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Myostatin regulates miR-431 expression via the Ras-Mek-Erk signaling pathway

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ABSTRACT

MicroRNAs (miRNAs) play critical regulatory roles in controlling myogenic development both *in vitro* and *in vivo*; however, the molecular mechanisms underlying transcriptional regulation of miRNA genes in skeletal muscle cells are largely unknown. Here, using a microarray hybridization approach, we identified myostatin-regulated miRNA genes in skeletal muscle tissues by systematically searching miRNAs that are differentially expressed between wild-type and myostatin-null mice during development. We found that 116 miRNA genes were differentially expressed in muscles between these mice across different developmental stages. We further characterized myostatin-regulated miR-431 was upregulated in skeletal muscle tissues of myostatin-null mice. In functional studies, we found that overexpression of miR-431 in C2C12 myoblast cells attenuated myostatin-induced suppression of myogenic differentiation. Mechanistic studies further demonstrated that myostatin acted through the Ras-Mek-Erk signaling pathway to transcriptionally regulate miR-431 expression C2C12 cells. Our findings provide new insight into the mechanisms underlying transcriptional regulation of miRNA genes by myostatin during skeletal muscle development.

1. Introduction

Myostatin (MSTN), also known as growth and differentiation factor 8 (GDF-8) [1], is a member of the transforming growth factor-β (TGF-β) superfamily. Initial reports identified MSTN as a negative regulator of skeletal muscle growth, showing that skeletal muscle mass was markedly increased in MSTN-knockout ($MSTN^{-/-}$) mice. A functional role for MSTN in regulating myogenic development was further supported by the phenotypes associated with naturally occurring mutations of the *MSTN* gene in cattle and humans [2,3]. Recent studies have demonstrated non-muscle functions of MSTN in the regulation of adipogenesis [4,5], cardiac myocyte hypertrophy [6] and glucose metabolism [7], and cancer cell apoptosis [8]. Given the profound biological significance of MSTN, there is considerable interest in identifying the molecular mechanisms underlying MSTN-regulated cellular responses.

microRNAs (miRNA) play critical regulatory roles in many different processes, including cell-fate determination, proliferation, differentiation and apoptosis, during normal and pathological development. An essential role for miRNAs in modulating skeletal muscle development is evidenced by the recent demonstration that deletion of a conditional Dicer allele in embryonic skeletal muscle results in perinatal lethality owing to skeletal muscle hypoplasia [9,10]. In particular, previous studies have demonstrated regulatory roles of the muscle-specific miRNAs, miR-1, miR-133 and miR-206, in modulating myogenesis during development [11,12]. In addition to muscle-specific miRNAs, some ubiquitously expressed miRNAs, such as miR-214 and miR-181a, also play critical roles in regulating myogenesis in muscle cells [13,14]. Moreover, it has been reported that dysregulation of miRNA expression in skeletal muscle disorders [15], suggesting that the precise regulation of miRNA expression in muscle cells must be tightly controlled by factors such as MSTN during development. The functional significance of miRNAs in regulating skeletal muscle development has been well documented, both *in vitro* and *in vivo*; however, little is known about the transcriptional regulation of miRNA genes during development. Given the critical roles of miRNAs in regulating myogenesis and the

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multiple targets of a given miRNA in cells, elucidating the molecular mechanisms underlying transcriptional regulation of miRNA genes is profoundly important for deciphering signaling pathways involved in the functional regulation of miRNAs.

Here, we report the identification and characterization of MSTN-regulated miRNAs in skeletal muscle during development in mice. Using MSTN$^{-/-}$ mice as a genetic system for screening MSTN-regulated miRNAs, we found that 63 miRNAs were upregulated and 53 were downregulated by MSTN in skeletal muscle across different developmental stages. We focused particular attention on miR-431, investigating the MSTN-mediated transcriptional regulation of this differentially expressed miRNA in muscle cells. We found that MSTN downregulated miR-431 expression both *in vitro* and *in vivo*, and further determined that overexpression of miR-431 in C2C12 myoblast cells attenuated MSTN-induced suppression of myogenic differentiation. Finally, we provided experimental evidence to show that MSTN transcriptionally regulates miR-431 expression through the Ras-Mek-Erk signaling pathway.

