1.

**Conclusions:** Acu/LFES increases protein synthesis through a mechanism related with exosome secretion. Acu/LFES on hindlimb decreases let-7-5p in serum exosomes leading to upregulation of the Igf1 signaling pathway and increasing protein synthesis in both hindlimb and forelimb skeletal muscles.

Key words: Acu/LFES, IGF-1 signaling pathway, mTOR, skeletal muscle, microRNA, exosome
Introduction:

Numerous studies have demonstrated that acupuncture with low frequency electrical stimulation (Acu/LFES) can correct muscle atrophy in human and animals with various diseases, including diabetes and chronic kidney disease-induced muscle wasting (1, 2), hindlimb suspension induced muscle loss (3), facioscapulohumeral muscle dystrophy (4), and amyotrophic lateral sclerosis or sciatic nerve injury caused muscle atrophy (5-7). Acu/LFES is widely used worldwide as a therapeutic intervention to reduce stress and other health problems (8). However, little is known regarding the precise mechanisms of this treatment on protein metabolism in muscle.

Skeletal muscle protein metabolism accounts for 75% of the change of the total body protein pool and a fine balance between protein synthesis and protein breakdown regulates skeletal muscle mass. Important determinants of protein synthesis are the key anabolic hormone insulin, growth factor Igf1 and the insulin/Igf1 pathway. Activation of this pathway will upregulate PI3K-Akt-mTOR leading to phosphorylation of mechanistic target of rapamycin complex 1 (mTORC1), and subsequent downstream 4E-binding protein-1 (4EBP1), and the ribosomal protein of 70-kDa S6 kinase 1 (p70S6K1) (9). As the name suggests, p70S6K’s target substrate is the S6 ribosomal protein. Phosphorylation of S6 initiates protein synthesis at the ribosome and proliferation of satellite cells results to increase muscle mass (10). The phosphorylation of p70S6K at threonine 389 has been used as a hallmark of activation by mTORC1. It is well known that resistance exercise stimulates mTORC1 activity, promoting increases in the rates of myofibrillar protein synthesis.

Muscle is recognized as an endocrinal organ. Contracting skeletal muscles have the capacity to communicate with other organs through the release of factors, such as myokines and exosomes for intercellular and inter-organ communication (11, 12). Exosomes are small membranous vesicles that are secreted from muscle fibers inside multivesicular bodies (MVB). The release of exosomes is a common cellular function in living biological systems (13). The exosome could act as messengers in tissue cross talk since these muscle-derived nano-sized vesicles have the ability to deliver useful or harmful molecules (such as cytokines, proteins and miRNAs) to distant organs as well as distant muscles. Both pre-miRNAs and mature miRNAs packaged in exosomes are quite stable (14). These miRs play an important regulatory roles in the mechanisms of adaptation to physiology and pathology conditions. Our previous studies of
exosomes following Acu/LEFS found that this treatment alters the expression of multiple miRNAs that are capable of regulating the physiology in distant organs, including decreased let-7 miRNA (15).

Lethal-7 (let-7) was the second microRNAs (miRNA) to ever be identified. It was originally discovered in the nematode elegans in 2000 (16). Later, let-7 miRNAs were found in various animal species, including human. Unlike the nematode and fruit fly, which have a single isoform, the let-7 family is composed of nine mature let-7 miRNAs encoded by 12 different genomic loci, some of which are clustered together in the human (17). Let-7 miRs have been the focus of a variety of approaches for therapy and diagnosis. For example, Let-7 has been widely studied in oncogene, cell cycle and immunology fields. Many studies showed that let-7 enhances antitumor responses by directly targeting the high mobility group A2 oncogenes and RAS genes (18, 19). Let-7 has been reported to be closely associated with regulation of cell cycle and leads to inhibitor cell proliferation (20). Studies have revealed that let-7 family members act as key regulators for immune response to pathogenic agents in various diseases (21, 22). All together, the let-7 family provides multiple possible strategies for developing approaches of diagnosis markers and therapy.

