Negative Auto-Regulation of Myostatin Expression is Mediated by Smad3 and MicroRNA-27

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Abstract
Growth factors, such as myostatin (Mstn), play an important role in regulating post-natal myogenesis. In fact, loss of Mstn has been shown to result in increased post-natal muscle growth through enhanced satellite cell functionality; while elevated levels of Mstn result in dramatic skeletal muscle wasting through a mechanism involving reduced protein synthesis and increased ubiquitin-mediated protein degradation. Here we show that miR-27a/b plays an important role in feed back auto-regulation of Mstn and thus regulation of post-natal myogenesis. Sequence analysis of Mstn 3’ UTR showed a single highly conserved miR-27a/b binding site and increased expression of miR-27a/b was correlated with decreased expression of Mstn and vice versa both in vitro and in vivo. Moreover, we also show that Mstn gene expression was regulated by miR-27a/b. Treatment with miR-27a/b-specific AntagomiRs resulted in increased Mstn expression, reduced myoblast proliferation, impaired satellite cell activation and induction of skeletal muscle atrophy that was rescued upon either blockade of, or complete absence of, Mstn. Consistent with this, miR-27a over expression resulted in reduced Mstn expression, skeletal muscle hypertrophy and an increase in the number of activated satellite cells, all features consistent with impaired Mstn function. Loss of Smad3 was associated with increased levels of Mstn, concomitant with decreased miR-27a/b expression, which is consistent with impaired satellite cell function and muscular atrophy previously reported in Smad3-null mice. Interestingly, treatment with Mstn resulted in increased miR-27a/b expression, which was shown to be dependent on the activity of Smad3. These data highlight a novel auto-regulatory mechanism in which Mstn, via Smad3 signaling, regulates miR-27a/b and in turn its own expression. In support, Mstn-mediated inhibition of Mstn 3’ UTR reporter activity was reversed upon miR-27a/b-specific AntagomiR transfection. Therefore, miR-27a/b, through negatively regulating Mstn, plays a role in promoting satellite cell activation, myoblast proliferation and preventing muscle wasting.


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Introduction
Myostatin (Mstn) is a secreted growth factor that belongs to the TGF-β super-family. Mstn is produced predominantly in skeletal muscle with lower levels of expression observed in white adipose tissue [1], heart [2] and mammary gland [3]. Analysis has revealed that Mstn is a profound negative regulator of muscle growth; while inactivation or mutation of the Mstn gene leads to increased skeletal muscle mass [1,4], enhanced myoblast proliferation [5], differentiation [6] and improved skeletal muscle regeneration [7], increased levels of Mstn result in severe cachectic-like muscle wasting [8–11].

The expression of Mstn is initially detected at embryonic day 9.5 in developing somites and continues to be detected postnatally in adult skeletal muscle fibers [1]. Furthermore, the mRNA expression of Mstn is developmentally regulated. While relatively abundant expression of Mstn is observed during fetal development, following birth the expression of Mstn rapidly decreases and remains quite low during postnatal development [12]. Previous work has also demonstrated that in adult skeletal muscle the expression of Mstn is greater in fast-twitch when compared to slow-twitch muscles [13,14] and thus is speculated to play a role in regulating muscle fiber type. Although we know that the expression of Mstn is regulated during myogenesis the exact mechanisms through which the abundance of Mstn is regulated remain to be fully determined. However, work from our lab has revealed that Mstn is transcriptionally regulated by the transcription factor MyoD through E-Box elements contained within the enhancer region of the Mstn gene [15]. In addition, further work
has demonstrated that Mstn is able to negatively auto-regulate its own expression through a Smad7-dependent mechanism [16]. In eukaryotes, transcriptional regulation is not the only mechanism through which gene expression is controlled. Most notably, post-transcriptional regulation of mRNA stability and translation through the action of microRNAs (miRNAs) plays a major role in regulating mRNA abundance. miRNAs are short single stranded RNAs that can bind specifically to complementary sequences found in 3’ untranslated regions (UTR) of target mRNAs, resulting in either repression of translation or degradation of mRNAs through the RNA-induced silencing complex (RISC) [17,18]. Previously published work has revealed that Mstn levels are regulated by miRNAs. Specifically, transgenic over expression of miR-208a in the heart has been shown to result in cardiac hypertrophy together with reduced expression of Mstn [19]. Furthermore, over expression of miR-499 leads to reduced Mstn 3’UTR activity, suggesting that Mstn is a target of miR-499 [20]. In addition, essential amino acids have been shown to promote muscle hypertrophy by not only suppressing Mstn levels but by inducing greater expression of miR-499, -206b, -23a, -1, and -206 [21]. Interestingly, a naturally occurring gain of function mutation in the 3’UTR region of the Texel sheep Mstn gene creates a miR-206 site causing translational inhibition of Mstn expression and a double muscling phenotype [22]. More recently, microRNA-27 (miR-27) has been shown to target and inhibit Mstn. Work by Allen and Loh revealed that miR-27a/b is able to reduce Mstn expression and mRNA stability and moreover indicated miR-27a/b may play a role in the increased expression of Mstn observed firstly, in Fast-twitch muscle, when compared to slow-twitch muscle and secondly, in response to Dexamethasone treatment [23]. Similarly, Huang et al revealed that over expression of miR-27a through addition of miR-27a mimics resulted in reduced Mstn mRNA expression and increased myoblast proliferation [24], consistent with known Mstn function.

Here we now show further evidence to support that Mstn expression is regulated by the miR-27a/b, as such AntagomiRs against miR-27a/b were able to increase Mstn expression, reduce myoblast proliferation and induce myotubular atrophy. Importantly, AntagomiR-27a/b-mediated myotube atrophy was due to increased Mstn function, as either blockade or complete absence of Mstn rescued the myotubular atrophy. Furthermore, results confirm a role for miR-27a/b in regulating muscle fiber type-specific and tissue-specific expression of Mstn and suggest that miR-27a/b may play a role in regulating Mstn expression and thus function during myogenic differentiation. We further show the utility of miR-27a/b in regulating Mstn expression and activity in vivo and that in Smad5-null mice there is increased expression of Mstn, which is due to reduced endogenous miR-27a/b expression in these mice. Results also reveal for the first time that Mstn up regulates the expression of miR-27a/b via Smad5, which in turn targets and represses Mstn, forming the basis of a novel microRNA-mediated Mstn negative auto-regulatory loop during myogenesis.