2. Materials and methods

2.1. Animal care and tissue collection

All animal procedures were approved by the Animal Ethics Committee of Peking Union Medical College, Beijing (China). The MSTN$^{-/-}$ mice used in the study are gifted from Braun at Max-Planck-Institut in Germany. See Supplemental Information for details.

2.2. miRNA microarray

Affymetrix GeneChip miRNA Arrays were used to analyze miRNA expression patterns in skeletal muscle tissues. Microarray hybridization experiments were performed by CapitalBio Corporation in Beijing. The differentially expressed miRNAs were analyzed using significance analysis of microarrays (SAM) [16]. See Supplemental Information for details.

2.3. Cell culture and treatment

Culture and treatments of C2C12 cells with recombinant myostatin or different inhibitors were performed as protocols described in Supplemental Information.

2.4. Isolating primary myoblasts

Primary myoblasts were isolated from hindlimb skeletal muscle, minced, and digested in a mixture of type I collagenase and dispase B (Roche Applied Science). Cells were filtered to remove debris, centrifuged, and cultured in growth medium (F-10 Ham's medium supplemented with 20% FBS, 4 ng/mL basic fibroblast growth factor, and 1% penicillin-streptomycin) on collagen-coated cell culture plates at 37 °C in 5% CO_2.

2.5. Western blot analysis

See Supplemental Information for details.

2.6. Immunohistochemistry

See Supplemental Information for details.

2.7. RT-PCR and real-time RT-PCR analysis

Expression of mature miRNAs was determined using the miRNA-specific TaqMan microRNA assay kit (Applied Biosystems) and the ABI IQ5 Sequence Detection System (Applied Biosystems). U6 was used for normalization. mRNA expression was analyzed with Fast EvaGreenqPCR Master Mix (Applied Biosystems) and normalized to that of GAPDH.

2.8. Identification of candidate transcriptional mediators

Candidate transcription factor mediators of MSTN-signaling–mediated miR-431 expression were identified using a TF Activation Profiling Plate Array I (Signosis FA-1001) as described by the manufacturer. See Supplemental Information for details.

2.9. Statistical analysis

Values are presented as means ± SEM. The statistical significance of the difference between two means was calculated using Student's t test. P-values < 0.05 were considered statistically significant.