In this study, we hypothesize that acupuncture increases protein synthesis not only in local, but also in distant muscle, through serum-derived exosomes-encapsulated microRNA. For proof of this hypothesis, we measured 1) protein synthesis in the muscle with or without Acu/LFES treatment and found increased synthesis with treatment; 2) exosome cargoes after Acu/LFES, and found that five members of the Let-7 family were significantly decreased by Acu/LFES; and 3) tested the impact of let-7 on Igf1 signaling pathway and protein synthesis.
Method:

**Animals:** These experiments were approved by the Emory University IACUC (protocol 4000152). The mice (C57BL/6J) were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and were housed with a 12-hour light/12-hour dark cycle.

**Acu-LFES treatment:** The mice were kept in specially designed restraints so that they would remain in a recumbent position during Acu-LFES treatment. Mice were awake without any anesthesia and appeared to be comfortable throughout the treatments. Acupuncture points selected were according to the WHO Standard Acupuncture guidelines (23). The positive point (anode: Yang-Ling-Quan, GB34) is in the hollow of the exterior-inferior of the caput fibulae about 6 mm deep. This position is close to the superficial fibular nerve and deep fibular nerve. The negative point (cathode: Zu-San-Li, ST36) is 5 mm beneath the capitulum fibulae and located laterally and posterior to the knee-joint about 7 mm deep and close to fibular nerve. The impulses were delivered between the two acupuncture needles. Disposable sterile needles with a diameter of 0.25 mm (Shen Li Medical & Health Material Co., Ltd., Wujiang, China) were used. The needles were connected into SDZ-II Electronic acupuncture instrument using consistent pulse, electric frequency 20Hz, electric current 1mA (1).

**Determination of protein synthesis by puromycin incorporation:** To determine the rate of protein synthesis we utilized surface sensing of translation (SUnSET) methodology (24). In vivo, 0.04μmol/g puromycin (Calibiochem, Catalog #: 540222) was injected intraperitoneally into mice before Acu/LFES. Muscle was harvested at 0, 6, 24, 48 and 72 hours after treatment and homogenized in Mueller’s Buffer (50mM HEPES, 0.1% Triton-X100, 4mM EGTA, 10mM EDTA, 15mM Na4P2O7, 100mM β-glycerophosphate, 20mM NaF, 5mM NaVO4 and 1% protease inhibitor cocktail). In vitro, C2C12 myotubes were grown in 6-well plates. Puromycin was added into cell culture medium (1μM final concentration) exactly 30 minutes before harvesting the cells. Cells were scraped into ice-cold RIPA buffer (100μl for one well of 6-well plate) followed by ultrasound sonication on ice. Puromycin-containing proteins were analyzed by Western Blot. Proteins were separated on 10% SDS-PAGE Gels. Anti-puromycin antibody was purchased from Millipore (MABE343; Burlington, MA).

**Isolation of exosome:** Purification and characterization of exosomes from serum, cell debris and organelles were eliminated by centrifugation at 1,000 g for 10 min, 4°C. The supernatant fraction was further centrifuged at 16,000 g for 30 min. The second supernatant
was sterile filtered through a 0.22 μm filter. Exosomes were pelleted at 120,000 g for 90 min at 4°C (L8-70M ultracentrifuge, Beckman-Coulter, Indianapolis IN). Finally, the exosome pellet was re-suspended in 100-400 μl RNA stabilization reagent (Qiagen, Germantown, MD). Exosomal size was verified by NanoSight instrument, and marker (TSG101) was assessed by Western blot (15).