Materials and Methods

Ethics Statement

All experiments involving animals were approved by the Nanyang Technological University Institutional Animal Care and Use Committee (NTU-IACUC), Singapore (Approval Number: ARF SBS/NIE-A 0057). All surgery was performed under Ketamine/Xylazine anesthesia and all efforts were made to minimize animal suffering.

Animals

Four-six-week-old C57BL/6J male wild type (WT) mice were obtained from the National University of Singapore Centre for Animal Resources, Singapore. Myostatin-null mice (Mstn-/-) were gifted by Prof. See-Jin Lee (Johns Hopkins University, Baltimore, MD, USA). All mice were housed in groups at a constant temperature (20°C) under a 12 h/12 h artificial light/dark cycle with free access to water. To study the effect of miR-27a blockade in vivo, 20 nM (25 µl total volume; in sterile nuclease free water) of each AntagomiR (AntagomiR-27a or AntagomiR Neg) oligonucleotides [25], was injected into the M. tibialis anterior (TA) muscle of anaesthetized WT mice (n = 3) using a 29-gauge syringe (Hamilton Co., Reno, NV, USA). The contralateral limb of each mouse was injected with negative control AntagomiR (AntagomiR Neg). In vivo transfection of plasmid DNA (pcDNA-miR-27a or pcDNA-miR-neg) was performed by intramuscular injection of 25 µg (25 µl total volume; in sterile PBS) of each plasmid DNA into the TA muscle of anaesthetized mice. The contralateral limb of each mouse was injected with the empty vector (pcDNA-miR-neg) as a control. Electrical pulses (50 Volts/cm, 5 pulses, 200 ms intervals) were then applied with two platinum electrodes placed on either side of the muscle belly, using the ECM 830 Electroporation system (BTX Instrument Division, Harvard Apparatus, Inc. MA, USA). Eight days post-injection, M. tibialis anterior (TA) muscles from both sides of the hind-limb were harvested for histological and molecular analysis. For staining of skeletal muscle sections, TA muscles were covered with OCT compound and then frozen in isopentane cooled with liquid nitrogen. Transverse sections (8 µm) were cut from the mid-belly of the muscle and mounted on slides for hematoxylin and 1% cosin (H&E) staining and for detection of MyoD and Pax7 by immunocytochemistry. Images were captured using the Leica CTR 6500 microscope equipped with the Leica DFC 420 camera and Image Pro Plus software (Media Cybernetics, Bethesda, MD). Muscle fiber size was measured as Cross Sectional Area (CSA) from 500 myofibers per mouse (n = 3).

C2C12 myoblast cell culture

Mouse C2C12 myoblasts, obtained from American Type Culture Collection (Manassas, VA, USA), were maintained as previously described [26]. Assessment of C2C12 myoblast proliferation was performed as previously described [6,26]. Briefly, C2C12 myoblasts were seeded at a density of 1000 cells/well in 96-well plates in proliferation medium (DMEM, 10% FBS and 1% P/S; Invitrogen, Carlsbad, CA, USA). After an overnight attachment period, myoblasts were transfected with 25 nM each of AntagomiRs specific for miR-27a (AntagomiR-27a), miR-27b (AntagomiR-27b) or negative control AntagomiR (AntagomiR Neg) (Dharmacon Inc, USA) using Lipofectamine 2000 (LF2000; Invitrogen, USA), as per the manufacturer’s guidelines. Following a further period of 72 h growth, proliferation was assessed using the methylene blue photometric end-point assay, as previously described [27], where absorbance at 655 nm is directly proportional to final cell number. To assess the effect of miR-27a/b blockade on differentiated myotubes and to study the effect of Mstn blockade on AntagomiR-27a/b-mediated myotube atrophy, C2C12 myoblasts were induced to differentiate on Thermofisher coverslips under low-serum conditions (DMEM, 2% Horse Serum) for 24 h. Following this, 24 h differentiated C2C12 myoblasts were transfected, using LF2000, with 50 nM each of AntagomiR-27a, AntagomiR-27b or AntagomiR Neg. Twelve hours following transfection, cells were then treated with either vehicle (Dialysis buffer; DB) or with a soluble form of the Activin receptor Type IIB (sActRIIB) at a final concentration of 3 µg/ml and allowed to
Primary myoblast culture

Mouse primary myoblasts were cultured from hind-limb muscles isolated from WT and Mstn−/− mice using a modified method of Partridge TA [29]. Briefly, hind-limb muscles were excised, minced and then digested in 0.2% collagenase type 1A for 90 min. Fibroblasts were removed by pre-plating the cells on excised, minced and then digested in 0.2% collagenase type 1A for 90 min. Fibroblasts were removed by pre-plating the cells on uncoated plates for 3 h at 37°C. Primary myoblasts were cultured on 10% Matrigel (BD Biosciences) coated plates and were allowed to adhere for 48 h before further treatment with either CMM or CCM for 12 h.

Specific Inhibitor of SMAD3 (SIS3) treatment

C2C12 myoblasts were differentiated as described above for 48 h followed by a further 24 h in the absence (0.05% DMSO) or presence of the SMAD3-specific inhibitor SIS3 (10 μM; Sigma-Aldrich). Cells were then harvested for total RNA isolation and subsequent quantitative real-time PCR (qPCR) analysis.