3. Results

3.1. Systematic identification of MSTN-regulated miRNAs

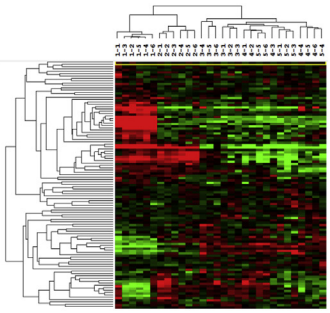
MSTN plays critical roles in regulating myogenesis during embryonic and postnatal development [1,17,18], however, our current understanding of cellular and molecular mechanisms underlying its functions is still incomplete. miRNAs are required for myogenesis during development, and MSTN, as a skeletal muscle-generated cytokine, may regulate myogenesis by modulating expression of miRNAs during skeletal muscle development. Therefore, we used wild-type (WT) and MSTN$^{-/-}$ mice as a genetic system to identify MSTN-regulated miRNAs during development. The growth features of WT and MSTN$^{-/-}$ mice during development demonstrated significant increase in body weight (Supplementary Fig. S1A) and muscle mass of both tibialis anterior (TA) (Supplementary Fig. S1B) and gastrocnemius (Gastro) (Supplementary Fig. S1C) muscles in MSTN$^{-/-}$ mice compared with WT mice. On the basis of this pattern of skeletal muscle growth in MSTN$^{-/-}$ mice, we chose various developmental stages, including fetal myogenesis (embryonic day 15.5 [E15.5]), adolescence (postnatal week 4), young adult (10 and 16 weeks) and aged (41 weeks), for use in identifying MSTN-regulated miRNAs in skeletal muscle (Fig. 1A). Microarray analysis identified 116 miRNAs with differential expression in skeletal muscle across different developmental stages between WT and MSTN$^{-/-}$ mice; 63 were upregulated by MSTN and 53 were downregulated (Fig. 1B, C). In addition, we also identified several miRNAs with no differences in expressional level between MSTN$^{-/-}$ and WT muscle that clearly demonstrated developmentally regulated expression patterns. For example, miR-26a expression gradually increased (Supplementary Fig. S2A) and miR-541 was downregulated in both genotypes during postnatal myogenesis (Supplementary Fig. S2B). Five miRNAs—miR-30e*, miR-467a, miR-335-3p, miR-339-3p and miR-142-3p—were significantly differentially expressed between WT and MSTN$^{-/-}$ mice only at E15.5 (Supplementary Fig. S3), suggesting that these miRNAs are involved in fetal myogenesis. Importantly, several miRNAs (miR-31, miR-133a, miR-133b, miR-206, miR-486, miR-27a) that were differentially expressed in muscle between MSTN$^{-/-}$ mice and their WT littermates (Supplementary Fig. S2C–2H) had previously been implicated in the regulation of muscle development [11,19–21], indicating that MSTN might

A

30 miRNA-microarray: Triplicates for two genotypic mice with 5 developmental stages

Stage	wild type (WT)			MSTN-/-		
	1	2	3	4	5	6
E15.5	1-1	1-2	1-3	1-4	1-5	1-6
4-week	2-1	2-2	2-3	2-4	2-5	2-6
10-week	3-1	3-2	3-3	3-4	3-5	3-6
16-week	4-1	4-2	4-3	4-4	4-5	4-6
41-week	5-1	5-2	5-3	5-4	5-5	5-6

B



Affymetrix® GeneChip® miRNA Arrays

- Total 609 mus musculus miRNAs on chip
- 116 (19%) differentially expressed miRNAs
- More DE miRNAs at postnatal stages than at embryonic E15.5, which is consistent with the known function of myostatin in regulating myogenesis

C

Stages	DE miRs	Up in MSTN$^{-/-}$	Down in MSTN$^{-/-}$
E15.5	5	2	3
4-week	33	19	14
10-week	25	14	11
16-week	24	13	11
41-week	29	15	14
Total	116	63	53

Fig. 1. Systematic identification of MSTN-regulated miRNAs. **A**. Strategy for identifying MSTN-regulated miRNAs. Total RNA isolated from whole embryos at E15.5 and gastrocnemius (Gastro) muscles at the indicated postnatal ages (weeks) was analyzed using microRNA microarrays. Three technical replicates of microarray experiments were performed using three male mice per genotype at every each age. **B**. Heatmap demonstrating differentially expressed (DE) miRNAs between MSTN$^{-/-}$ mice and WT littermates. **C**. Numbers of differentially expressed miRNAs (DE miRs) upregulated (Up) and downregulated (Down) in MSTN$^{-/-}$ mice compared with WT littermates.

regulate the transcription of these miRNAs as part of its role in governing muscle growth and hypertrophy.