**Reverse transcription and quantitative PCR (q-PCR) for microRNA and mRNA:** Total RNA was extracted using Tri-Reagent (Molecular Research Inc., Cincinnati, OH). For miRNA, the miRCURY LNA™ Universal cDNA Synthesis kit (Exiqon INC., Woburn, MA) was used for reverse transcription of miR. The primers were purchased from Exiqon. The miRCURY LNA microRNA PCR SYBR Green master mix (Exiqon INC) was used for qPCR with the following cycle parameters: 95°C for 10 minutes and 40 cycles at 95°C for 10 seconds and 60°C for 60 seconds. Expression of individual microRNA was standardized to the mouse U6 gene (tissue) or miR103 (serum) (1, 25). For mRNA, the reverse transcription was used Thermoscript RT-PCR kit (Invitrogen Carlsbad, CA). Real-time qPCR was performed with SYBR Green PCR Reagents (Bio-Rad, Hercules, CA) using the following cycle parameters: 94°C for 2 minutes and 40 cycles at 94°C for 15 seconds, 55°C for 30 seconds, 72°C for 30 seconds with final extension at 72°C for 10 minutes. The quantification cycle (Cq) values was defined as the number of cycles required for the fluorescence signal to exceed the detection threshold. Individual miRNA or mRNA expression was calculated as the difference between the threshold values of the two genes (2^-Δcq). Melting curve analysis was routinely performed to verify the specificity of the reaction. Let-7-5p (YP00204767) was ordered from Qiagen (Germantown, MD).

**miRNA-Seq Library Preparation and Sequencing:** Qualitative and quantitative analysis of the total RNA was performed using the Thermo Nanodrop 2000 and Agilent 2100 Bioanalyzer respectively. Small RNA libraries were prepared using the SeqMatic tailormix miRNA sample preparation kit (SeqMatic. Union City, CA, USA) as per manufacturer’s instructions. Briefly, 100 ng of total RNA was used for library preparation. Small RNA’s were ligated with Illumina compatible adapters and each sample was tagged with a unique barcode to allow multiplexing. The adapter-ligated libraries were then enriched using PCR amplification followed by gel enrichment for mature miRNA library. The amplified library was validated using a High Sensitivity DNA chip on the Agilent Bioanalyzer. The libraries were further quantified on Qubit® 2.0 Fluorometer (Life Technologies, Grand Island, NY, USA) using the High Sensitivity dsDNA
assay. Libraries from all the samples were multiplexed and run in a single lane of Illumina 3K flowcell. PhiX was used as an internal control on each lane to monitor the error statistics. Cluster generation was performed on the v3 flowcell on the Illumina cBot. The clustered flowcell was sequenced on the Illumina HiSeq3000 system as a 100-cycle single read multiplexed run.

**Cell culture:** C2C12 cells (ATCC, Manassas, VA), studied between passages 3 and 9, were cultured in growth medium (Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% fetal bovine serum, 10% cow serum, 25 mM glucose, 100 u/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine). Myotube differentiation was induced by replaced growth medium to differentiation media (FBS and cow serum was replaced by 2% horse serum).

**Luciferase reporter assay and transfection:** Effectene transfection reagent was used for transfection (Qiagen). Firefly and Renilla luciferase activities were measured by dual-luciferase assays (Promega) using TD-20/20 Luminometer (Turner designs, Sunnyvale, CA) (26). The luciferase report vectors (pMIR-REPORT Luciferase) were purchase from Applied BIOSYSTEMS (Waltham, MA) and constructs were made by Emory Integrated Genomics Core.

**Statistical analysis:** Data were presented as mean ± se. To identify significant differences between two groups, comparisons were made by using the Student’s t-test. When multiple treatments were compared, ANOVA was performed with a post hoc analysis by the Student-Newman-Keuls test. Differences with P values < 0.05 were considered significant.
Results:

