Category: Letter

Stress responsive miR-23a attenuates skeletal muscle atrophy by targeting MAFbx /atrogin-1

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Abstract

Muscle atrophy occurs in many pathological states and results primarily from accelerated protein degradation by the ubiquitin-proteasome pathway. We used dexamethasone to induce muscle wasting and investigated the role of a microRNA (miRNA) in the control of muscle-specific E3 ubiquitin ligase MAFbx/atrogin-1. Here we show that miR-23a suppresses MAFbx/atrogin-1 translation by binding to 3' UTR of the mRNA. Furthermore, ectopic expression of miR-23a is sufficient to protect myocytes from atrophy *in vitro* and *in vivo* in response to dexamethasone treatment, and heat stress-induced miR-23a protects muscle from dexamethasone-induced muscle atrophy. Our surprising discovery of the physiological role of miR-23a in preventing the atrophy program should lay the basis not only for further understanding of the mechanisms of muscle wasting in diverse diseases, but also for developing novel therapies for these debilitating conditions.

INTRODUCTION

Adult skeletal muscle has tremendous plasticity at least in part due to a dynamic balance between protein synthesis and degradation. Increased protein synthesis leads to muscle hypertrophy, while increased protein degradation leads to muscle atrophy. Increased physical activity, particularly resistance training, induces muscle hypertrophy which is characterized by growth of the existing myofibers and often brings about health benefits. On the other hand, various chronic diseases result in skeletal muscle atrophy exacerbating the disease condition and compromising life quality. Therefore, preventing and attenuating skeletal muscle atrophy is one of important clinical issues.

Muscle atrophy occurs in response to fasting, chronic diseases (e.g., cancer, diabetes, AIDS, sepsis, and sarcopenia) and disuse. Under these diverse conditions, the atrophying muscles undergo accelerated protein degradation primarily through activation of the ubiquitin-proteasome pathway (1) and a common series of transcriptional adaptations that together constitute an “atrophy program” (2). In multiple models of skeletal muscle atrophy, the muscle-specific F-box protein MAFbx/atrogin-1 is upregulated and appears to be essential for accelerated muscle protein loss (3). Overexpression of MAFbx/atrogin-1 in skeletal myotubes leads to atrophy, and mice deficient in MAFbx/atrogin-1 are resistant to various models of skeletal muscle atrophy (4, 5).

Sandri et al. reported that members of the class O of forkhead box transcription factors (FoxO) regulated MAFbx/atrogin-1 transcription (6). Atrophic factors induce FoxO expression and initiate its nuclear transport to enhance MAFbx/atrogin-1 expression, which leads to muscle atrophy. On the other hand, PI3K/Akt signaling pathway, the major pathway of muscle hypertrophy, can prevent muscle atrophy by phosphorylating FoxO to inhibit its nuclear transport (7).

Recent studies have revealed a new aspect of muscle gene regulation in which small, noncoding RNAs, known as microRNAs (miRNAs), play fundamental roles in diverse biological and pathological processes, including differentiation and morphogenesis of skeletal muscle (8). miRNAs are approximately 22 nucleotides in length and inhibit translation of specific mRNA targets by interacting with the 3’ untranslated regions (3’ UTR) of the targets (9). miRNAs are primarily transcribed as pri-miRNAs, ranging in length from a few hundred to thousands of nucleotides with a characteristic hairpin secondary structure, and cleaved as pre-miRNAs by RNase III enzyme Drosha. Then

pre-miRNAs are exported from nucleus by Exportin-5 and proccesed into ~22 nucleotide miRNAs by RNase III enzyme Dicer. Finally, a single strand miRNAs are integrated into RNA-induced silencing complex (RISC), which directs miRNA binding the 3' UTR of target mRNAs.

It has been reported that some miRNAs are expressed in a tissue-specific manner, which suggests roles of the miRNAs in the specification of the tissue during development. In the skeletal muscle, several muscle-specific miRNAs have been reported to play multiple roles in the control of muscle growth and differentation. Chen et al. (10) showed that muscle specific miRNAs, miR-1 and miR-133, down regulate serum responsive factor (SRF) and histone deacetylase 4 (HDAC4), respectively to control muscle differentiation. It has also been shown that myogenic regulatory factor, MyoD, induces miR-206 to promote myogenesis (11, 12). These previous studies have revealed the importance of muscle-specific miRNAs in skeletal muscle development; however there has no report on the function of miRNAs in adult skeletal muscle plasticity. Here we first report MAFbx/atrogin-1 gene regulation by miRNA to elucidate involvement of miRNA in muscle plasticity. Bioinformatics approach revealed potential target sites in the 3' UTR of MAFbx/atrogin-1. We show that miR-23a binds to 3' UTR of MAFbx/atrogin-1 to inhibit its translation, and forced expression of miR-23a in myotubes and myofibers results in resistance to muscle atrophy. Furthermore, we demonstrated that miR-23a is upregulated in response to physiological heat stress, which is potent in rendering protection against muscle atrophy *in vitro* and *in vivo*. These findings strongly support a functional role of miR-23a in muscle physiology and suggest a new therapeutic target in the setting of skeletal muscle atrophy.