3.2. Expression of the miR-431 gene is downregulated by MSTN in skeletal muscle cells

We recently showed that miR-431, which is predominantly expressed in skeletal muscle, promotes myogenic differentiation by fine-tuning Pax7 (paired box 7) levels during muscle development (our unpublished data). Our microarray analyses indicated that expression levels of the developmentally downregulated miR-431 gene were higher in skeletal muscles of MSTN$^{-/-}$ mice compared with those in WT mice (Fig. 2A); this finding was further corroborated by a real-time polymerase chain reaction (PCR) analysis (Fig. 2B). We also examined miR-431 expression levels in primary myoblasts isolated from skeletal muscle of MSTN$^{-/-}$ mice and WT littermates. Consistent with the results of tissue analyses, we found that miR-431 expression levels were higher in both proliferating and differentiating myoblasts isolated from MSTN$^{-/-}$ than in those from WT littermates (Fig. 2C). Taken together, our data suggest that expression of the miR-431 in muscle cells is downregulated by MSTN. To further confirm this, we treated C2C12 myoblast cells with different concentrations of recombinant MSTN protein (rMSTN) and analyzed the expression of the miR-431 gene by real-time PCR. Consistent with the *in vivo* results shown in Fig. 2A–C, rMSTN induced a significant concentration- and time-dependent decrease in miR-431 expression levels in C2C12 cells (Fig. 2D, E). In addition, we further demonstrated that MSTN-mediated downregulation of miR-431 expression was partially attenuated by the MSTN inhibitor follistatin (Fig. 2F). Collectively, our *in vivo* and *in vitro* evidence support the conclusion that MSTN downregulates expression of the miR-431 gene in skeletal muscle cells.

3.3. MSTN-mediated inhibition of myogenic differentiation is attenuated by miR-431 overexpression in skeletal muscle cells

MSTN inhibits myogenic differentiation [22,23], and we have recently reported that overexpression of miR-431 promotes myogenic differentiation both *in vitro* and *in vivo* (our unpublished data). Thus, it is conceivable that MSTN might inhibit myogenic differentiation by downregulating miR-431 expression in muscle cells. To test this hypothesis, we generated C2C12 cells stably overexpressing miR-431 (Fig. 3A). Consistent with our previous report [23], the rMSTN suppressed differentiation in control cells (NC) as evidenced by decrease in MyoG mRNA (Fig. 3B) and protein levels (Fig. 3C), however, rMSTN-induced differentiation inhibition was significantly attenuated in miR-431–overexpressing C2C12 cells (Fig. 3B, C). We further immunostained miR-431–overexpressing C2C12 cells treated with rMSTN in differentiation medium with an antibody against the myogenic differentiation marker, myosin heavy chain (MHC) (Fig. 3D), and counted the numbers of MHC-positive (MHC^{+}) cells. As expected, MSTN treatment remarkably reduced the numbers of MHC^{+} cells in control NC stable cells (Fig. 3D, E), and overexpression of miR-431 in C2C12 cells significantly attenuated rMSTN-induced differentiation inhibition as evidenced by more numbers of MHC^{+} cells than that in MSTN-treated control C2C12 cells (Fig. 3D, E). The results were further supported by an examination of MHC expression at both mRNA (Fig. 3F) and protein (Fig. 3G) levels. Taken together, our findings indicate that miR-431 plays a critical role in MSTN-mediated inhibition of myogenic differentiation.

3.4. MSTN regulates miR-431 via the Ras/Raf-Mek-Erk signaling pathway

We have previously shown that activation of the Mek-Erk signaling pathway is required for MSTN function in muscle cells [23]. Here, we also demonstrated that miR-431 expression was downregulated by MSTN in muscle cells, and overexpression of miR-431 partially attenuated the inhibitory effect of MSTN on myogenic differentiation. Given these observations, we reasoned that MSTN might regulate miR-431 expression through the Mek-Erk signaling pathway. To test this possibility, we treated C2C12 cells with rMSTN in the presence or absence of the Mek inhibitor PD98059. As previously reported [23], rMSTN activated the Erk signaling pathway (Supplementary Fig. S4A and S4B). An