1. Acu/LFES in hindlimb increased protein synthesis in both gastrocnemius and triceps brachii muscles. Our previous studies have found that Acu/LFES decreases CKD-induced protein degradation and improves muscle function (1). To investigate whether Acu/LFES alters protein synthesis, we verified the protein synthesis in normal mice after Acu/LFES. Acu/LFES mice received needles in point GB34 and S36 of hindlimb using a consistent pulse for a one-time 30 minutes of electrical stimulation. Sham mice had acupuncture needles inserted in close proximity to the ACU/LFES insertion position, needles were connected to the LFES device but electrical stimulation was not applied. In gastrocnemius muscle, which is close to the electrical stimulation area, Acu/LFES increased protein synthesis by 1.7-fold immediately after treatment and protein synthesis continued to increase up to the last experimental reading at 72 hours (Figure 1A). Interestingly, protein synthesis also significantly increased in triceps brachii muscle, which is not near the electrically stimulated area (Figure 1B). The protein synthesis related proteins phosphorylated mTORC1 and p70S6 also significantly increased, apparent at 6 hours after treatment through the terminal 24 hour time point (Figure 1C). In normal mice, Acu/LFES did not change the protein degradation markers TRIM63/MuRF1, FBXO32/atrogin-1 and myostatin (Figure 1D).

2. Blocking exosome secretion limited the Acu/LFES-induced increase in protein synthesis. Our previous research found that Acu/LFES increases exosome secretion (15). To explore whether the increasing protein synthesis in triceps brachii is due to exosome-mediated regulation, we used GW4869 to inhibit exosome secretion. Mice were injected with 1 μg/g body weight of GW4869 16 hours before Acu/LFES. In gastrocnemius muscle, the increase of protein synthesis was maintained from 6-hours to 24-hours after Acu/LFES treatment, but was not apparent in animals after 48- and 72-hours (Figure 2A). In normal mice without exosome blockers, the increase in protein synthesis was maintained up to 72 hours. In triceps brachii muscle from mice treated with GW4869, Acu/LFES-induced protein synthesis was apparent at the 6-hour point but not at any later time points (Figure 2B). These results suggest that Acu/LFES-induced increase in muscle protein synthesis in distant muscles might be associated with exosome secretion.

3. Acu/LFES decreased let-7 in serum exosomes and skeletal muscles. The Acu/LFES induced increase in protein synthesis in distant muscle could be due to circulating exosomes
carrying microRNAs. To explore this possibility, miRNA deep sequencing was performed on serum-derived exosomes from both sham- and Acu/LFES-treated mice. We found that five members of the let-7 miRNA family were significantly decreased by Acu/LFES (Table 1). The largest change is Let-7c-5P, which was 79% decreased by Acu/LFES treatment. In addition, the expressions of Let-7b-5p, let-7e-5p, let-7a-5p and let-7f-5p were also significantly decreased by 78%, 71%, 78% and 75% respectively (Table 1). To verify the deep-sequence data, we isolated RNA from serum exosomes of sham and Acu/LFES mice and measured let-7c-5p miRNA by real-time qPCR. The expression of let-7c-5p was decreased 75% in the serum exosomes from Acu/LFES mice vs. sham mice (Figure 3A). To validate whether Acu/LFES also altered let-7 miRNA in muscle tissue, the expression of let-7c-5p was measured in skeletal muscle of mice. In gastrocnemius, the expression of let-7c-5p was significantly decreased at 0, 6, 24 and 48 hours after treatment (Figure 3A). Similar changes were observed in triceps brachii muscle (data not shown).

According to a miRNA database search, Let-7-5p targets insulin-like growth factor 1 (Igf1), Igf2 and insulin receptor (insr), Igf1 receptor (Igf1r), Igf2 receptor (Igf2r), Igf1 binding protein 5 (Igf1bp5), and insulin receptor substrate (IRS) 1 and 2 (27). To identify whether Acu/LFES alters the mRNA of these targets, qPCR was performed. The expression of all these mRNAs were sharply increased immediately after Acu/LFES with the exception of Igf1bp5, which showed a delayed increase. The most significant change was in Igf1, Igf2, Igf1r and Igf2r, which maintained their increase until 48 hours after Acu/LFES (Figure 3B). Igf1bp5 expression was significantly increased at 24 to 48 hours after acupuncture. Protein level of Igf1 also significantly increase from 0-hours to 24-hours after Acu/LFES treatment by Western blot measurement (Figure 3C). Increasing the level of Igf1rα and Igf1rβ also increased from 6 to 24 hours (Figure 3C).