RESULTS

miR-23a interacts with 3' UTR of MAFbx/atrogin-1 mRNA. To investigate miRNA function in muscle atrophy, we employed bioinformatics to search for miRNAs that could potentially interact with MAFbx/atrogin-1. By using the online databases "*Human miRNA targets"* (http://cbio.mskcc.org/mirnaviewer/) and "*miRBASE Targets"* (http://microrna.sanger.ac.uk/targets/), we found miR-23a to be a strong candidate. miR-23a has been previously reported as a miRNA that is upregulated in the hypertrophy heart of mice with transgenic expression of activated calcineurin A (13). Our bioinformatics analysis revealed that the putative miR-23a binding site in MAFbx/atrogin-1 3' UTR is highly conserved in mammals including human and mouse (**Fig. 1a**). To investigate functional interaction of miR-23a and MAFbx/atrogin-1, the 3' UTR of MAFbx/atrogin-1 mRNA was subcloned into down stream of a luciferase reporter vector (**Fig. 1b**). miR-23a over expression vector (pCXbG-miR-23a) and the reporter vector (pLuc2EXN-atrogin-1 3' UTR) were co-transfected into HeLa cells, and luciferase activity was measured 24 hours after the transfection. We also constructed a reporter vector with mutations on the putative miR-23a binding site of the 3' UTR of MAFbx/atrogin-1 mRNA (pLuc2EXN-atrogin-1 3' UTRΔ) and transfected as well as pLuc2EXN-atrogin-1 3' UTR and pLuc2EXN. As expected, the luciferase activity from the pLuc2EXN-atrogin-1 3' UTR was markedly reduced when co-transfected with miR-23a expression vector but not form the mutant vector (**Fig. 1c**), suggesting that miR-23a could interact with the 3'UTR to down regulate its expression.

miR-23a leads to resistance to dexamethasone induced muscle atrophy *in vitro* and *in vivo*. We investigated miR-23a function by using an *in vitro* model of muscle atrophy with forced expression of miR-23a. A miR-23a experession vector, pCXbG-miR-23a, was transfected into a mouse myoblast cell line (C2C12) followed by induction of myogenesis (**Supplementary Fig. 1**). The myotubes were then treated with 10 μM dexamethasone (Dex) for 24 hours and the diameters were measured by visualization of GFP as a transgene marker. In mock transfected cells, myotube diameter decreased significantly in response to Dex treatment. (**Fig. 2a**). On the other hand, the diameter of miR-23a expressing myotube did not decrease in response to Dex treatment. Dexamethasone-induced upregulation of MAFbx/atrogin-1 at transcriptional level was not affected by miR-23a expression (**Fig. 2b**), suggesting miR-23a mediates its function

through other mechanisms than transcription. Furthermore, when we used electric pulse mediated gene transfer to transfect pCXbG-miR-23a into adult skeletal muscle in mice, we found that miR-23a-tranfected muscle showed less muscle weight loss than mock-transfected muscle in response to 7-day Dex treatment (daily with i.p. injection) (**Fig. 3a**) because miR-23a transfected myofibers became resistant to atrophy (**Fig. 3b**). Here, we measured cross sectional area of fast-twitch muscle fibers because glucocorticoid is known to induce muscle atrophy in fast twitch fibers selectively (14). In mock transfected muscle, cross sectional area of the fibers decreased significantly due to dexamethasone treatment, while miR-23a positive fibers were obviously resistant to muscle atrophy (**Fig. 3b**). These results suggest collectively that miR-23a expression is sufficient to attenuate skeletal muscle atrophy *in vitro* and *in vivo*.

Heat stress activates miR-23a expression and attenuates muscle atrophy.

miR-23a was reported as one of stress responsive miRNAs in cardiac muscle (13) and, therefore, it is possible that miR-23a is also induced by stress in skeletal muscle. We screened several physical stresses to induce miR-23a *in vitro* and found that heat stress has most striking effect to induce miR-23a in myoblasts (data not shown). This preliminary finding prompted us to investigate the potential role of heat stress-induced miR-23a in protection against muscle atrophy. We obtained exciting new evidence that heat stress renders myotubes resistant to dexamethasone-induced atrophy (**Fig. 4a, b**) without affecting MAFbx/atrogin-1 mRNA expression (**Fig. 4b**). To knockdown heat stress-induced miR-23a, locked nucleic acid (LNA) antisense against miR-23a was transfected into C2C12. The LNA antisense was reported to bind to miRNAs selectively and down-regulate their function by forming rigid duplex with their complementary miRNAs (15). LNA antisense down-regulated miR-23a expression (**Supplementary Fig. 2**) and counteracted heat stress-induced atrophy resistance (**Fig. 4b, c**). These results showed that miR-23a was concerned with resistance against muscle atrophy induced by heat stress.

To ascertain the functional role of heat stress-induced resistance to muscle atrophy *in vivo*, we subjected mice to heat stress followed by dexamethasone treatment for 7 days. In fast-twitch plantaris muscle, muscle weight loss was significantly lower in heat stress exposed group than that of unexposed group (**Fig. 4d**). The cross sectional areas of fast-twitch fibers were also measured to reveal that the fiber size decrease was

significantly lower in heat stress exposed group than that of unexposed group (**Fig. 4e**). Again, dexamethasone-induced MAFbx/atrogin-1 mRNA expression was not affected by heat stress (**Supplementary Fig. 3**). Consistent with our findings in cultured myotubes, miR-23a expression in skeletal muscle was significantly upregulated in mice exposed to heat stress (**Fig. 4f**). These results suggest that heat stress-induced miR-23a represses MAFbx/atrogin-1 expression post-transcriptionally to attenuate muscle atrophy.

