

Original Article

MURF contributes to skeletal muscle atrophy through suppressing autophagy

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Abstract: Introduction: Muscle atrophy due to denervation, immobilization, or unweighting inflicts large number of patients, and provides a huge challenge to modern medicine. MuRF-1 (muscle RING Finger 1), an E3 ubiquitin ligase, has been shown to be upregulated in atrophy muscle, while how MuRF-1 contributes to muscle atrophy remain elusive. Method: In the present study, we compared MuRF expression in skeletal muscle from denervation muscle atrophy patients or health control. Results: When MuRF is overexpressed, autophagy is considerably inhibited in skeletal cell C2C12, while autophagy is reversed if MuRF knocked down. Further analysis demonstrated that MuRF dampens skeletal muscle cell proliferation through downregulation of autophagy, which is an essential event for muscle atrophy. Discussion: Our study comes up with a new mechanism of MuRF for muscle atrophy, which potentially invoking a novel therapeutic approach for the disease.

Keywords: MuRF, autophagy, muscle atrophy

Introduction

Skeletal muscle atrophy results from an array of conditions, such as aging, starvation, cancer, diabetes, bed rest, hormone dysfunction, and denervation [1]. As skeletal muscle fibers are multinucleated structures, protein turnover, cellular turnover, as well as nuclear turnover regulate muscle mass and fiber size. Any factor affecting protein, cellular and nuclear turnover may contribute to muscle atrophy. Muscle atrophy due to denervation, immobilization, or unweighting inflicts large number of patients, and provides a huge challenge to modern medicine [2-4].

MuRF-1 (muscle RING Finger 1), an E3 ubiquitin ligase, together with MAFbx (muscle atrophy F-box), another E3 ubiquitin ligase, have been shown to be upregulated in atrophy muscle due to denervation, immobilization, or unweighting [5]. Working with ubiquitin-activating enzyme (E1) and ubiquitin-conjugating enzyme (E2), E3 ubiquitin ligase transfers ubiquitin to the target protein, leading to degradation of target protein or other biological consequences. Multiple

proteins have been suggested to be targets of MuRF-1, such as contractile and structural proteins as titin, troponin1, myosin heavy and light chains, myosin-binding protein C [6, 7], and may contribute to muscle atrophy through diverse mechanism, including interrupting glycolysis and glycogen metabolism [8]. However, the fundamental mechanism remains to be elucidated.

Autophagy is an evolutionary conserved cellular process to maintain cellular autonomous homeostasis via "self-eating" cell's own cytosolic components, such as synthesized protein, even organelles. Besides starvation, a myriad of extrinsic and intrinsic effectors, including growth factors and intracellular signaling, can initiate autophagy. Autophagy signaling, such as ULK1 and Atg 7, has been shown to be involved in skeletal muscle growth [9, 10], and dysregulation of autophagy has been observed in mice model of spinal muscular atrophy [11]. In addition, enhanced autophagy by simvastatin improves physiological function in muscular atrophy [12], highlighting the essential role of autophagy for maintaining muscular