4. Overexpressing let-7c-5p decreased protein synthesis in cultured C2C12 myotubes. To examine whether let-7 could change protein synthesis, we transfected let-7c-5p miRNA mimic or its inhibitor into cultured C2C12 cells. First, we tested whether let-7c-5p was successfully overexpressed. The expression of let-7 was increased 10.2-fold in the cells transfected with let-7c-5p mimic, and 15% decreased following transfection of the let-7 inhibitor compared with the control mimic group (Figure 4A). Second, we measured protein synthesis using puromycin incorporation and found that overexpressing let-7 significantly decreased protein synthesis.
Inhibition of endogenous let-7c-5p increased protein synthesis 1.28-fold (Figure 4B). Providing let-7c-5p also decreased the ratio of phospho to total mTORC1 and p70S6, two major protein synthesis related molecules. Inhibition of let-7c-5p did not alter the phosphorylation of these two proteins (Figure 4C).

5. **Provision of let-7 inhibited insulin/IGF-1 signaling pathway components in cultured C2C12 myotubes.** Since decreasing let-7-5p showed upregulation of insulin/Igf1 signaling pathway in animals, the protein and mRNA levels of these targets were investigated in the cultured cells. Providing let-7c-5p to the C2C12 cells in culture resulted in a decline in the expression of Igf1, Igf1r, Igf2r and Irs1. Conversely, transfection of the let-7 inhibitor enhanced the levels of Igf1, Igf2, Igf1r, Igf2r, Igfbp5 and Irs2 (Figure 5A). These results were further supported by Western blot analysis (Figure 5B). Increasing the level of let-7c-5p suppressed Igf1. Decreasing endogenous let-7 expression raised the amount of Igf1, Igf receptor α, but not in Igf receptor β (Figure 5B). The 3'-UTR of Igf1 contains a conserved binding site for Let-7 miRNA according to a consensus sequence search. To experimentally confirm that let-7-5p interacts with the Igf1 mRNA, the Igf1 target site of let-7 (1360 to 1367 nt on Igf1 3'-UTR) was cloned into a luciferase reporter construct (pLUC-Igf1/3UTR). When cells were transfected with pLUC-Igf1/3UTR along with let-7c-5p miRNA, luciferase activity was decreased. When cells were transduced with an Igf1-luciferase construct in which the binding site had been mutated (pLUC-Igf/mut), this decrease was abolished (Figure 5C). These results confirmed that let-7-5p directly targets Igf1 and blocks its translation.
Discussion:

In this study, we provide evidence showing that Acu/LFES administered to the hind limb muscles results in enhanced protein synthesis in both hindlimb and forelimb of mice. In addition, we showed that the treatment decreases circulation of let-7 miRNA in exosomes, which has the potential to influence distant muscles. A decrease in let-7 would increase the production of multiple insulin/Igf1 related mRNAs; therefore, the consequence of let-7 inhibition would be increased protein synthesis.

It is well known that resistance exercise increases muscle mass through upregulation of the IGF-1 signaling pathway. Many tissues secrete IGF-1, including liver and skeletal muscle. Circulating Igf1 is largely contributed by the liver. However, skeletal muscle protein synthesis does not depend on plasma Igf1 (28). Instead, intrinsic secretion of muscle IGF-1 is a key determinant for activation of protein synthesis in muscle. Activation of the IGF-1 pathway results in phosphorylation of Akt, followed by upregulation of the mechanistic target of rapamycin complex 1 (mTORC1) (29). In our study, we found that acupuncture mimics resistance exercise, in that it upregulates the IGF-1 signaling pathway for at least 48 hours leading to increased protein synthesis. The half-life of IGF-1 is only 5-10 minutes (30), so how is the increased protein synthesis supported for 48 hours? We believe that key is the acupuncture-induced decrease in let-7 (Figure 3A) that results in increased levels of IGF-1 for 48 hours and the consequent activation of protein synthesis (Figure 1A). In addition, the lower level of circulating let-7 would remove a repressive effect resulting in activation of IGF-1/Akt/mTORC1 signaling in distant muscles. Patients with severe diseases that have muscle wasting frequently are unable to exercise to stimulate protein synthesis so it is important to explore other treatments that will provide them with a similar benefit. Acupuncture will help them to increase muscle protein synthesis and prevent muscle wasting.