DISCUSSION

This study demonstrates that miR-23a is a novel regulator of MAFbx/atrogin-1 through post-transcriptional regulation of the gene, playing a substantial role in protection against skeletal muscle atrophy. We showed here miR-23a interacts with MAFbx/atrogin-1 3' UTR and forced expression of miR-23a in cultured myotubes and adult skeletal muscle results in resistance to glucocorticoid-induced muscle atrophy. We have also linked these new findings to a physiological setting of heat stress-induced protection against muscle atrophy. These findings strongly support that miR-23a functions in skeletal muscle by regulating the MAFbx/atrogin-1 gene in response to wasting and present a viable therapeutic target for this important clinically relevant problem that could be affected by and exacerbate many chronic diseases.

Recently, people start to pay more and more attention to the functional role of stress responsive miRNAs (16). Various types of stresses have been reported to induce miRNAs (17, 18), which have been clearly demonstrated in the cardiac muscles. Pressure overload or calcineurin activation induces pathological cardiac hypertrophy (20, 21), in which miR-195 and miR-208 play important roles (13, 19). Upon such hypertrophic stress, several miRNAs including miR-23a were upregulated, and miR-23a overexpression in cardiomyocytes evoked myocyte hypertrophy (13). In the skeletal muscle, overexpression of miR23a did not lead to hypertrophy, but resistance against dexamethasone-induced atrophy (**Fig. 3**). Together with findings in this study, it remains formally possible that miR-23a plays an anti-atrophic effect on skeletal muscle. The apparent protection observed *in vivo* in our study could be a result of the mechanism.

We screened several physiological stresses to identify the one that heat stress could profoundly upregulate miR-23a expression in C2C12 myoblasts. Since miR-23a expression was sufficient to induce hypertrophy in cardiomyocyte, it is also possible that miR-23a induces significant muscle hypertrophy. Our results in culture myotubes clearly demonstrate that miR-23a does not promote muscle hypertrophy in vitro, but prevent myotubes from atrophy (**Fig. 2a**). Notably, heat stress renders myotubes resistant to glucocorticoid-induced atrophy without affecting the induction of MAFbx/atrogin-1 mRNA. LNA antisense mediated miR-23a silencing counteracted heat stress-induced resistance of muscle atrophy. In addition, heat stress also activated miR-23a expression *in vivo* and protected skeletal muscle from atrophy. These results suggest that stress inducible miR-23a down regulates MAFbx/atrogin-1

post-transcriptionally and protect muscle from atrophy.

Previous study suggested that heat shock proteins (HSPs) play a role in muscle atrophy attenuation by heat stress (22). HSPs serve as molecular chaperones in refolding, disaggregation, and degradation of damaged polypeptides induced by various stresses including heat stress. Our results suggest that heat stress-induced miR-23a inhibits protein degradation by suppressing the proteasome system via down-regulation of the MAFbx/atrogin-1expression.

Regulations of miRNAs are attractive targets for clinical therapies. Several miRNAs have been shown to be important in disease development, such as cancer (23，24), heart disease (13) and cholesterol/lipid metabolism (25). Our results suggest that miR-23a has the potency to attenuate muscle atrophy. Therapeutic manipulation of miR-23a expression could potentially maintain skeletal muscle function by suppressing MAFbx/atrogin-1 expression.

Although we have shown that miR-23a is induced by heat stress, its transcriptional control has yet to be determined. It has been reported that miR-23a is included in a polycistronic miRNA cluster, in which two or three miRNAs are generated from a common pri-miRNA, together with miR-27a and miR-24-2 (26), which is conserved from zebrafish to human. Such unique transcriptional control is thought to contribute to a highly coordinated regulation of miRNA target genes. Interestingly, miR-23b, which has similarity with miR-23a, is also in this polycistronic miRNA cluster. Elucidation of transcriptional control this super cluster miRNA might offer significant insights into the physiological meaning of stress responsive miRNAs.

METHODS

Cell culture. C2C12 (American Type Culture Collection) mouse skeletal myoblasts were maintained at subconfluent densities in 20% FBS in DMEM (GM). At 80-100% confluent density, medium was changed to 2% horse serum (HS) in DMEM (DM) and myogenesis was induced. After 4 days incubation with DM, more than 80% cells formed multi-nuclear myotube. To induce muscle atrophy in vitro, cells were incubated with 10 μM dexamethasone (Dex) in DM for 12 hours or 24 hours. After the treatment, cells were harvested with appropriate buffers or used for morphological analysis.

Myotube morphological analysis. After the Dex treatment, myotubes were stained with Hoechist 33342 (Invitrogen Co., Carlsbad, CA) for nucleus and cell tracker green fluorescent (Lonza Group Ltd., Basel, Switzerland) for cytoplasm and photographed directly in culture plates by using IX71 fluorescent microscope (Olympus, Tokyo, Japan) with EM-CCD digital camera (Hamamatsu Photonics K.K., Shizuoka, Japan). More than 50 myotubes' diameter was measured by AquaCosmos software (Hamamatsu Photonics K.K.).