MuRF negatively regulates autophagy

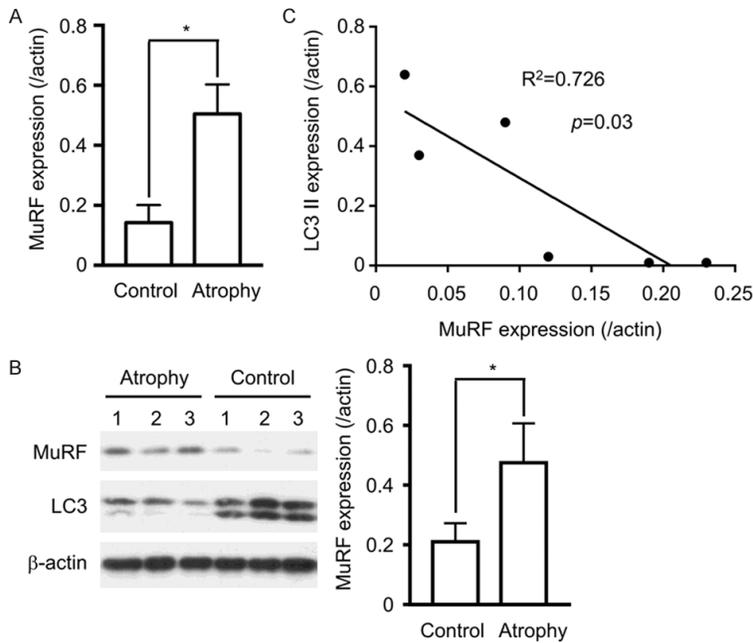


Figure 1. MuRF expression and autophagy in atrophy muscle. Muscle from patients or health control were homogenized, followed by preparation of total RNA with Trizol, or preparation of protein lysates with RIPA. (A) Total RNA was reverse-transcribed and then real time PCR was performed using specific primer pairs. Data are mean \pm SEM of three independent experiments. Protein lysates were analyzed by immunoblotting with specific antibodies for the expression of MuRF, LC3. Densitometry quantitation of MuRF bands over β -actin or LC3 II over β -actin using ImageJ (B). (C) Correlation analysis between expression of MuRF and LC3 II. Data are mean \pm SEM of three independent experiments and analyzed with Student's *t* test, **P* < 0.05 was considered as statistically significant.

homeostasis. Other than muscle cells, autophagy also maintain stemness of muscle stem cell, which is essential for muscle regeneration [13]. However, how autophagy is regulated in muscular atrophy remains to be elucidated.

In the present study, our results shows that MuRF-1 expression is reciprocally associated with autophagy in skeletal muscle and further data demonstrate that MuRF-1 negatively regulates autophagy in skeletal muscle cells, suggesting a novel mechanism of MuRF-1 in atrophy, which potentially put up a new therapy for muscle atrophy.

Materials and methods

Patients and samples

Muscle tissues were from denervation muscle atrophy patients or health control, with written consents from all involved persons. This study was approved by the Ethics Committee of Huashan hospital, Fudan University.

Reagents and antibodies

All antibodies for Western blotting were from Abcam if not specified (Cambridge, MA). Antibodies for β -actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against MuRF was obtained from Biologends (San Diego, CA). HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA).

Cell culture and transfection

Myoblast C2C12 cell line was purchased from American Type Culture Collection (ATCC, Bethesda, MD) and was cultured in DMEM medium supplemented with 10% FBS.

siRNAs targeting indicated genes and scrambled siRNA were purchased from Life Technologies (Grand Island, NY). C2C12 cells were plated in 24-well plates (2×10^5 cells/well) and transfected the next day with 30 pmol siRNA and 2

μ l lipofectamine 2000 according to the manufacturer's instructions. After 48 h, the siRNA-transfected cells were analyzed or used for other experiments.

RT-PCR

RT-PCR for MuRF isoforms was performed using specific primer pairs. Total RNA was extracted from C2C12 cells using TRIzol[®] reagent (Invitrogen, Carlsbad, CA), and the RNA was reverse transcribed using uperscript II (Invitrogen) and random hexamer primers. The cDNA as used as template for PCR with specific primer pairs, and PCR products were analyzed by agarose gel electrophoresis and visualized under V light with ethidium bromide.

Immunoblotting

Immunoblotting was performed as previously described [14]. Cells were collected and lysed with lysis buffer [50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.5% Nonidet P-40, plus complete

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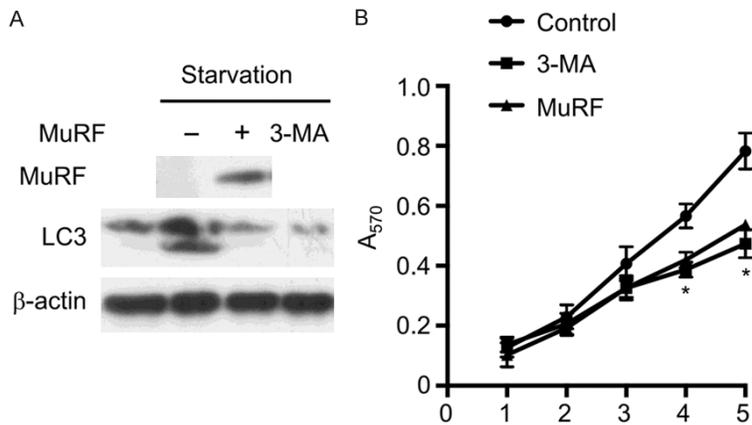