examination of miR-431 expression in C2C12 cells showed that miR-431 expression was downregulated by MSTN in the absence PD98059 (Fig. 4A), consistent with the results shown in Fig. 2. However, in the presence of PD98059, MSTN-mediated downregulation of miR-431 expression was attenuated (Fig. 4A), suggesting that MSTN-induced activation of Mek/Erk is required for MSTN-mediated downregulation of miR-431 expression in muscle cells. Next, we further examined the involvement of Ras kinase in MSTN-regulated downregulation of miR-431 in C2C12 cells overexpressing dominant-negative Ras (DN-Ras, Ras17N) [23], MSTN-induced activation of Mek/Erk was completely blocked in DN-Ras C2C12 cells (Supplementary Fig. S4C and S4D). In agreement with the results shown in Fig. 4A, MSTN-mediated downregulation of miR-431 was attenuated in DN-Ras C2C12 cells (Fig. 4B). It has been previously reported that phosphoinositide 3-kinase (PI3K) also plays a role in mediating MSTN function [24]. However, we found that MSTN-mediated downregulation of miR-431 was not blocked by the PI3K inhibitor wortmannin in C2C12 cells (Supplementary Fig. S5). Taken together, these findings indicate that the Ras-Mek-Erk signaling pathway is required for MSTN-mediated downregulation of miR-431 expression in muscle cells.

To identify transcriptional mediators of miR-431 gene transcription regulated by MSTN signaling, we sought to identify MSTN-responsive transcriptional factors (TF) using a TF activation profiling array. These experiments demonstrated that binding of signal transducer and activator 1 (Stat1) and IFN-stimulated response element (ISRE) was significantly enriched in response to MSTN treatment (Supplementary Fig. S6). These results suggest that Stat-1 and interferon-stimulated factors are candidate transcriptional factors involved in MSTN signaling-mediated regulation of miR-431 expression. In support of this, a sequence analysis of an 8-kb region upstream of the miR-431 precursor revealed the presence of multiple Stat1 and ISRE sites.

4. Discussion

The functional significance of miRNAs in controlling normal development and diseases has been well established; however, little is known about the molecular mechanisms underlying the transcriptional regulation of miRNA genes during development. The fact that a given miRNA has multiple targets in a cell implies that the specificity of the miRNA function is tightly regulated at either transcriptional or post-transcriptional levels. It has been well documented that miRNAs are engaged in muscle development through regulation of myogenic cell functions [11–14,20]. MSTN plays a critical role in controlling myogenesis, and it has been recently reported that TGF-β regulates miRNA gene expression [25]. Thus, it is conceivable that MSTN, as a member of the TGF-β superfamily, might regulate myogenesis by fine-tuning expression of muscle-related miRNA genes during development. Through a systematic analysis of miRNAs that are differentially expressed in skeletal muscle tissues between MSTN$^{-/-}$ and WT mice, we identified several myogenesis-related miRNAs (miR-31, miR-133a, miR-206, miR-486, miR-27, and miR-486), suggesting that the expression of such miRNA genes is potentially regulated by MSTN. Recently, Hitachi et al. also demonstrated that MSTN signaling regulates Akt activity via the regulation of miR-486 expression [21]. Taken together, these results indicate that miRNAs play a critical role in MSTN function during development.