Detection of MyoD and Pax7 by immunofluorescence

Muscle sections were fixed in 4% paraformaldehyde for 5 min and then permeabilized in 0.2% PBS-Tween 20. After this, sections were blocked in a solution containing 6% mouse IgG blocking reagent (MOM Immunodetection kit; Vector laboratories, Inc, CA, USA) and 3% bovine serum albumin (BSA) in PBS for 1 h, followed by 5 min in MOM protein diluent with 1.5% BSA in PBS, as per the manufacturer’s instruction. Muscle sections were then stained with mouse monoclonal anti-Pax7 (Developmental Studies Hybridoma Bank; DSHB, Iowa City, IA, USA; 1:1000) primary antibody in 1.5% BSA in PBS overnight at 4°C. Following incubation with horse biotinylated anti-mouse IgG (Vector laboratories, Inc, CA, USA; 1:500), rabbit polyclonal anti-Laminin (Sigma-Aldrich, Singapore; 1:1000) and rabbit polyclonal anti-MyoD (Santa Cruz, USA; 1:40) for 3 h, the sections were then washed and stained with Streptavidin conjugated Alexa Fluor 488 (Invitrogen; 1:1000) and goat anti-rabbit Alexa Fluor 594 (Invitrogen; 1:1000) for 30 min. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen) before mounting with prolong gold anti-fade mounting medium (Invitrogen). Pax7+ cells and activated myoblasts (MyoD7+) that lie underneath the basal lamina, as detected through Laminin staining, were counted and expressed as the percentage of positive nuclei per 100 myofibers. Images were captured using either the Nikon A1Rs confocal microscope equipped with Photometrics CoolSNAP HQ2 camera, or the Leica CTR 6500 microscope equipped with Leica DFC-420 camera and Image Pro Plus software (Media Cybernetics, Bethesda, MD).

Plasmids

The 3′-UTR of murine Mstn (1,448 bp) was PCR amplified using the following primers 5′-AAG CTT GCT TTG CAT TAG GTT-3′, 5′-AAG CTT GCC TTT CAA AAA TG-3′ and cloned as a HindIII fragment into the pmIR-REPORTTM miRNA Expression Luciferase Reporter Vector system (Life Technologies). The predicted miR-27a/b binding site within the Mstn 3′ UTR was mutated using a PCR-based mutagenesis approach with combinations of the primers above and the following megaprimer 5′-CCC CTC AAT TTC GAA GTC ACA GGT TCA AGC ACC ACA GG-3′, as per the protocol by Picard et al 1994 [30]. The mutated Mstn 3′ UTR was then cloned as a HindIII fragment into the pmIR-REPORTTM expression reporter vector, sequence verified to confirm mutation of the miR-27a/b binding region and named Mstn 3′ UTR-mut.

The pcDNA 6.2-GW/EmGFP vector containing mature miR-27a (pcDNA-miR-27a) was used for miR-27a overexpression studies. The pcDNA 6.2-GW/EmGFP vector (pcDNA-miR-neg) was used as a control.

The miR-27a promoter (miR-27a pro), miR-27b promoter (miR-27b pro) and the mutant miR-27b promoter reporter construct (miR-27b pro-mut) used in this study were kindly gifted by Dr Xiao Yang (State Key Laboratory of Proteomics, Genetic Laboratory of Development and Diseases, Institute of Biotechnology, Beijing, China) and have been described previously [31,32].

Transfections and Luciferase assay

For co-transfection of reporter plasmids and miR-27a over expressing vectors, C2C12 myoblasts were seeded into 24-well plates at a density of 15,000 cells/cm2 24 h before transfection. Proliferating C2C12 myoblasts were co-transfected with 0.1 μg Mstn 3′ UTR or Mstn 3′ UTR-mut reporter plasmids and 0.4 μg pcDNA-miR-27a or pcDNA-miR-neg, as a negative control. Transfection was carried out with LF2000 (Invitrogen, USA) according to the manufacturer’s protocol. For co-transfection of reporter plasmids and AntagomiRs against miR-27a/b, 50 nM of AntagomiR-27a, AntagomiR-27b or AntagomiR Neg were co-transfected with Mstn 3′ UTR or Mstn 3′ UTR-mut using LF2000 (Invitrogen, USA) as per the manufacturer’s protocol. After 48 h of transfections, cells were lysed, and luciferase assays were performed on protein extracts using the Dual-Luciferase reporter system (Promega, USA), according to the manufacturer’s recommendations. To assess for miR-27a and miR-27b promoter reporter activity, miR-27a pro, miR-27b pro and miR-27b pro-mut constructs were electroporated (GenePulsar MXcell, Bio-rad, Hercules, CA, USA) into 1 million C2C12 cells and grown to confluence. The electroporated C2C12 myoblasts were then plated at a density of 15,000 cells/cm2 in 24 well plates and treated with (10 μM) or without (0.05% DMSO) SIS3 in the presence (CMM; 10 ng/ml) or absence (CCM) of Mstn for 24 hrs.

Following transfections, cells were lysed, and luciferase assays were performed on protein extracts using the Dual-Luciferase reporter system (Promega, USA), according to the manufacturer’s recommendations. Renilla and firefly luciferase signals were detected using the GloMax luminometer (Promega, USA). AntagomiRs (AntagomiR-27a, AntagomiR-27b and AntagomiR Neg) were synthesized by Dharmacon Inc, USA. Synthetic miR-27b mimic and non-targeting miRNA negative control mimic were obtained from Dharmacon. A final concentration of 50 nM each of miR-
were normalized to GAPDH and U6, respectively. The following
expression was performed using the CFX96 Real-Time System
(Invitrogen, USA). Each qPCR reaction (10 µl) contained 3 µl of diluted
cDNA, 5 µl of 2 X SsoFast Evagreen (Bio-rad) and primers at a
final concentration of 200 nM. All reactions were performed using
the following thermal cycle conditions: 98°C for 3 min followed by
45 cycles of a two step reaction, denaturation at 98°C for 3 sec and
annealing at 60°C for 20 sec, followed by a denaturation curve
from 60°C to 95°C in 5 sec increments of 0.5°C to ensure
amplification specificity. To assess for the expression of mature
miR-27a and miR-27b, cDNA was synthesized from extracted
RNA using the miScript II RT kit (Cat.# MS00001351 and
Cat.# MS00001356 respectively; Qiagen), miScript universal
reverse primer (Qiagen) and miScript SYBR Green PCR Kit
(Cat.# 218075; Qiagen). The expression of miRNAs and miRNAs
were normalized to GAPDH and U6, respectively. The following
mouse-specific primers were used for qPCR analysis: precursor-miR-27a/b (pre-miR-27a/b) Forward 5′-GCA GGG CTT AGC
TGc TGC TTG-3′; Reverse 5′-GGG GCC ACT TAG CCA CCT T
-3′; Mstn Forward 5′-AGT GGA TCT AAA TGA GGG CAG T
-3′; Reverse 5′-GTG TCC AGC GGC AGC AGC TTA C-3′; U6
Forward 5′-CTC GCT TCG GCC GCA CA-3′ Reverse 5′-AAC
GCT TCA CAG ATT TGC GTG CT-3′ GPDH Forward 5′-ACA
ACT TGG GTA TGG AA-3′ Reverse 5′-GAT GCC GGG ATG ATG TGG T-3′.

