International Journal of Biochemistry and Cell Biology 90 (2017) 145–154

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Research paper

miR-18a induces myotubes atrophy by down-regulating *Igf1*

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ARTICLE INFO

Keywords:
Atrophy
miR-18a
Myotubes
Igf1

ABSTRACT

Muscle atrophy occurs when there is a net loss of muscle mass, leading to a change in the balance between protein synthesis and protein degradation. *Igf1* is important for protein synthesis in muscle cells and can induce local skeletal muscle hypertrophy and attenuate age-related skeletal muscle atrophy via the PI3K/Akt pathway in mice, consequently restoring and improving muscle mass and strength. In this study, we show that miR-18a expression is down-regulated during C2C12 myoblast differentiation and mouse tibialis anterior muscle postnatal development. Functional studies show that forced expression of miR-18a induces myotubes atrophy and increases the expression of MuRF1, Atrogin-1 and CTSL. miR-18a also decreases the phosphorylation of both Akt and FoxO3, and an inhibitor of the PI3K/Akt pathway blocks the function of miR-18a. An analysis of miR-18a targets reveals that Igf1 is regulated by miR-18a. miR-18a suppresses the expression of Igf1 in a 3′UTR-dependent manner. These findings strongly support the idea that miR-18a has a functional role in muscle physiology and suggest that miR-18a is a potential novel therapeutic target for skeletal muscle atrophy.

1. Introduction

Muscle atrophy occurs in response to fasting, chronic disease and disuse. Some genes are dramatically overexpressed during atrophy, and they may influence the physical properties of muscles and the pathological progression of atrophy. There is evidence that ubiquitin-mediated proteolysis plays an important role in skeletal muscle protein degradation. The muscle-specific E3 ubiquitin ligases MuRF1 (also called trim63) and Atrogin-1 (also called MAFbx or Fbxo32) are dramatically up-regulated in multiple types of skeletal muscle atrophy (Lecker et al., 2004; Glass, 2010a; Cohen et al., 2009). In addition to MuRF1 and Atrogin-1, cathepsin L (CTSL) expression is also increased at both the mRNA and protein levels in the muscles wasting (Deval et al., 2001). All three genes described above can be considered as marker genes of muscle atrophy.

Insulin-like growth factor 1 (*Igf1*) signaling is one of the best described mechanisms for inducing hypertrophy (Banerjee and Guttridge, 2012). The pathway that mediates hypertrophy downstream of Igf1 activation is PI3K/Akt (Shi et al., 2011). The *Igf1*/PI3K/Akt pathway induces skeletal muscle hypertrophy by both increasing protein synthesis and blocking protein degradation (Glass, 2010b).

miR-18a belongs to the miR-17-92 cluster, which comprises a group of seven microRNAs on chromosome 13 that are transcribed as a single polycistronic unit (Mendell, 2008; Ota et al., 2004). The miR-17-92 cluster has been defined as a common microRNA signature in several solid tumors (Komatsu et al., 2014; Dews et al., 2006).

Here we report that miR-18a is down-regulated in differentiated C2C12 myotubes as well as during skeletal muscle development. The overexpression of miR-18a can target loss of *Igf1*. In turn, lower expression of *Igf1* increases the dephosphorylation of Akt, leading to the dephosphorylation of FoxO3 and the overexpression of MuRF1, Atrogin-1 and CTSL. Consequently, these events cause skeletal muscle atrophy. These findings reveal that miR-18a is involved in skeletal muscle atrophy. The results strongly support the idea that miR-18a has a functional role in muscle physiology and suggest that miR-18a might be a new therapeutic target for skeletal muscle atrophy.

2. Results

2.1. miR-18a induces myotubes atrophy

We measured the level of miR-18a in the mouse tibialis anterior muscle from 1 day to 7 months of age. The results showed that the miR-18a expression level was deceased by ~70% at 1 month and ~80% at 2

Abbreviations: Igf1, Insulin-like growth factor 1; MuRF1, muscle RING finger 1; CTSL, cathepsin L; MYHC, myosin heavy chain

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http://dx.doi.org/10.1016/j.biocel.2017.07.020
Received 29 October 2016; Received in revised form 5 July 2017; Accepted 31 July 2017
Available online 04 August 2017
1357-2725/