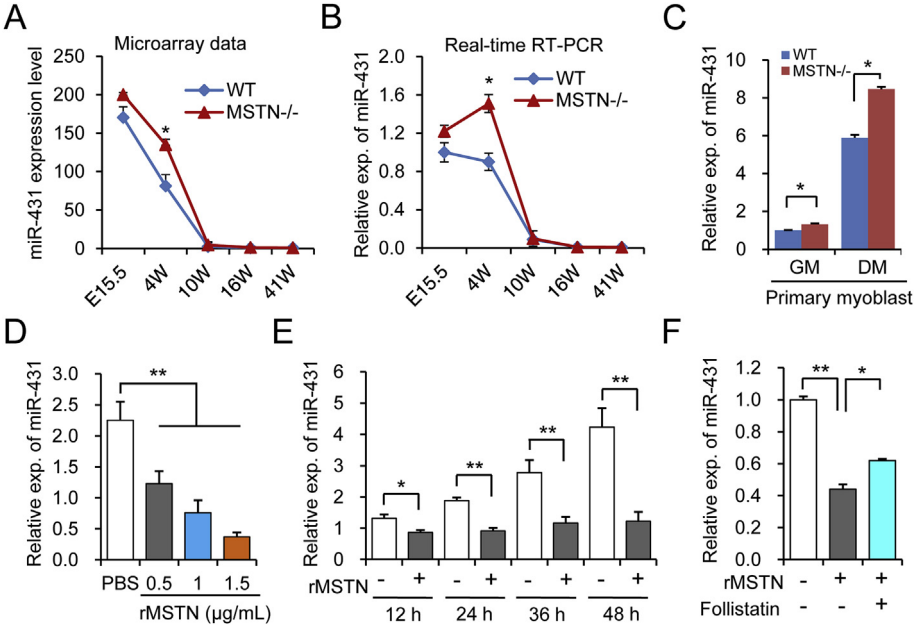


Fig. 2. miR-431 is downregulated by MSTN in a concentration- and time-dependent manner. **A**. miR-431 expression level in gastrocnemius muscle of MSTN$^{-/-}$ mice and WT littermates based on signal intensities in microarray analyses. Values are means ± SEM (*$P < 0.05$; n = 3 per time point for each genotype). **B**. miR-431 expression was validated by TaqMan quantitative RT-PCR using the same RNA samples as used in microarray experiments. U6 snRNA served as an internal control. **C**. The expression of miR-431 in primary myoblasts isolated from skeletal muscle of MSTN$^{-/-}$ mice and WT littermates was examined by TaqMan quantitative RT-PCR. GM, growth medium for culturing proliferating myoblasts; DM, differentiation medium for inducing myoblast differentiation. U6 snRNA served as an internal control. Values are means ± SEM of three separate experiments (*$P < 0.05$). **D**. miR-431 expression was analyzed in C2C12 myoblast cells treated with the indicated concentration of rMSTN or PBS (control) in differentiation medium (DM) for 24 h. U6 snRNA served as an internal control in real-time RT-PCR analyses. Values are means ± SEM of the three separate experiments performed in triplicate (**$P < 0.01$). **E**. miR-431 expression in C2C12 cells treated with 0.5 μg/mL of rMSTN or PBS (control) in DM for the indicated times. U6 snRNA served as an internal control. Values are means ± SEM of three separate experiments performed in triplicate (*$P < 0.05$, **$P < 0.01$). **F**. miR-431 expression in C2C12 cells treated with 0.5 μg/mL of rMSTN in DM for 24 h, with or without pretreatment with the MSTN inhibitor follistatin (100 ng/mL) for 30 min. U6 snRNA served as an internal control. Values are means ± SEM of three separate experiments performed in triplicate (*$P < 0.05$, **$P < 0.01$).