The let-7 family is involved with maintenance of muscle mass. An increase in let-7 is often associated with muscle loss. Oculopharyngeal muscle dystrophy patients have significantly increased expression of let-7 (31). Muscle biopsy studies found that the expression of let-7s was increased in the skeletal muscle in humans with lower limb immobilization (32). However, there is some controversy about this role. In healthy men with 21-days bed rest (33) let-7 was increased; but in another study, the expression of let-7 was decreased in skeletal muscle at 10-days of bed rest in healthy man (34). Our current study found that seven members
of the let-7 family were decreased in normal mice after acupuncture. These changes were associated with an increase in protein synthesis and upregulation of the IGF-1 signaling pathway.

The impact of let-7 on the IGF1 signaling pathway could differ in various tissues. Our current study indicates that let-7c-5p directly targets Igf1 and inhibits its translation in skeletal muscle. The consequence of inhibiting IGF1 is inhibition of the downstream signaling pathway. For example, decreasing the activity or availability of mTORC1 would reduce protein synthesis, which is what we see in our acupuncture mice. The mTORC1 complex is a key player of nutrient status, and when activated, mTORC1 promotes protein synthesis, lipogenesis and energy metabolism (35). Dubinsky et al showed that let-7 plays a critical role in nutrient homeostasis and proteostasis regulation by repression of mTORC1 in brain, in this case, without influencing upstream the IGF1 signaling pathway. This paper demonstrates that let-7 only represses mTOR activation without turning off the insulin-signaling pathway in brain (36). However, other recent articles describe let-7 directly targeting Igf-1 and/or Igf1 receptor in human colorectal cancer cell (37), in endometrial stromal cells (38) and in cultured testicular fragments (39). Another study showed that elevated let-7 expression increased insulin resistance while inhibition of the let-7 reduced insulin resistance and improved glucose uptake in the diabetic myocardium through Akt and mTOR pathways (40).

Acupuncture involves the insertion of very thin needles through the skin at strategic points on the body; it is part of the ancient practice of Traditional Chinese Medicine. The most common role of acupuncture is to treat pain, for stress management and for overall wellness. Traditional Chinese Medicine practitioners believe the human body has more than 2,000 acupuncture points connected by channels or meridians, named Jing-Luo (collaterals). These channels create an energy flow (Qi, pronounced "chee") through the body that is responsible for overall health. Disruption of the energy flow can cause disease. By applying acupuncture to certain points, it is thought to improve the flow of Qi, thereby improving health for distant organs. However, no anatomy study has identified structural components of the Jing-Luo in human body. In this study, we found that acupuncture changes the exosome concentration, size, and cargo, such as microRNA, that is in the circulation. We believe that muscle-derived exosomes in circulation play the role of Jing-Luo and exosome-carried cargo plays the role of Qi. This is a new concept to explain ancient medicine.
Conclusions: Acu/LFES in the hindlimb releases exosomes into the circulation where it can move to and influence distant muscle to increase protein synthesis. The increase in protein synthesis in response to Acu/LFES is a consequence of the decrease in exosome-carried let-7 miRNA. Since let-7 targets and inhibits multiple Igf1 signaling pathway components, limitation of let-7 upregulates this pathway and leads to increased protein synthesis. Our study provides strong mechanistic insights for understanding the benefits of treating muscle atrophy with Acu/LFES.