Figure 2. MuRF overexpression inhibits autophagy in C2C12 cells. A. C2C12 cells were transiently transfected with MuRF plasmid. 48 h later, the cells were subjected to starvation for 4 hours and lysed, followed by SDS-PAGE and transfer to nitrocellulose membrane. After blotting with LC3 antibody, the membrane was incubated with HRP-conjugated secondary antibody, and visualized with an ECL chemiluminescence kit. B. C2C12 cells with or without MuRF overexpression were plated in 24-well plates, and MTT assay was used to determine the proliferation of the cells. Data are mean \pm SEM of three independent experiments and analyzed with Student's *t* test, **P* < 0.05 was considered as statistically significant.

protease inhibitor mixture (Roche Applied Science, 04693116001)]. After brief vortexing and rotation, cell lysates were subjected to SDS-PAGE and then transferred to PVDF membranes. Following by incubation with primary antibody and then with proper HRP-conjugated secondary antibody. The immunoreactive bands were detected with ECL plus immunoblotting detection reagents (Amersham Pharmacia Biotech).

Autophagy analyses

Autophagy was analyzed by immunoblotting as described previously [15]. In the immunoblotting analysis, cells were treated as indicated, and cell lysates were immunoblotted with anti-MAP1LC3A antibody to monitor the MAP1LC3A-II generated during the formation of autophagosomes. Each experiment was performed at least 3 independent times.

MTT cell proliferation assay

Vybrant MTT cell proliferation assay kit (Invitrogen, Eugene, OR) was used to determine the cell proliferation according to manufacturer's instructions.

Statistical analysis

The two-tailed Student's *t* test was used for all statistical analyses in this study. A *p* value less

than 0.05 was considered as statistically significant.

Results

Association between MuRF expression and autophagy

In order to understand the possible association between MuRF-1 expression and autophagy, we prepared total RNA and protein lysates of skeletal muscles from muscle atrophy patients or healthy controls. We performed real time PCR and western blot for MuRF, and western blot for LC3, a commonly used marker for autophagy, and Atg5, a key regulator for autophagy. Our results showed that MuRF was downregulated in atrophy muscles, both in mRNA and protein level (Figure 1A and 1B), while

autophagy was inhibited in atrophy muscles based on LC3 (Figure 1B). Further analysis revealed a strong association between MuRF expression (protein) and autophagy (based on LC3 II) (Figure 1C). Thus, our data shows a potential association between MuRF-1 and autophagy in muscle.

Overexpression of MuRF inhibits autophagy

To explore whether MuRF regulates autophagy in muscle cells, we determined to overexpress MuRF in myoblast cell C2C12, which display a low or no expression of MuRF (Figure 2A). We constructed a MuRF-expressing plasmid and transfected C2C12 cells, which considerably upregulated the expression of MuRF in C2C12 cells (Figure 2A). Starvation strongly induces autophagy in C2C12 cells, while overexpression of MuRF obviates the induction of autophagy by starvation, roughly the same as treatment with autophagy inhibitor, 3-MA (Figure 2A). As autophagy regulates cellular proliferation, which plays essential role in muscular atrophy, so we would like detect whether overexpression of MuRF affects C2C12 proliferation. As shown in Figure 2B, autophagy inhibition with 3-MA inhibits C2C12 proliferation, and overexpression of MuRF showed a similar effect, strongly suggesting essential role of autophagy in MuRF-mediated proliferation. So our results support the idea that MuRF restrains