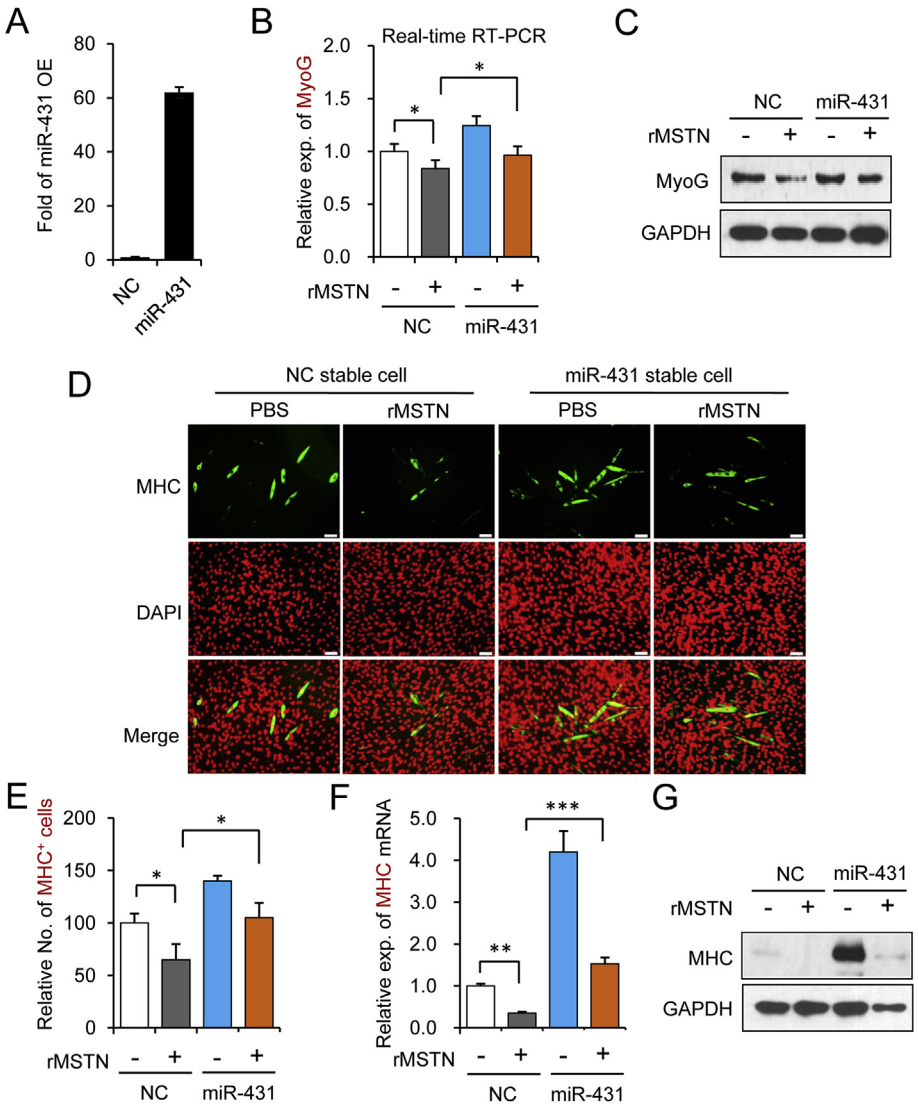


Fig. 3. Overexpression of miR-431 attenuates MSTN-induced inhibition of differentiation in C2C12 cells. **A**. Fold overexpression (OE) of miR-431 in C2C12 cells stably expressing miR-431 relative to that in control cells (NC). **B**. Myogenin (MyoG) mRNA was examined in C2C12 cells stabling overexpressing miR-431 and in control C2C12 cells (NC) treated with 0.5 μg/mL rMSTN or PBS (control) for 12 h in differentiation medium (DM). GAPDH served as an internal control for quantitative RT-PCR. Values are means ± SEM of three separate experiments performed in triplicate (*$P < 0.05$). **C**. MyoG protein was examined in C2C12 cells treated as described in panel B. GAPDH served as a control for equal loading in Western blot analyses. **D**. MHC staining in C2C12 cells stably overexpressing miR-431 and NC control C2C12 cells treated with 0.5 μg/mL of rMSTN for 36 h in DM. **E**. MHC-positive cells described in panel D were calculated and expressed as a percentage of that in the PBS-treated control (NC) group (*$P < 0.05$). **F**. MHC mRNA levels in samples treated as in panel D were examined by real-time RT-PCR. GAPDH served as an internal control. Values are means ± SEM of three separate experiments performed in triplicate (**$P < 0.01$, ***$P < 0.001$). **G**. MHC protein levels in samples treated as in panel D were analyzed by Western blotting. GAPDH served as a control for equal loading.