Vectors. pCXbG-miR-23a; A primary miR-23a region was amplified by PCR using human genomic DNA with primers: 5'-AAA GTC GAC CTT TCT CCC CTC CAG GTG C-3' and 5'-AAG ATA TCT CGA GAC AGG CTT CGG GGC CTC TC-3'. The PCR product was digested with SalI plus EcoRV and introduced into plasmid pCXbG at XhoI plus EcoRV sites. The pCXbG was generated from plasmid pCX-EGFP (27). The DNA fragment including the EGFP fused with blasticidin resistant gene and XhoI and EcoRV sites was amplified by PCR using the pTracerEF-Bsd (Invitrogen) and pEGFP (Clontech Laboratories, Inc., Mountain View, CA) as templates and it was inserted into the EcoRI sites of pCX-EGFP to yield the pCXbG. pLuc2EXN-atrogin-1 3'UTR; A 300 bp nucleotide fragment of the MAFbx/atrogin-1 3'UTR, including a putative miR-23a binding site, was amplified from mouse cDNA by PCR using Ex Taq HS (TaKaRa, Osaka, Japan). Primer sequences are as follows; forward primer: 5'-TCT CTA GAA TCC CAG CAC ACG ACA ACA CTT C-3'; reverse primer: 5'-CTC TCG AGG ACT CCG TTT CCA TGG CTG AC-3'. Amplified fragments were cloned into the EcoRI and XbaI sites of pLuc2EXN plasmid. The pLuc2EXN was generated from a plasmid pGL3-control to clone Luc2 gene (Promega Co., Madison, WI) ahead of additional

EcoRI and XbaI sites. pLuc2EXN-atrogin-1 3'UTRΔ was generated by site-direct mutagenesis from pLuc2EXN-atrogin-1 3'UTR as described previously (28). Briefly, a forward primer, 5'- GCC GAT GGA AAT TTA CAC ACT ATA ATT CCA CAT G -3', and a reverse primer, 5'- CAT GTG GAA TTA TAG TGT GTA AAT TTC CAT CGG C -3', (mutated nucleotides are underlined) were used in a PCR-based site-directed mutagenesis using the QuickExchange lightning sited-directed mutagenesis kit (Stratagene). All plasmid DNAs were transformed and amplified in DH-5α bacteria, purified using the plasmid Midi kit (Qiagen).

Transfection. C2C12 were seeded in 6-well plates 24 hours before transfection. Cells were transfected with 1 μg of each vector or 50 ng of locked nucleic acid (LNA; underlined) antisense, 5'- AUC ACA UUG CCA GGG AUU UCC -3', with Lipofectamine 2000 (Invitrogen) in serum free Opti-MEM (Invitrogen). Medium was changed to medium containing FBS after 6 hours incubation.

Reporter assay. HeLa cells (ATCC) were seeded in 6-well plates 24 hours before transfection. Cells were transfected with 0.1 μg of pLuc2EXN-atrogin-1 3'UTR, 0.1 μg of LacZ and 1.8 μg of pCXbG-miR-23a with Lipofectamine 2000. Cells were lysed 24 hours after the transfection, and luciferase activity was measured using luciferase reporter assay system (Promega Co). Luciferase activity was normalized by LacZ activity as described (29).

RNA analysis. According to manufacturers' protocols, total RNA was extracted from cells and animal tissues by using ISOGEN (WAKO, Osaka, Japan) and 10 μg of RNA was subjected to reverse transcription (RT) by using SuperScript III (Invitrogen). Oligo dT primers were used to generate cDNA and an aliquot of the RT reaction was used directly for PCR with Ex Taq HS (TaKaRa, Osaka, Japan) and gene-specific primers. The primer sequences are as follows: MAFbx/atrogin-1, 5'-GGG AGG CAA TGT CTG TGT TT-3' and 5'-TTG TGA AAA AGT CCC GGT TC-3'; GAPDH, 5'-GTG GCA AAG TGG AGA TTG TTG CC-3' and 5'-GAT GAT GAC CCG TTT GGC TCC-3'. GAPDH was used as an internal standard.

MicroRNA analysis. TaqMan MicroRNA Reverse Transcription Kit and TaqMan

MicroRNA assays (Applied Biosystems, Foster City, CA) were used to quantify expression of mature miRNAs according to manufacturer's protocol. In miRNA quantification, each reverse transcriptase (RT) reaction contained 10 ng of purified total RNA. The reactions were incubated for 30 min at 16 °C, 30 min at 42 °C, and 5 min at 85°C. Real-time PCR reactions for each miRNA (10 μl volume) were performed in triplicate, and each 10 μl reaction mixture included 2 μl of diluted RT product. Reactions were incubated in an Applied Biosystems 7500 Fast Real-Time PCR system in 96-well plates at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

Animal experiments. Adult (8 weeks of age) male C57BL/6 mice (Clea Japan, Inc., Tokyo, Japan) were housed in temperature-controlled quarter (21˚C) with a 12-h light/12-h dark cycle and provided with water and chow *ad libitum*. To induce skeletal muscle atrophy, 1 mg Dex/kg body weight was injected intraperitonealy for 7 days. After Dex treatment, animals were sacrificed and muscles were harvested with appropriate buffer or embedded with O.C.T. compound. The animal protocols were approved by the Animal Care and Use Committees, the University of Tokyo and Waseda University.

Immunohistochemistry. Anti-myosin heavy chain (MHC) 2a (SC-71) and 2b (BF-F3) monoclonal antibodies were used as primary antibodies for immunostaining of O.C.T. -embedded cross sections from mice skeletal muscle (30). Mouse anti-dystrophin antibody (D8043, Sigma, St. Louis, MO) with FITC-conjugated secondary antibody was used to identify shapes of muscle fibers. Cross sections were fixed in PBS with 4% paraformaldehyde, then permeabilized in PBS with 0.3% Triton X-100, and incubated with each primary Ab for overnight. The secondary antibodies were either FITC conjugated anti-mouse IgG or R-PE conjugated anti-mouse IgM (for MHC 2a and MHC 2b, respectively; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Finally, cover slips were enclosed with VECTA SHIELD (Vector Laboratories, Ltd., Burlingame, CA). Cross sectional area was measured by AquaCosmos software (Hamamatsu Photonics K.K.).