Statistical analysis
Statistical analysis was performed using two-tailed Student’s t-test
and ANOVA. Data are expressed as mean ± SEM and p<0.05
were considered significant. Experimental replicates are described
in relevant figure legends.

Results
miR-27a/b targets and represses Mstn through a miR-
27a/b-specific target site in the 3′ UTR of the Mstn gene

In agreement with previously published reports [23,24], analysis
with the TargetScan5.1 (http://www.targetscan.org/) algorithm
revealed the presence of a single target site for microRNA-27a/b
(miR-27a/b) in the 3′UTR of the murine Mstn gene (Figure 1A).
Importantly, TargetScan analysis revealed an 8 mer seed match,
defined as a perfect match to positions 2–8 of the mature miRNA
followed by an adenosine, between the miR-27a and miR-27b seed
sequence and the miR-27a/b binding site in the 3′UTR of the
murine Mstn gene (Figure 1A). Furthermore the mstn 3′UTR miR-
27a/b target site was found to be flanked by AU-rich sequences,
which act to boost miRNA efficacy [33].

To further study miR-27a/b regulation of Mstn, we cloned the 3′
UTR region of the murine Mstn gene into the pMIR-
REPORT™ miRNA Expression Luciferase Reporter Vector and
co-transfected together with a miR-27a over expression vector
(pcDNA-miR-27a). A significant reduction in Mstn 3′UTR
reporter luciferase activity was observed in myoblasts co-
transfected with Mstn 3′UTR and pcDNA-miR-27a, when
compared with myoblasts co-transfected with Mstn 3′UTR and
control (pcDNA-miR-Neg) (Figure 1B). To identify if miR-27a/b
target site was responsible for the reduced luciferase activity
observed, the putative miR-27a/b binding site in the Mstn 3′UTR
was mutated. When pcDNA-miR-27a was co-transfected with the
mutant Mstn 3′UTR reporter (Mstn 3′UTR-mut), no
significant reduction in luciferase activity was observed (Figure 1C).
Furthermore co-transfection of Mstn 3′UTR with a miR-27a-
specific AntagomiR (AntagomiR-27a) resulted in significant
increase in Mstn 3′UTR reporter luciferase activity, over and
above that observed in control AntagomiR Neg transfected cells
(Figure 1B). The effect of AntagomiR-27a appeared to be specific to
the miR-27a/b site in the Mstn 3′UTR site, since addition of
AntagomiR-27a failed to increase luciferase activity in Mstn
3′UTR-mut reporter transfected myoblasts (Figure 1C). These
data strongly suggest that miR-27a/b is able to negatively regulate
Mstn miRNA and that the miR-27a/b target site found within the
Mstn 3′UTR is critical for miR-27a/b regulation of Mstn.

Correlation between Mstn and miR-27a/b expression in vivo and in vitro

While high levels of Mstn are detected in skeletal muscle, lower
levels of Mstn are expressed in white adipose tissue, heart and
mammary gland [1–3]. Furthermore, Mstn expression is higher in
fast-twitch muscles, when compared to slow-twitch muscles
[13,14]. To investigate whether or not miR-27a/b plays a role in
regulating tissue-specific Mstn expression, we analyzed precur-
sor-miR-27a/b (pre-miR-27a/b) and Mstn expression profiles in
different tissues. When compared to liver and biceps femoris (BF)
muscle, we noted reduced expression of Mstn and increased
expression of pre-miR-27a/b in the heart (Figure 1D and 1E). On
the other hand in tissues where relatively higher levels of Mstn were
observed, such as liver and BF muscle, lower pre-miR-27a/b
expression was detected (Figure 1D and 1E). Consistent with the
data published by Allen and Loh [23], we also observed a
difference in Mstn and pre-miR-27a/b expression between fast-
twitch and slow-twitch muscles. qPCR analysis revealed signifi-
cantly increased Mstn miRNA expression, concomitant with
reduced pre-miR-27a/b expression in the predominantly fast-
twitch BF muscle, when compared to the slow-twitch soleus (Sol)
muscle (Figure 1D and 1E).

Next we also compared the expression of Mstn and miR-27a/b
during differentiation in C2C12 myoblasts. Subsequent qPCR
revealed that there was relatively higher expression of Mstn at 24 h
differentiation, which sharply declined from 48 h differentiation
ongoing in C2C12 myoblasts (Figure 1F). However in contrast, we
observed a gradual increase in miR-27b expression from 24 h
through to 96 h differentiation (Figure 1G). Thus, the expression of
Mstn appears to be inversely associated with miR-27a/b
expression during C2C12 myoblast differentiation.

AntagomiR-mediated blockade of miR-27a/b leads to
enhanced Mstn activity

Mstn, as a negative regulator of skeletal muscle growth, has been
previously demonstrated to inhibit myoblast proliferation and
therefore induce severe myotubular atrophy in vitro [5,8,9].
Therefore, we next assessed the effect of AntagomiR-27a and AntagomiR-27b transfection on Mstn function during myogenesis. As predicted, transfection of AntagomiR-27a or AntagomiR-27b resulted in reduced expression of miR-27a (Figure S1A) and miR-27b (Figure S1B) respectively, when compared to control (AntagomiR Neg) transected cells (Figure 2A).

In addition, transfection of either AntagomiR-27a or AntagomiR-27b into differentiating C2C12 myotubes resulted in noticeable myotubular atrophy when compared to AntagomiR Neg transected myotubes (Figure 2B). Subsequent quantification revealed a significant 24% and 26% decrease in average myotube area in AntagomiR-27a and AntagomiR-27b transfected myotubes respectively, when compared to AntagomiR Neg transected myotubes (Figure 2C). Consistent with blockade of miR-27a/b and with the development of myotube atrophy a significant increase in Mstn expression was observed following transfection of C2C12 myotubes with AntagomiR-27a or AntagomiR-27b (Figure S1D).