List of abbreviations
Acu/LFES: acupuncture with low frequency electrical stimulation
SUnSET: surface sensing of translation
mTORC1: mechanistic target of rapamycin complex 1
p70S6K1: ribosomal protein of 70-kDa S6 kinase 1
let-7: lethal-7
miRNA: microRNAs
IRS: insulin receptor substrate
IGF-1: Insulin-like growth factor 1
3’-UTR: three prime untranslated region
4EBP1: 4E-binding protein-1

Declarations
The Emory University Institutional Animal Care and Use Committee (IACUC) approved all experiments (protocol 4000152).

Consent for publication:
Not applicable

Availability of data and materials:
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.
**Competing interests:**
The all authors declare that they have no competing interests.

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**Authors’ contributions**
XHW designed experiments. ZS, MY, AK, FH FM performed experiments and analyzed data. ZS and MY prepared initial manuscript. XHW and JDK wrote the manuscript. All authors read and approved the final manuscript.

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**Figure legend:**

**Figure 1. Acu/LFES in hindlimb increased protein synthesis in both gastrocnemius and triceps brachii muscles:**
Experiments were performed in the sham and Acu/LFES treated mice. Protein was isolated from the muscle of mice immediately (0), 6-, 24-, 48- and 72-hours after Acu/LFES. Puromycin was injected 30 minutes before treatment. Puromycin in tissue lysates was measured by Western blots. The point graph of incorporate puromycin into gastrocnemius muscle (A) and triceps brachii muscle (B) show the change of the density of puromycin protein normalized to their corresponding GAPDH protein (n = 6/group; * = p<0.05 vs. sham). (C) The proteins mTORC1 and P70S6 were measured by Western blotting in sham and Acu/LFES mice. The bottom point graphs show the ration of phosphate protein to total protein normalized to the same sample GAPDH (n = 6/group; * = p<0.05 vs. sham). (D) The proteins TRIM63/MuRF1, FBXO32/atrogen-1 and myostatin were measured by Western blotting in sham and Acu/LFES mice.

**Figure 2. Blocking exosome secretion limited the Acu/LFES-induced increase in protein synthesis:**
Experiments were performed in the sham and Acu/LFES treated mice. GW4869 was injected 16 hours and puromycin was injected 30 minutes before treatment. Protein was isolated from the muscle of mice immediately (0), 6-, 24, 48 and 72 hours after Acu/LFES. Puromycin in tissue lysates was measured by Western blots. The point graph of incorporate puromycin into gastrocnemius muscle (A) and triceps brachii muscle (B) show the change of the density of puromycin protein normalized to their corresponding GAPDH protein (n = 6/group; * = p<0.05 vs. sham).

**Figure 3. Acu/LFES decreased let-7 in serum exosome and skeletal muscles:**
Total RNA was isolated from serum exosomes (A), gastrocnemius muscle (B) of sham and Acu/LFES treated mice. The expression of let-7c-5p was assayed by real time qPCR. The bar graph shows microRNA from the exosomes of Acu/LFES mice compared with levels in shams (defined as 1-fold). Results are normalized to miR-103a for serum and U6 for muscle (Bars: mean ± s.e.; n = 6/group; *= p<0.05 vs. sham). (C) Protein was isolated from gastrocnemius
muscle. Igf1, Igfrα and Igfrβ measured by Western blots. The point graph of protein showed the change of the density of proteins normalized to their corresponding GAPDH protein (n = 6/group; * = p<0.05 vs. ctrl/mimic).

Figure 4. Overexpressing let-7c-5p decreased protein synthesis in cultured C2C12 myotubes: The C2C12 myotubes were transfected with control/mimic, let-7-5p/mimic and let-7/inhibitor. (A) Total RNA was isolated from myotubes and the expression of let-7-5p miRNA was assayed by real time qPCR. The bar graph shows the miRNA from let-7/mimic and let-7/inhibitor compared with levels in control/mimic (defined as 1-fold). Results are normalized to miR-103a (Bars: mean ± s.e.; n = 9/group; *= p<0.05 vs. ctrl/mimic). (B) Protein was isolated from myotube and puromycin in cell lysates were measured by Western blots. The point graph of incorporate puromycin into myotubes showed the change of the density of puromycin protein normalized to their corresponding GAPDH protein (n = 6/group; * = p<0.05 vs. ctrl/mimic). (C) The ration of phosphate protein to total protein (p/t) of mTORC1 and p70S6 were measured by Western blotting in myotubes transfected with control/mimic, let-7-5p/mimic. The protein results were normalized to the GAPDH (n = 6/group; * = p<0.05 vs. ctrl/mimic).