Electric pulse-mediated gene transfer. Electric pulse-mediated gene transfer was

performed as described (28). Briefly, both tibialis anterior (TA) muscles were injected with 20 μg of plasmid DNA under anesthesia (50 mg/kg i.p. pentobarbital sodium). Within 1 min after injection, electrical field was delivered to the injected TA muscle by SEN-3401 electric stimulator (Nihon Kohden, Tokyo, Japan) through a model 532 two-needle array (BTX Instrument Division Harvard Apparatus, Inc., Holliston, MA). Eight pulses at 100 ms in duration, 1 Hz in frequency, and 100 V in amplitude (200 V/cm) were applied to the muscle by placing the needle arrays on the medial and lateral sides of the TA muscle so that the electrical field was perpendicular to the long axis of the myofibers. The mice were allowed to recover for 4 days before dexamethasone injection.

Heat stress exposure. For *in vitro* study, C2C12 myotubes were exposed to heat stress on a water bath (42°C) for 30 mins. To induce muscle atrophy *in vitro*, medium was changed to DM containing 10 μM Dex for 12 hours after the heat stress exposure. For *in vivo* study, the mice were exposed to heat stress at 42°C for 1 hour. After 24 hours of heat stress exposure, 1 mg Dex/kg body weight was injected intraperitonealy to induce skeletal muscle atrophy. Dex was injected once a day for 7 days.

URLs. "*Human miRNA targets"*: http://cbio.mskcc.org/mirnaviewer/. "*miRBASE Targets"*: http://microrna.sanger.ac.uk/targets/.

Statistics. Data are expressed as the means ± standard errors of the means. Statistical significance ($P < 0.05$) was determined by Student's t-test or ANOVA followed by the Dunnett test for comparisons between two groups or multiple groups, respectively.

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AUTHOR CONTRIBUTIONS

S. W. and T. A. designed research; S. W., M. O. and T. A. performed research; Y. K., S. M., H. A. contributed new reagents/analytical tools; S. W., K. S., T. U. and T. A. analyzed data; S. W., Z. Y. and T. A. wrote the paper.

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Figure Legends

Figure 1

miR-23a targets MAFbx/atrogin-1 3' UTR. (**a**) Sequence alignment of putative miR-23a binding site in 3' UTR of MAFbx/atrogin-1 shows a high level of complementarity and sequence conservation. (**b**) A reporter vector construct of Luc-atrogin-1 3' UTR. To analyze miR-23a function, MAFbx/atrogin-1 3' UTR including miR-23a putative binding site was cloned into the down stream of luciferase expression vector. (**c**) HeLa cells were cotransfected with either Luc-atrogin-1 3' UTR (WT), Luc-atrogin-1 3' UTRΔ, a mutant reporter vector (mutant) or original vector (control), along with miR-23a expression vector or mock vector. Luciferase activity was measured (n = 6). Values indicate luciferase activity relative to mock-cotransfected condition. * $P < 0.001$. Error bars mean ±SEM.

Figure 2

Forced expression of miR-23a counteracted dexamethasone-induced muscle atrophy. (**a**) miR-23a expression vector was transfected into C2C12 cells and myogenesis was induced. After 10 μM dexamethasone treatment (Dex), myotube diameter was measured (n > 50). The bar indicates 10 μm. (**b**) MAFbx/atrogin-1 mRNA expression was quantified by using real-time PCR (n = 6). Values are relative to mock control. * $P < 0.05$, ** $P < 0.01$. Error bars mean ±SEM.

Figure 3

Forced expression of miR-23a in adult muscle fibers counteracted dexamethasone-induced muscle atrophy. (**a**) Muscle weight (n = 8) and (**b**) cross sectional areas (CSA) of fast-twitch muscle fibers (MHC2b; Red) with or without miR-23a expression vector (Green; n > 80) were measured. Expression vectors for miR-23a or mock were transfected into TA muscle by the electric pulse-mediated gene transfer. The bar indicates 10 μm. * $P < 0.001$. Error bars mean ±SEM.

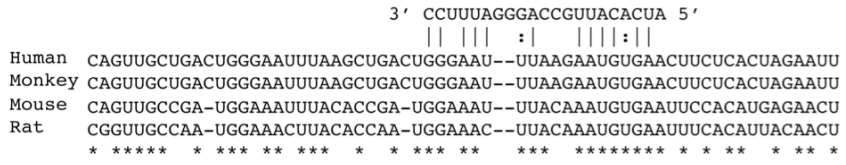
Figure 4

Heat stress exposed myotubes/muscle fibers show resistance against muscle atrophy. After heat stress exposure, myotubes were treated with Dex. (**a**) Myotubes