To confirm whether or not the myotube atrophy observed following AntagomiR-mediated blockade of miR-27a/b was due to enhanced Mstn function, we next assayed myotube area in AntagomiR-27a and AntagomiR-27b transfected C2C12 myotubes cultures treated together with soluble Activin type IIb receptor (sActRIIB) Mstn antagonist. Treatment of AntagomiR-27a and AntagomiR-27b transfected C2C12 myotubes with sActRIIB rescued the myotubular atrophy observed in the AntagomiR only transfected myotubes (Figure 2B). Subsequent quantification revealed an ~40% and ~30% increase in average myotube area, which was similar to that observed in AntagomiR Neg transfected myotubes, in sActRIIB treated AntagomiR-27a and AntagomiR-27b transfected myotubes respectively, when compared to respective vehicle control (Dialysis buffer; DB) treated transfected myotubes (Figure 2C).

In agreement with the results above, AntagomiR-mediated reduction of miR-27a expression (Figure S1E) did not result in any appreciable myotube atrophy in Mstn-null mice-derived primary myotube cultures, when compared to AntagomiR Neg transfected primary myotubes (Figure 2D & 2E). However, in contrast, AntagomiR-mediated reduction of miR-27a in primary myotubes cultures isolated from WT mice (Figure S1G) led to elevated Mstn expression (Figure S1G) and observable myotubular atrophy (Figure 2D & 2E), with an ~32% decrease in average myotube area observed in AntagomiR-27a transfected myotubes, when compared to AntagomiR Neg transfected myotubes (Figure 2E).

These results confirm that blockade of miR-27a/b results in myotube atrophy through a mechanism dependent on Mstn.

miR-27a/b regulates myofiber size and SC function through targeting endogenous Mstn expression in skeletal muscle

To investigate whether miR-27a/b regulates endogenous Mstn levels in skeletal muscle, M. tibialis anterior (TA) muscles of WT mice were intramuscularly injected and in vivo electroporated with either the pcDNA-miR-27a over expression construct or control (pcDNA-miR-neg). The expression of Mstn was quantified by qPCR 8 days post-injection and as shown in Figure 3A overexpression of miR-27a in vivo resulted in a significant reduction in Mstn expression in TA muscle. Since Mstn is a potent negative regulator of skeletal muscle mass and satellite cell (SC) function [1,4,7,34], we also stained TA muscle serial sections with H&E (Figure 3B) and quantified myofiber cross sectional area (CSA), as well as the percentage of Pax7+ and MyoD+ cells in TA muscle following miR-27a overexpression. An ~30% increase in average myofiber CSA was observed in miR-27a overexpressing TA muscle, when compared to the control transfected contralateral TA muscle (Figure 3C). Furthermore we also observed an ~52% increase in the number of very large myofibers (>2500 μm²) and an ~60% decrease in the number of very small myofibers (<1500 μm²) upon miR-27a overexpression of miR-27a (Figure 3D), which is quite consistent with the increased myofiber CSA and loss of Mstn function. Interestingly, detection of Pax7 and MyoD by immunofluorescence (Figure 3E & S1H) revealed a significant ~12% and ~7% increase in the pool of Pax7+ cells and activated myoblasts (MyoD+) respectively in pcDNA-miR-27a transfected TA muscle, when compared with control-transfected contralateral TA muscle (Figure 3E & 3F).

To further confirm regulation of endogenous Mstn expression by miR-27a, we also injected either an AntagomiR specific for miR-27a (AntagomiR-27a) or a non-silencing negative control AntagomiR (AntagomiR Neg) into TA muscle of WT mice. The expression of Mstn was assessed 8 days post-injection and consistent with reduced miR-27a, Mstn expression was significantly up regulated, albeit modestly, upon AntagomiR-mediated blockade of miR-27a in vivo, when compared to AntagomiR Neg transfected contralateral TA muscle. (Figure 4A). We further stained TA muscle serial sections with H&E (Figure 4B) and although no significant difference was noted in average myofiber CSA (Figure 4C), we did observe an ~25% increase in the number of very small myofibers (<1500 μm²) and an ~10% decrease in the number of very large myofibers (>2500 μm²) (Figure 4D), which is quite consistent with the elevated Mstn.
expression detected. Furthermore, detection of Pax7 and MyoD by immunofluorescence (Figure 4E & S1H) revealed a significant ~11% and ~4% decrease in the pool of Pax7+ cells and activated myoblasts (MyoD+) respectively in AntagomiR-27a transfected TA muscle, when compared with AntagomiR Neg-transfected muscle (Figure 4E & 4F).

Increased Mstn expression observed in the absence of Smad3 is due to reduced miR-27a/b expression

Smad3-null mice display severe muscle atrophy, which has been attributed to elevated endogenous levels of Mstn detected in Smad3-null mice [35]. Therefore we next wanted to test whether or not the increased Mstn levels observed in Smad3-null mice was due to reduced miR-27a/b expression. Consistent with previously published data, qPCR analysis revealed a significant increase in Mstn expression in TA, GAS and QUAD muscles isolated from Smad3-null mice, when compared to WT controls (Figure 5A).

Importantly, the elevated Mstn expression was associated with a significant decrease in both mature miR-27a and miR-27b expression in all muscle tissues isolated from Smad3-null mice, as compared to WT mice (Figure 5B & 5C). Similarly, C2C12 myotubes treated with Specific Inhibitor of Smad3 (SIS3), a compound previously shown to specifically inhibit Smad3 function via suppressing Smad3 phosphorylation [36], displayed significantly increased Mstn expression concomitant with reduced pre-miR-27a/b (Figure 5D & 5E). To confirm that the reduced expression of miR-27 was responsible for the elevated Mstn expression detected in Smad3-null mice we next assessed Mstn expression between WT and Smad3-null mouse primary myoblast cultures following transfection of a miR-27b-specific mimic. As expected Mstn expression was significantly elevated in Smad3-null cultures, when compared to WT cultures (Figure 5F). Importantly, transfection of the miR-27b mimic reduced the expression of Mstn back to levels comparable to that observed in WT controls (Figure 5F), suggesting that reduced miR-27a/b expression may be responsible for the increased levels of Mstn observed in Smad3-null mice.