Figure 5. Provision of let-7 inhibited insulin/IGF-1 signaling pathway components in cultured C2C12 myotubes: (A) Total RNA was isolated from C2C12 myotubes transfected with control/mimic, let-7-5p/mimic and let-7/inhibitor. The expression of indicated mRNA was assayed by real time qPCR. The bar graph shows mRNA from different group compared with levels in control/mimic (defined as 1-fold). Results are normalized to 18S (Bars: mean ± s.e.; n = 6/group; *= p<0.05 vs. control/mimic). (B) Protein was isolated from the C2C12 myotubes transfected with control/mimic, let-7-5p/mimic and let-7/inhibitor. Igf1, Igfrα and Igfrβ in myotube lysates was measured by Western blots. The point graph showed the change of the density of each protein normalized to their corresponding GAPDH protein (n = 6/group; * = p<0.05 vs. control/mimic). (C) C2C12 cells were transfected with luciferase pMIR-ctrl vector (white bars) or the vector containing the 3'-UTR of Igf1 (pMIR-Igf: black bars). Cells were co-transfected with control mimic (miR-ctrl), let-7c-5p or let-7 inhibitor. Luciferase activity in cells that received the pMIR-ctrl with miR-ctrl was designated as the 100% (far left white bar). The other bars show the response to
let-7 expressed as a percent of this control. Triplicate determinations were made in each condition and each experiment was repeated twice; the firefly luciferase (FFL) results were normalized by renilla luciferase (RL) activity. Data: mean ± s.e.; n=6; * = p<0.05 vs. pMIR-ctrl+miR-ctrl.
Figure 1A & B: Acu/LFES in hindlimb increased protein synthesis in both gastrocnemius and triceps brachii muscles:
Experiments were performed in the sham and Acu/LFES treated mice. Protein was isolated from the muscle of mice immediately (0), 6-, 24-, 48- and 72-hours after Acu/LFES. Puromycin was injected 30 minutes before treatment. Puromycin in tissue lysates was measured by Western blots. The point graph of incorporate puromycin into gastrocnemius muscle (A) and triceps brachii muscle (B) show the change of the density of puromycin protein normalized to their corresponding GAPDH protein (n = 6/group; * = p<0.05 vs. sham).
Figure 1C & D: Acu/LFES in hindlimb increased protein synthesis in both gastrocnemius and triceps brachii muscles:
Experiments were performed in the sham and Acu/LFES treated mice. Protein was isolated from the muscle of mice immediately (0), 6-, 24-, 48- and 72-hours after Acu/LFES (C) The proteins mTORC1 and P70S6 were measured by Western blotting in sham and Acu/LFES mice. The bottom point graphs show the ration of phosphate protein to total protein normalized to the same sample GAPDH (n = 6/group; * = p<0.05 vs. sham). (D) The proteins TRIM63/MuRF1, FBXO32/atrogin-1 and myostatin were measured by Western blotting in sham and Acu/LFES mice.
Figure 2. Blocking exosome secretion limited the Acu/LFES-induced increase in protein synthesis: Experiments were performed in the sham and Acu/LFES treated mice. GW4869 was injected 16 hours and puromycin was injected 30 minutes before treatment. Protein was isolated from the muscle of mice immediately (0), 6-, 24, 48 and 72 hours after Acu/LFES. Puromycin in tissue lysates was measured by Western blots. The point graph of incorporate puromycin into gastrocnemius muscle (A) and triceps brachii muscle (B) show the change of the density of puromycin protein normalized to their corresponding GAPDH protein (n = 6/group; * = p<0.05 vs. sham).
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