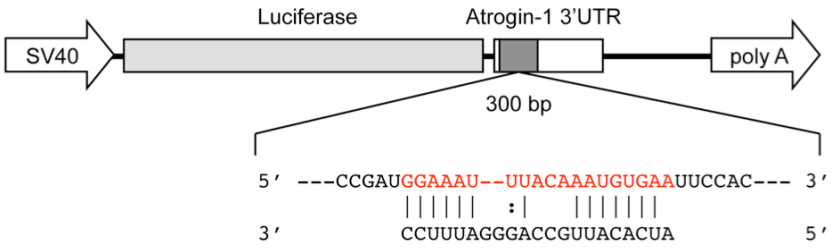
were stained with cell tracker green (Green) and Hoechist 33342 (Blue), then their diameters were measured (n > 100). (**b**) Heat stress-induced MAFbx/atrogin-1 mRNA expression was measure in C2C12 cells with/without Dex-treatment. (**c**) Heat stress upregulated miR-23a expression *in vitro*. (**d**) For *in vivo* experiment, mice were injected dexamethasone for 7 days (H/Dex) 24 hours after heat stress, and sacrificed to measure muscle weight. (**e**) Cross sectional area (CSA) of fast-twitch fibers (MHC2b; Red) was measured. Bar indicates 200 μm. (**f**) Heat stress upregulated miR-23a expression in mice skeletal muscle. miR-23a expression was measured by using real-time PCR. Values are relative to control. * $P < 0.05$, ** $P < 0.01$. Error bars mean ±SEM.

Fig. 1

a


3′ CCUUUAGGGACCGUUACACUA 5′
Human CAGUUGCUGACUGGGAAUUUAAGCUGACUGGGAAU--UUAAGAAUGUGAACUUCUCACUAGAAUU
Monkey CAGUUGCUGACUGGGAAUUUAAGCUGACUGGGAAU--UUAAGAAUGUGAACUUCUCACUAGAAUU
Mouse CAGUUGCCGA-UGGAAAUUUACACCGA-UGGAAAU--UUACAAAUGUGAAUUCCACAUGAGAACU
Rat CGGUUGCCAA-UGGAAACUUACACCAA-UGGAAAC--UUACAAAUGUGAAUUUCACAUUACAACU

b


Luciferase
Atrogin-1 3'UTR
SV40
poly A
300 bp
5′ ---CCGAUGGAAAU--UUACAAAUGUGAAUUCCAC--- 3′
3′ CCUUUAGGGACCGUUACACUA 5′