Mstn upregulates miR-27a/b expression through a Smad3-dependent mechanism to negatively auto-regulate its own expression

The data presented above suggested to us that Smad3 may play an important role in regulating basal miR-27a/b expression in muscle. Since Mstn is known to activate Smad3, we further hypothesized that Mstn may signal to up regulate the expression of miR-27a/b in muscle. To determine whether Mstn regulates miR-27a/b expression, C2C12 myoblasts and 48 h differentiated myotubes were treated with conditioned medium containing euukaryotic produced CHO-cell secreted Mstn protein (CMM) for 12 h. Pre-miR-27a/b expression was quantified by qPCR and as can be seen in Figure 6A & 6B, the expression of pre-miR-27a/b was significantly increased in both C2C12 myoblasts and myotubes upon treatment with CMM, when compared with cells treated with conditioned medium collected from control CHO cells (CCM) (Figure 6A & 6B). These data confirm that Mstn is able to positively regulate miR-27a/b expression in muscle.

To confirm whether Smad3 is involved in Mstn regulation of miR-27a/b, C2C12 myoblasts were transfected with either the miR-27a promoter (miR-27a pro), miR-27b promoter (miR-27b pro) or a mutant miR-27b promoter reporter construct, where the smad binding site has been mutated (miR-27b pro-mut) and subjected to treatment with CMM. Treatment with CMM resulted in a significant increase in promoter-reporter luciferase activity in myoblasts transfected with either the miR-27a or miR-27b promoter constructs (Figure 6C); however, no significant increase in luciferase activity was observed in C2C12 myoblasts transfected with the mutated miR-27b promoter construct following CMM treatment (Figure 6C). Transfected myoblasts were also subjected to treatment with both CMM and SIS3. As shown in Figure 6C, addition of SIS3 was able to partially rescue the increased miR-27a- and miR-27b-promoter-reporter luciferase activity observed following treatment with CMM alone (Figure 6C). Therefore these data confirm that Smad3 plays a critical role in the ability of Mstn to up regulate miR-27a/b expression.

Next we assessed whether or not the increased miR-27a/b, due to CMM treatment, would in turn target and repress Mstn expression. To test this C2C12 myoblasts transfected with the Mstn 3’UTR reporter were subjected to treatment with CMM. As shown in Figure 6D, treatment of Mstn 3’UTR reporter transfected myoblasts with CMM resulted in an ~50% reduction in Mstn 3’UTR reporter activity. The ability of CMM to reduce Mstn 3’UTR reporter activity appeared to be dependent on miR-27a/b function, as AntagomiR-mediated inhibition of miR-27a/b, as well as mutation of the miR-27a/b binding site in the Mstn 3’UTR, prevented CMM-mediated inhibition of Mstn expression (Figure 6D & 6E). Taken together these data highlight a novel negative auto-regulatory mechanism through which Mstn signals to regulate it’s own expression (Figure 6F).

Discussion

In the present study we have further characterized the role of miR-27a/b in regulating Mstn expression and activity. Evidence presented here confirms that Mstn is indeed a target of miR-27a/b both in vitro and in vivo. Consistent with previous reports [23,24], we show that over expression of miR-27a results in reduced Mstn 3’UTR reporter activity, which is blocked upon mutation of the miR-27a/b binding site in the Mstn 3’UTR. Furthermore, over expression of miR-27a in vivo led to decreased Mstn expression concomitant with myofiber hypertrophy and increased numbers of Pax7+ cells and activated myoblasts (MyoD+); quite consistent with the fact that loss of Mstn leads to increased muscle mass and
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Figure 3. Over expression of miR-27a targets and represses endogenous Mstn expression and function in vivo. (A) qPCR analysis of Mstn mRNA expression in TA muscle isolated from WT mice (n = 3) 8 days post intramuscular injection and in vivo transfection of either control (pcDNA-miR-neg) or miR-27a (pcDNA-miR-27a) over expression constructs. p<0.01 (**). (B) Representative images of H&E stained pcDNA-miR-neg and pcDNA-miR-27a in vivo transfected TA muscle cross sections from WT mice. Scale bars = 100 μm. (C) Graph showing average myofiber cross sectional area (CSA; μm²) in pcDNA-miR-neg and pcDNA-miR-27a in vivo transfected TA muscle from WT mice. Average myofiber area was calculated from 10 random images per coverslip (n = 3). p<0.001 (**). (D) Frequency distribution of myofiber area (μm²) in pcDNA-miR-neg and pcDNA-miR-27a in vivo transfected TA muscle from WT mice as calculated from 10 random images per coverslip (n = 3). (E) Left: Representative merged immunofluorescence image showing a Pax7⁺ cell (Green; white arrowhead) in a pcDNA-miR-27a in vivo transfected TA muscle cross section from WT mice. Sections were also stained for Laminin (Red) and nuclei were counterstained with DAPI (Blue). Scale bar = 10 μm. Right: Graph showing the number of Pax7⁺ cells in pcDNA-miR-neg and pcDNA-miR-27a in vivo transfected TA muscle from WT mice. Bars represent mean number ± S.E.M of Pax7⁺ cells, per 100 myofibers, from 3 sections each collected from pcDNA-miR-neg and pcDNA-miR-27a transfected WT mice (n = 3). p<0.01 (**). (F) Graph showing the number of MyoD⁺ cells in pcDNA-miR-neg and pcDNA-miR-27a in vivo transfected TA muscle from WT mice. Bars represent mean number ± S.E.M of MyoD⁺ cells, per 100 myofibers, from 3 sections each collected from pcDNA-miR-neg and pcDNA-miR-27a transfected WT mice (n = 3). p<0.01 (**).