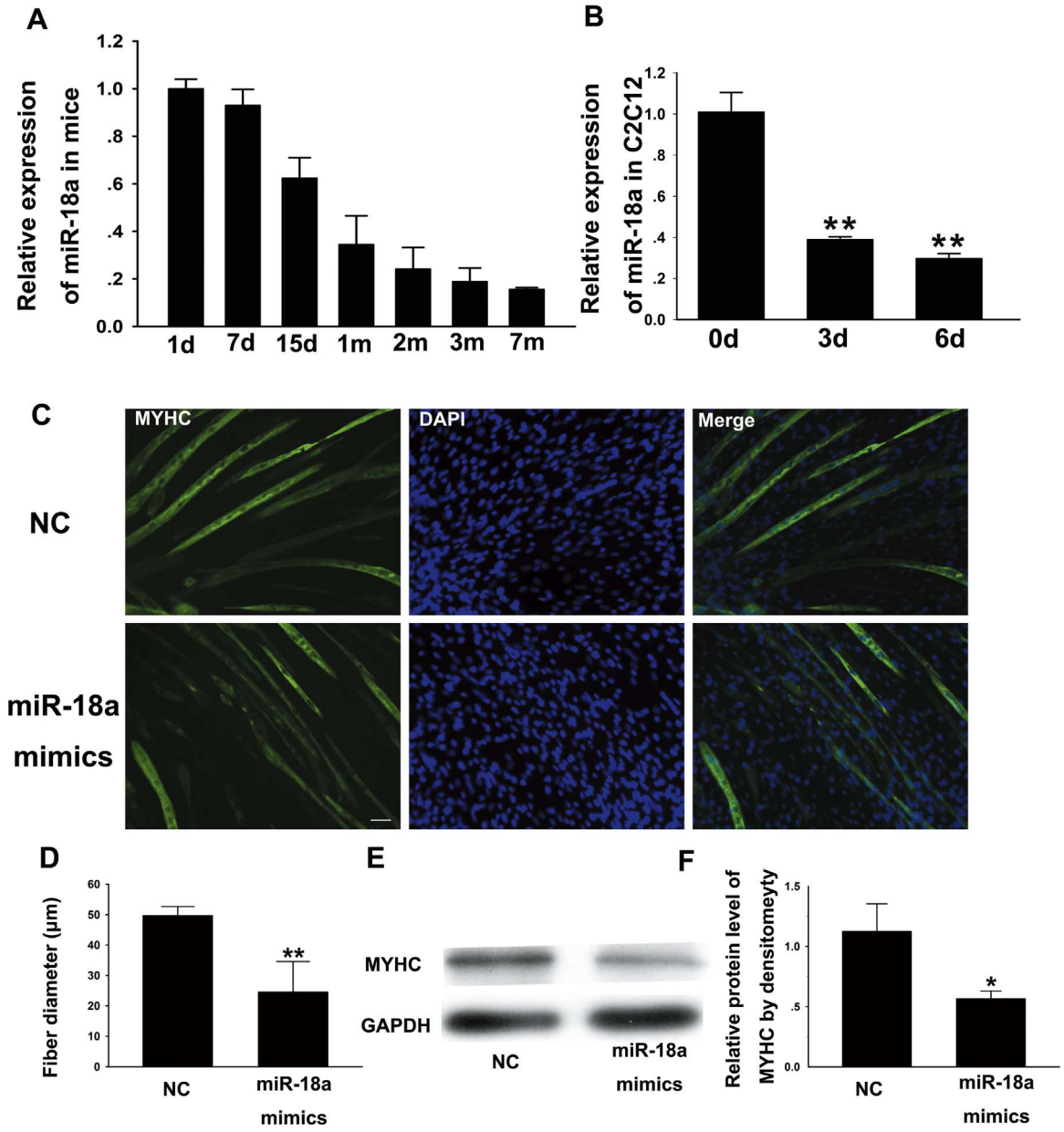

Fig. 1. miR-18a Induces Myotubes Atrophy A, the relative expression of miR-18a in the tibialis anterior muscle of mice at different ages were analyzed by real-time PCR with U6 small nuclear RNA as an internal reference for normalization. The values are the mean ± S.E. (n = 3). d, day(s); m, month(s). B, the relative expression of miR-18a during C2C12 myoblasts differentiation was analyzed by real-time PCR with U6 small nuclear RNA as an internal reference for normalization. The values are the mean ± S.E. (n = 3); d, day(s); **, $p < 0.01$ vs 0 d. C, representative images of myosin immunofluorescence staining in C2C12 myoblasts that were differentiated for 4 d and then transfected with miR-18a mimics or NC for 36 h. Scale bars = 50 μm. D, analysis of the myotubes diameters in the transfected cells. The values are the mean ± S.E. (n > 50); **, $p < 0.01$. E, analysis of myosin protein expression. The myotubes were transfected with miR-18a mimics or NC for 36 h, and the protein extracts were analyzed by Western blot. F, quantification of the myosin levels from E. The values are mean the ± S.E. (n = 3); *, $p < 0.05$.

months relative to its level on day 1 (Fig. 1A). We also found that miR-18a expression was down-regulated by ~70% in differentiated C2C12 myotubes (6 days) (Fig. 1B). To determine the effect of miR-18a in myotubes, the myotubes were transfected with the synthetic miR-18a mimics or the scrambled negative control (NC) on day 4 of differentiation. At 36 h after transfection, the miR-18a mimics-transfected myotubes showed a reduced diameter, as measured by immunofluorescence (Fig. 1C, D) compared with the control myotubes. Concurrently, the protein expression of MYHC (myosin heavy chain) was down-regulated by ~50% in the myotubes that were transfected with the synthetic miR-18a mimics (Fig. 1E, F).

Furthermore, Q-PCR analysis showed that the miR-18a mimics induced high mRNA expression of MuRF1 and CTSL (Fig. 2A). Additionally, Western blot analysis revealed that miR-18a increased the protein expression of MuRF1, Atrogin-1 and CTSL (Fig. 2B, C). Data from these experiments confirmed that miR-18a induced myotubes atrophy.

2.2. miR-18a induces C2C12 atrophy via the PI3K/Akt pathway

Next, we investigated the mechanism that miR-18a regulated atrophy. The *Igf1*/PI3K/Akt pathway play a key role in muscle hypertrophy and this pathway can regulate the expression of MuRF1, Atrogin-1 and CTSL. Interestingly, *Igf1* is the predicted target of miR-18a (TargetScan database). To test the functional relationship between miR-18a and the PI3K/Akt pathway, C2C12 myoblasts were transfected with miR-18a mimics for 36 h. Q-PCR and Western blot analysis revealed that a high miR-18a level was associated with high MuRF1,