c

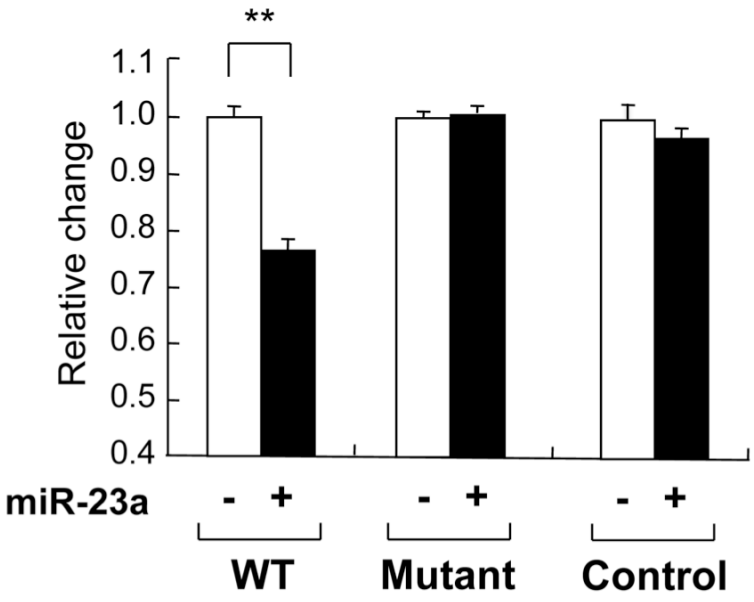

**
Relative change
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
miR-23a
- + - + - +
WT
Mutant
Control

Fig. 2

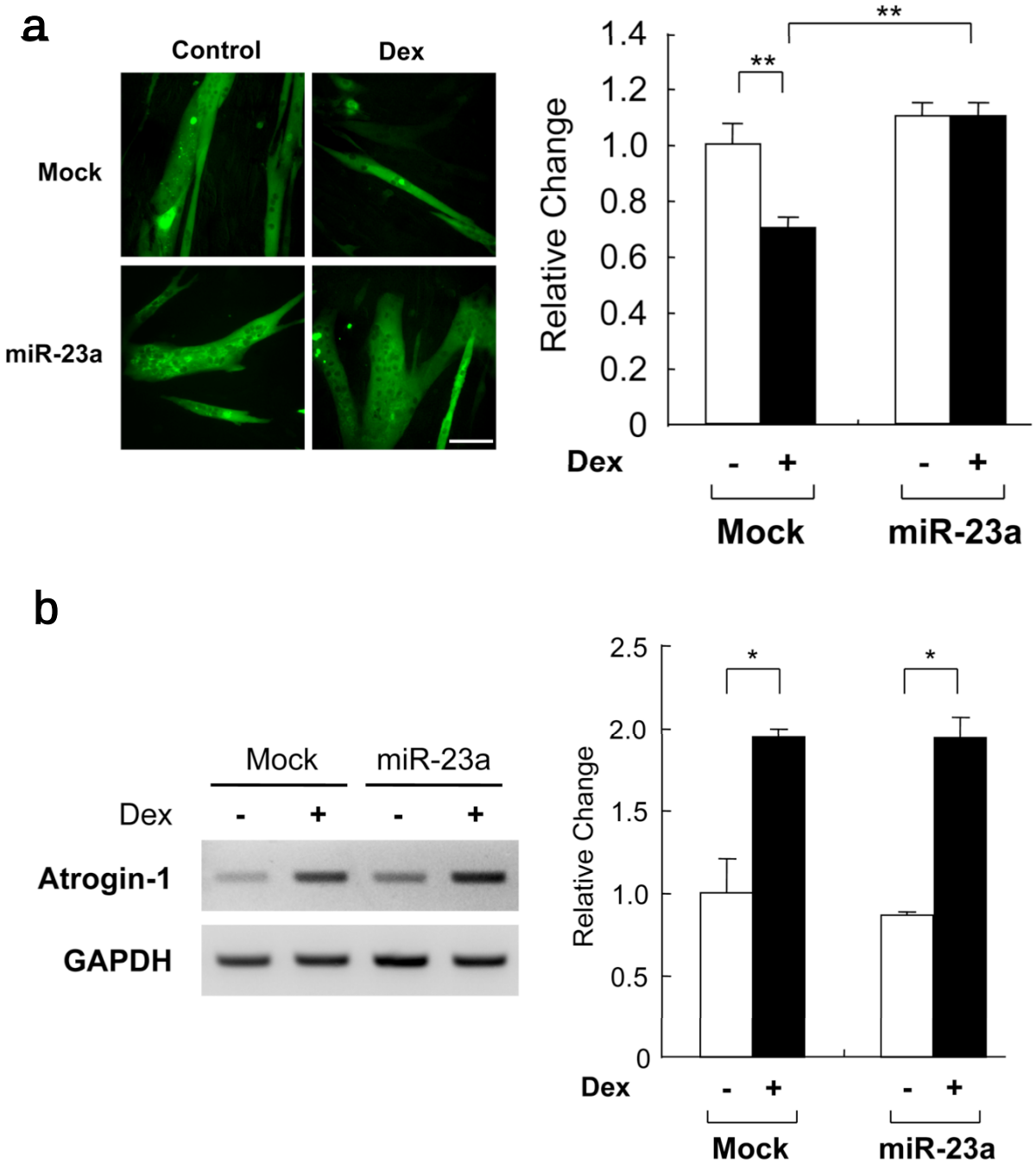
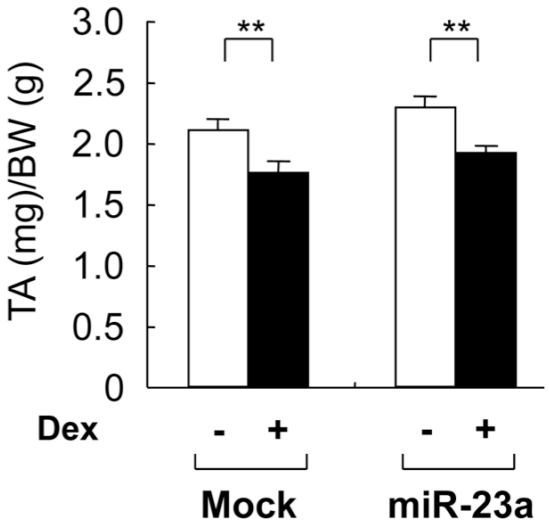
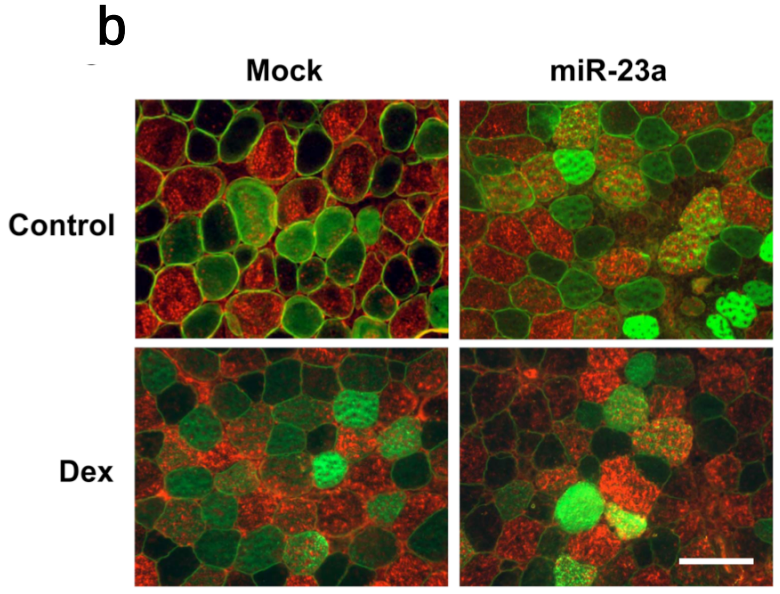

a
Control
Dex
Mock
miR-23a
Relative Change
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
**
**
Dex
-
+
-
+
Mock
miR-23a
b
Mock
miR-23a
Dex
-
+
-
+
Atrogin-1
GAPDH
Relative Change
2.5
2.0
1.5
1.0
0.5
0
*
*
Dex
-
+
-
+
Mock
miR-23a