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enhanced satellite cell number, activation and self-renewal [1,4,34]. Previously published work has revealed that hypertrophy of skeletal muscle may occur independent of satellite cell function [37]. Consistent with this, hypertrophy of skeletal muscle induced upon blockade of Mstn, was also shown to occur in the absence of satellite cells [38,39]. Nevertheless, as satellite cells play a critical
role during skeletal muscle regeneration [37] and that loss of Mstn leads to enhanced satellite cell activation, self-renewal and accelerated skeletal muscle regeneration [7,34] we strongly believe that the increased numbers of Pax7+ and MyoD+ cells observed following over expression of miR-27a is due to loss of Mstn. Recent work from Crist et al has shown that miR-27 is able to down regulate Pax3 protein levels, without affecting the levels of Pax7 [40]. However, we now show that over expression of miR-27
in vivo leads to increased numbers of Pax7+ cells. It is important to highlight that previously published work from our lab revealed that Mstn is a potent negative regulator of Pax7 expression during myogenesis [26]. Therefore, the increase in Pax7+ cells observed in response to overexpression of miR-27 is most likely due to miR-27-mediated inhibition of Mstn as opposed to direct regulation of Pax7 by miR-27. In addition to overexpression studies, we now show that blockade of miR-27a, through addition of an AntagomiR specific for miR-27a, results in enhanced Mstn expression and significantly reduced C2C12 myoblast proliferation. AntagomiR-mediated blockade of miR-27a and miR-27b not only upregulated Mstn expression but also significantly reduced C2C12 myoblast proliferation. These data are consistent with previously published reports demonstrating that excess Mstn inhibits myoblast proliferation [5] and with a recent report, which shows that addition of miR-27a mimics results in decreased Mstn mRNA concomitant with an increase in the number of proliferating C2C12 myoblasts [24]. In addition to controlling myoblast growth excess Mstn has been shown to promote skeletal muscle wasting in vivo and in vitro [10,11]. In agreement with this, we observed significantly increased expression of Mstn together with pronounced myotubular atrophy upon AntagomiR-mediated inhibition of miR-27a and miR-27b. This effect appeared to be dependent on increased Mstn as an ActRIIB-mediated blockade of Mstn rescued the atrophy phenotype and moreover primary myotube cultures isolated from Mstn-null mice were resistant to AntagomiR-induced myotube atrophy. It is important to mention that we noted differences in the effects of AntagomiR-27a and AntagomiR-27b mediated blockade of Mstn on myoblast cultures [24, SI1C to SI1D]. Although we do observe a significant increase in Mstn expression in both C2C12 myoblasts and myotubes transfected with either AntagomiR-27a or AntagomiR-27b the increase in Mstn was more significant in AntagomiR transfected myotube cultures, when compared to proliferating myoblasts. At this stage we do not know why there are differences in AntagomiR-27a- and AntagomiR-27b-mediated...
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(A) Myoblasts

(B) Myotubes

(C) Fold Change (Luciferase/Renilla)

(D) Main 3’UTR

(E) Main 3’UTR-mut

(F) Diagram of Myostatin and p-Smad3/4 Complex

CCM vs CMM

CMM+DMSO

CMM+DMSO

CMM+SIS3

CCM

AntagomiR Neg

AntagomiR-27a

AntagomiR-27b

CCM

AntagomiR Neg

AntagomiR-27a

AntagomiR-27b

NS
regulation of Mstn between myoblasts and myotubes, however we speculate that the Antagomir effect might be more persistent in myotube cultures when compared to proliferating myoblasts. Blockade of miR-27a/b in vivo also resulted in significantly increased Mstn expression, which was associated with decreased myofiber CSA. Unlike the dramatic phenotype observed in vitro, Antagomir-mediated blockade of miR-27a only resulted in minor muscle atrophy. However, it is important to mention that we only observed a slight increase in Mstn expression in vivo upon Antagomir injection, which we suggest may account for the subtle atrophy phenotype observed. Nevertheless, we did note a significant reduction in the numbers of Pax7+ cells and activated myoblasts (MyoD+) upon Antagomir-mediated blockade of miR-27a/b in vivo, further confirming that miR-27 is able to regulate Mstn.

Taken together these data presented here strongly support that miR-27a/b negatively regulates both Mstn expression and function in vitro and in vivo. Importantly, in our current experiments we did not observe any significant difference in the ability of miR-27a or miR-27b to regulate Mstn expression or activity. Given that miR-27a and miR-27b have the same “seed” sequence, UGACACU, which recognizes complementary sequences in the 3’UTRs of target genes, it is not surprising that we found no difference in the ability of miR-27a or miR-27b to regulate Mstn.

To date several studies have shown that Mstn expression is relatively higher in fast twitch muscle fibers, when compared to slow twitch muscle fibers [13,14]. Recently published evidence suggests that miR-27 may play a role in regulating skeletal muscle fiber type-specific expression of Mstn [23]. Specifically, work from Allen and Loh revealed that miR-27a and miR-27b fast-twitch and slow-twitch muscle-specific expression was complementary to that of Mstn. In the current manuscript we also found a similar trend in miR-27a/b and Mstn expression between fast and slow muscle fiber types. Interestingly, here we further show that miR-27a/b and Mstn expression was inversely associated between different tissues. Specifically, when compared to heart tissue, we noted higher Mstn expression, concomitant with reduced expression of miR-27a/b in liver tissue. It is noteworthy to mention that although expression of Mstn has been detected in Liver previously [41,42], high expression of Mstn in the Liver, as shown here, has not been previously reported. We speculate that variations in the expression of Mstn detected in Liver tissue between various studies may be due to differences in the sensitivity of the techniques used to assess for Mstn expression. In addition to regulating fiber type- and tissue-specific Mstn expression, we also show that miR-27a/b could potentially regulate Mstn mRNA levels during myogenic differentiation in vitro. As differentiation ensued we noted a decrease in Mstn expression, consistent with previous reports [43,44], concomitant with a steady increase in miR-27 expression.