MuRF negatively regulates autophagy

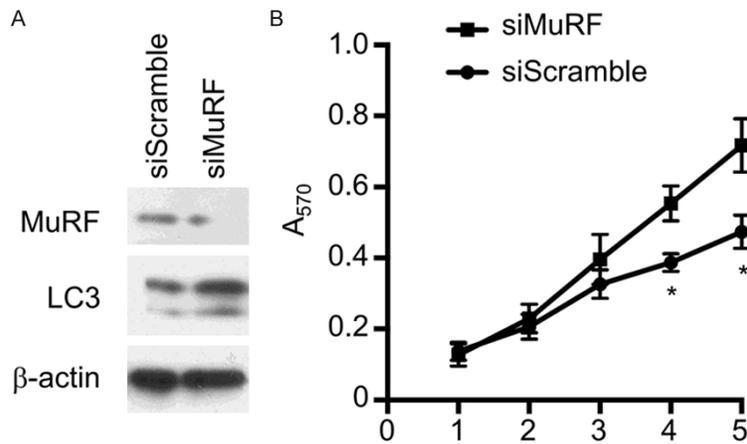


Figure 3. MuRF knockdown reverses autophagy in C2C12 cells. A. C2C12 cells overexpressed MuRF were transiently transfected with MuRF specific siRNA. 48 h later, the cells were subjected to starvation for 4 hours and lysed, followed by SDS-PAGE and transfer to nitrocellulose membrane. After blotting with MuRF antibody, the membrane was incubated with HRP-conjugated secondary antibody, and visualized with an ECL chemiluminescence kit. B. C2C12 cells with or without MuRF knockdown were plated in 24-well plates, and MTT assay was used to determine the proliferation of the cells. Data are mean \pm SEM of three independent experiments and analyzed with Student's *t* test, **P* < 0.05 was considered as statistically significant.

proliferation of skeletal muscle cells through inhibiting autophagy.

Knockdown of MuRF promote autophagy

To confirm the effect of MuRF on autophagy, we further knocked down MuRF with siRNA specifically targeting on MuRF in skeletal muscle cell line C2C12 overexpressed MuRF, and our western blot results confirmed the efficiency of the knockdown on MuRF (**Figure 3A**). Next we induced autophagy by starvation in C2C12 cells with MuRF knockdown; we observed knockdown of MuRF reversed autophagy in C2C12 cells (**Figure 3A**). Similarly, knockdown of MuRF also restored the proliferation of C2C12 cells (**Figure 3B**). Collectively, our study confirmed that MuRF regulates skeletal muscle cells proliferation via dampening autophagy.

Discussion

In 2001, overexpression of MuRF-1 has been found in different muscle atrophy, and plays multiple role in the process of skeletal muscle atrophy [5]. In the present study, we observed that autophagy is inhibited in atrophy skeletal muscles, which has a strong correlation with MuRF-1. Further analysis reveals that MuRF-1

dampens proliferation of skeletal muscle cells through inhibiting autophagy, and provides a potential approach for atrophy therapy.

Autophagy has been shown to be involved in multiple biological events of both muscle cells and muscle stem cells [13, 16]. There is no surprise to observe that autophagy goes away in muscle atrophy, as shown in the present study. Our data here also demonstrate that autophagy is indispensable for muscle cell proliferation, which is a cellular turnover for muscle growth. Autophagy promotion improves physiological function in muscle atrophy supports the feasibility for muscle atrophy therapy through regulation of autophagy. As overexpression of

MuRF-1 is key event for muscle atrophy, it is reasonable to assume that MuRF regulates autophagy.

Although it is commonly recognized that autophagy is inhibited in muscle atrophy, there are only sporadic studies on how is autophagy regulated in muscle atrophy. For example, mitochondria dysfunction leads to reactive oxygen species production and contributes to muscle atrophy [16]. Until now, there are no reports that MuRF regulates autophagy. In this study, taking advantage of specific overexpression and knockdown of MuRF in skeletal muscle cells, our results confirms the negative regulatory effect of MuRF on autophagy.

However, there are some interesting questions remain to be answered regarding the regulation of autophagy by MuRF-1 during the process of muscle process. What is the target protein of MuRF in regulating autophagy? What is the ubiquitination module of MuRF, mono-ubiquitination or poly-ubiquitination? At K48, K63 or on other lysine? Does the ubiquitination lead to degradation (positive autophagy regulator, such as Beclin 1, Atg5) or signaling (negative regulator such as Bcl2)? Answers to these questions are critical for understanding how

MuRF negatively regulates autophagy

MuRF-1 regulates autophagy and contributes to muscle atrophy.

In summary, our study gains insight into how MuRF upregulation contributes to muscle atrophy, and reveals that MuRF negatively regulates autophagy in skeletal muscle cells, thus come up with a new mechanism of MuRF for muscle atrophy, which potentially invoking a novel therapeutic approach for the disease.

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Disclosure of conflict of interest

None.

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