Interestingly, we also identified some differentially expressed miRNAs whose function has not previously been implicated in muscle development, including miR-431. We found miR-431 particular interesting because it is not only downregulated by MSTN, it is also predominantly expressed in skeletal muscle and brain tissues [26] (also see our unpublished data). The unique expression pattern of miR-431 suggests its functional significance in MSTN-regulated myogenesis during development. In support of this, we provided experimental evidence that miR-431 is downregulated by MSTN in a concentration- and time-dependent manner. Our unpublished data (currently under revision in *Nature Communication*) also show that miR-431 regulates satellite cell heterogeneity and function by directly targeting Pax7, an observation that aligns well with the idea that MSTN promotes satellite cell self-renewal by increasing Pax7 expression [27]. We further found that overexpression of miR-431 in C2C12 cells attenuated MSTN-mediated inhibition of differentiation in C2C12 cells, indicating that MSTN suppresses muscle cell differentiation through regulation of miR-431 expression.

Several reports, including those by our laboratory, have documented that MSTN functions through multiple signaling pathways [23,24]. Accordingly, we used chemical inhibitors of different pathways, including the MEK1 inhibitor PD98059 (Fig. 4A), PI3K inhibitor wortmannin (Supplementary Fig. S5), p38 inhibitor SB203580 and JNK inhibitor SP600125 (data not shown), to investigate which signaling pathway is involved in MSTN-mediated miR-431 expression. We found that only PD98059 abolished the downregulation of miR-431 by MSTN, indicating that MSTN acts through MEK1-dependent Erk1/2 activation to regulate miR-431 expression. Consistent with previous reports that the kinase activity of Ras is important in MSTN-mediated Mek1-Erk1/2 activation [23], we further found that rMSTN failed to negatively regulate

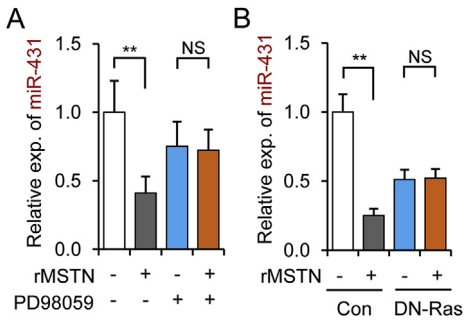


Fig. 4. MSTN regulates miR-431 via the Ras-dependent Mek-Erk signaling pathway. **A.** Expression of miR-431 was examined in C2C12 cells treated with 0.5 μg/mL of rMSTN or PBS (negative control [−]) in DM for 24 h, with or without pretreatment with PD98059 for 30 min. Data are presented relative to expression in the PBS-treated group. U6snRNA served as an internal control for TaqMan quantitative RT-PCR. **B.** Expression of miR-431 was examined in C2C12 cells overexpressing dominant-negative Ras (DN-Ras, Ras17N) and control C2C12 cells treated with 0.5 μg/mL of rMSTN in DM for 24 h (**$P < 0.01$).

miR-431 in C2C12 cells stably expressing dominant-negative Ras (DN-Ras). Collectively, these findings indicate that MSTN negatively regulates miR-431 through a Ras-dependent Mek1-Erk signaling pathway.

Interestingly, our preliminary analysis of potential transcriptional mediators of MSTN regulation of miR431 identified Stat1 and ISRE. Notably, both Stat1 and ISRE have been implicated in myogenesis regulation, as evidenced by studies in mdx mice demonstrating that a primary deficiency of dystrophin leads to the aberrant activation of nuclear transcription factors, including Stat-1 and ISRE, which might further contribute to the muscle pathogenesis observed in these mice [28]. Further investigation of the MSTN-Stat1 and MSTN-ISRE axes will provide additional molecular details about the mechanisms underlying MSTN function and the transcriptional regulation of the miR-431 gene.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.150.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.150.

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