These data are in agreement with a recently published report by Chen et al, which shows a similar increase in miR-27 expression and associated decrease in Mstn expression during myogenic differentiation [45]. As Mstn is a potent negative regulator of myoblast differentiation [6], we speculate that the elevated miR-27 expression may function in an autocrine/paracrine manner allowing for myogenic differentiation to proceed. Although it is tempting to suggest that epigenetic mechanisms (such as miR-27) could be responsible for regulating Mstn expression during differentiation, in our current experiments we noted that the increase in miR-27 expression during differentiation might not be enough to account for the dramatic drop in Mstn expression observed. These data suggest that additional factors may play a role in inhibiting Mstn during differentiation. One likely candidate, that may be responsible for regulating Mstn expression during differentiation, is Smad7. Consistent with this, previously published work from our lab clearly demonstrates that Smaod7 is able to inhibit Mstn expression [16]. Moreover, Kollia et al have previously demonstrated that the expression of Smad7 is elevated during differentiation and that over expression of Smad7 results in enhanced myogenic differentiation and rescue of Mstn-mediated inhibition of differentiation [46]. However, further work will need to be performed to confirm this. A role for miR-27 in regulating myogenic differentiation is not novel, in fact Crist et al recently demonstrated that miR-27b is able to negatively regulate PAX3 protein levels in adult muscle satellite cells to allow for timely entry into the myogenic differentiation program [40]. Therefore, taken together these data suggest that posttranscriptional regulation of Mstn mRNA by miR-27 plays a critical role in controlling timely tissue-specific expression/activity of Mstn during development.

Recently, we have shown that Smaod3-null mice have elevated expression of Mstn and not surprisingly pronounced skeletal muscle atrophy [35]. Here we have investigated if miR-27a/b could be responsible for the increased Mstn expression observed in Smaod3-null mice; and in agreement with increased Mstn expression we found reduced miR-27a and miR-27b expression in Smaod3-null mice. More importantly, mimic-mediated over expression of miR-27b in Smaod3-null mice was able to reduce Mstn expression back to levels comparable to WT mice. Therefore we speculate that loss of Smaod3 leads to reduced miR-27a/b expression, which in turn increases Mstn mRNA stability and or translation leading to enhanced skeletal muscle wasting. These data, together with the fact that specific inhibitor of Smaod3 (SIS3) treatment was able to significantly reduce miR-27a/b expression, suggest that Smaod3 plays an important role in regulating endogenous miR-27a/b expression. Further support for Smaod3 regulation of miR-27 is seen in published work from Sun et al, which revealed the presence of a Smaod binding element in the miR-24-2/miR-23a/miR-27a
cluster upstream regulatory sequence and that the Smad binding site was critical for TGF-β1-mediated inhibition of miR-24-2/miR-29a/miR-27a [31].

Interestingly, we now show for the first time that Mstn is able to up regulate the expression of miR-27a/b. Furthermore, Smad3 is critical for Mstn regulation of miR-27a/b as either mutation of the Smad binding site or treatment with SIS3 ablated the Mstn-mediated response. Mstn has been previously shown to negatively feedback to block its own expression/activity, through mechanisms involving inhibition of Mstn proteolytic processing and Smad7-dependent inhibition of Mstn expression [12,16]. Here we now describe an independent mechanism through which Mstn regulates its own expression. Specifically, addition of exogenous Mstn resulted in increased miR-27a/b expression, which in turn led to reduced Mstn 3’ UTR activity. This mechanism was dependent on miR-27a/b, as either blockade of miR-27a/b or mutation of the miR-27a/b binding site in the Mstn 3’ UTR prevented Mstn feedback regulation. These data, together with previously published work, suggest that there are independent auto-regulatory mechanisms through which Mstn regulates its own activity; given the fact that Mstn is a potent negative regulator of skeletal muscle myogenesis, we speculate that such mechanisms are in place to allow for timely regulation of myogenesis.

In summary, we provide further evidence to support a role for miR-27 in regulating Mstn expression. Evidence suggests that miR-27a and miR-27b play an important role in controlling tissue-specific and muscle fiber type-specific expression of Mstn and regulating Mstn function during myogenesis. Furthermore, we now show that miR-27a/b forms the basis of a novel negative auto-regulatory mechanism through which Mstn inhibits its own expression in muscle.

Supporting Information

Figure S1 AntagomiR-mediated inhibition of miR-27a/b and enhanced expression of Mstn. (A) qPCR analysis of miR-27a expression in C2C12 myoblasts following transfection of AntagomiR Neg or AntagomiR-27a. Bars represent fold change (relative to AntagomiR Neg control) ± S.E.M (n = 3) normalized to U6 expression. p < 0.001 (**). (B) qPCR analysis of miR-27b expression in C2C12 myoblasts following transfection of AntagomiR Neg or AntagomiR-27b. Bars represent fold change (relative to AntagomiR Neg control) ± S.E.M (n = 3) normalized to U6 expression. p < 0.001 (**). (C) qPCR analysis of Mstn expression in C2C12 myoblasts following transfection of AntagomiR Neg, AntagomiR-27a or AntagomiR-27b. Bars represent fold change (relative to AntagomiR Neg control) ± S.E.M (n = 3) normalized to GAPDH expression. p < 0.001 (**). (D) qPCR analysis of Mstn expression in differentiated C2C12 myotubes following transfection of AntagomiR Neg, AntagomiR-27a or AntagomiR-27b. Bars represent fold change (relative to AntagomiR Neg control) ± S.E.M (n = 3) normalized to GAPDH expression. p < 0.001 (**). (E) qPCR analysis of miR-27a expression in differentiated primary myoblast cultures from Mstn-null mice following transfection of AntagomiR Neg or AntagomiR-27a. Bars represent fold change (relative to AntagomiR Neg control) ± S.E.M (n = 3) normalized to U6 expression. p < 0.001 (**). (F) qPCR analysis of miR-27a (F) and Mstn (G) expression in differentiated primary myoblast cultures from WT mice following transfection of AntagomiR Neg or AntagomiR-27a. Bars represent fold change (relative to AntagomiR Neg control) ± S.E.M (n = 3) normalized to U6 (F) or GAPDH (G) expression. p < 0.001 (**). (H) Upper panel: Representative immunofluorescence images showing Pax7+ cells (Green; white arrowheads) in an in vivo transfected TA muscle cross section from WT mice. Nuclei were counterstained with DAPI (Blue) and a MyoD/DAPI merged image is also shown. Scale bars = 10 μm. Lower panel: Representative immunofluorescence images showing MyoD+ cells (Red; white arrowheads) in an in vivo transfected TA muscle cross section from WT mice. Nuclei were counterstained with DAPI (Blue) and a MyoD/DAPI merged image is also shown. Scale bars = 100 μm. (TIF)

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Author Contributions

Conceived and designed the experiments: CM AV HA XG SB RM RK MS. Wrote the paper: CM AV HA XG SB RK MS.

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