Fig. 3

a


3.0
2.5
2.0
1.5
1.0
0.5
0
TA (mg)/BW (g)
**
**
Dex
-
+
-
+
Mock
miR-23a

b


Mock
miR-23a
Control
Dex

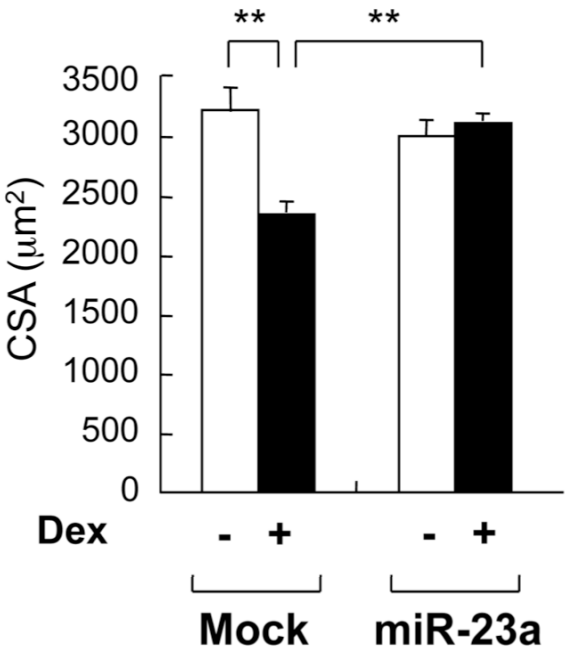
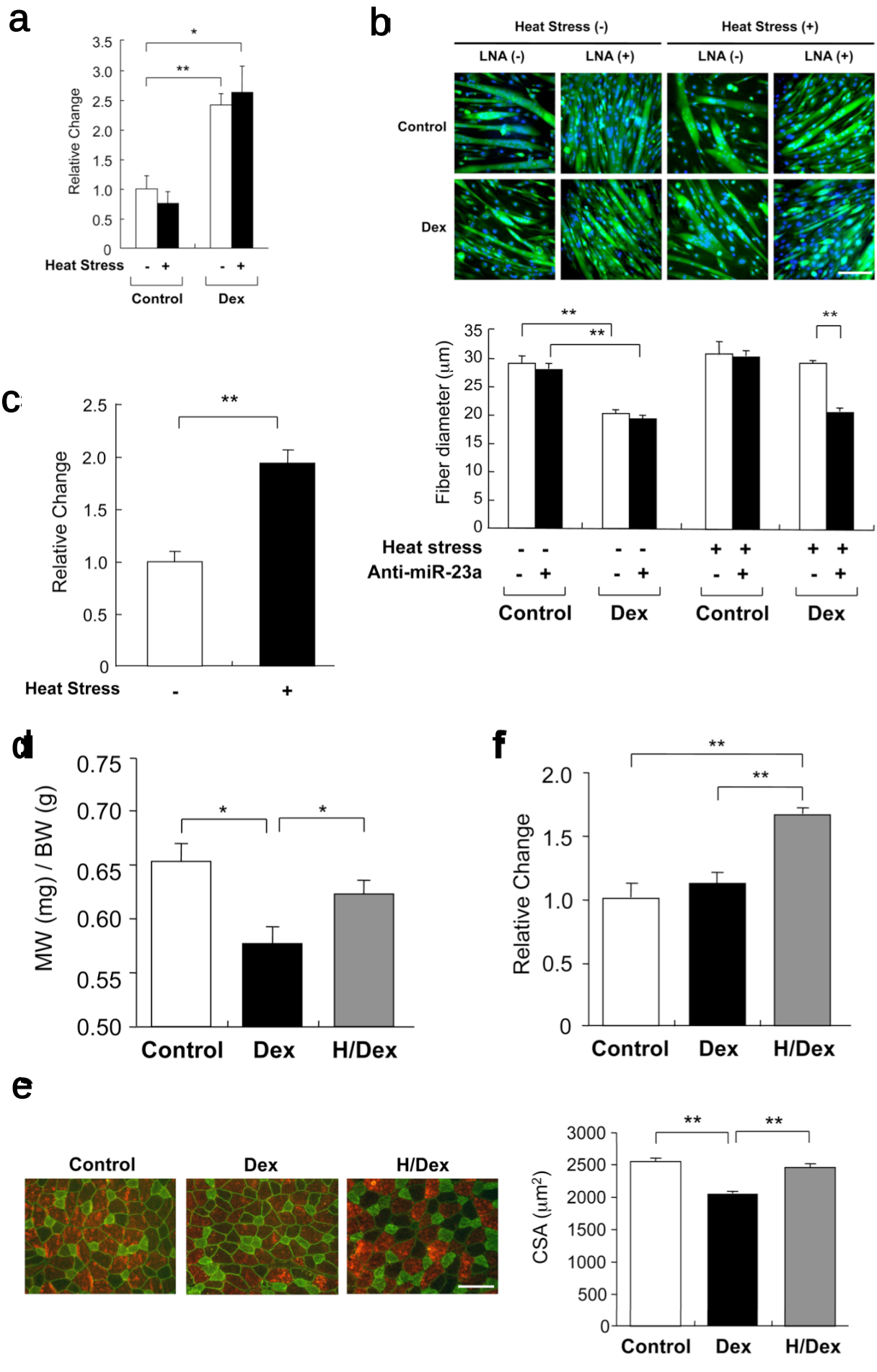

**
**
3500
3000
2500
2000
1500
1000
500
0
CSA (μm²)
Dex
-
+
-
+
Mock
miR-23a

Fig. 4


a
Relative Change
Heat Stress
Control
Dex
b
Heat Stress (-)
Heat Stress (+)
LNA (-)
LNA (+)
Control
Dex
Fiber diameter (μm)
Heat stress
Anti-miR-23a
c
Relative Change
Heat Stress
d
MW (mg) / BW (g)
Control
Dex
H/Dex
e
Control
Dex
H/Dex
f
Relative Change
CSA (μm²)