Citation: Molecular Therapy–Nucleic Acids (2012) 1, e23; doi:10.1038/mtna.2012.15

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Small RNA-Mediated Epigenetic Myostatin Silencing

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Myostatin (Mstn) is a secreted growth factor that negatively regulates muscle mass and is therefore a potential pharmacological target for the treatment of muscle wasting disorders such as Duchenne muscular dystrophy. Here we describe a novel Mstn blockade approach in which small interfering RNAs (siRNAs) complementary to a promoter-associated transcript induce transcriptional gene silencing (TGS) in two differentiated mouse muscle cell lines. Silencing is sensitive to treatment with the histone deacetylase inhibitor trichostatin A, and the silent state chromatin mark H3K9me2 is enriched at the Mstn promoter following siRNA transfection, suggesting epigenetic remodeling underlies the silencing effect. These observations suggest that long-term epigenetic silencing may be feasible for Mstn and that TGS is a promising novel therapeutic strategy for the treatment of muscle wasting disorders.

Molecular Therapy–Nucleic Acids (2012) **1,** e23; doi:10.1038/mtna.2012.15; published online 15 May 2012

Introduction

Myostatin (Mstn) (formerly growth and differentiation factor 8) is an endogenous, secreted protein from the transforming growth factor-β superfamily that negatively regulates skeletal muscle growth and differentiation.[1] Mstn null mutations result in increased musculature in cattle,[2] mice[1,3] and humans.[4] Mstn inhibition has been shown to induce functional improvement in the *mdx* mouse model[5–10] of Duchenne muscular dystrophy, an X-linked, monogenic muscle wasting disorder caused by loss-of-function mutations in the gene encoding dystrophin. Impaired dystrophin function results in progressive muscle degeneration leading to death, typically in the third decade of life by cardiac or respiratory failure.[11] Previous studies have demonstrated that the combination of Mstn blockade with dystrophin restoration in *mdx* mice resulted in a greater functional improvement than either treatment alone.[12] Consequently, the Mstn signaling pathway is a promising pharmacological target for the treatment of muscle wasting conditions such as Duchenne muscular dystrophy. A number of strategies have been used to achieve Mstn blockade including Mstn neutralizing antibodies,[13] endogenous Mstn antagonists (Mstn propeptide,[5] follistatin,[7] and soluble Acvr2b (the Mstn receptor)),[14] destructive exon-skipping,[15] and RNA interference (RNAi).[16]

An alternative to the canonical RNAi pathway (also known as post-transcriptional gene silencing, PTGS) is transcriptional gene silencing (TGS).[17,18] TGS is a homology-dependent gene silencing pathway mediated by small interfering RNAs (siRNAs), viral/plasmid expressed short hairpin RNAs[19] or expressed antisense RNAs[20,21] with complementarity to target gene promoters. These small RNA effector molecules target low copy-number promoter-associated RNA transcripts[22] in order to recruit chromatin-remodeling factors[20] to the complementary promoter and, in some cases, induce promoter DNA methylation.[19,22] The induction of targeted epigenetic changes enables long-term therapeutic gene silencing. To this end Suzuki *et al.* showed that transient transfection of an siRNA targeting the *human immunodeficiency virus*-1 5′ long terminal repeat was able to suppress *human immunodeficiency virus* replication for 31 days in the HeLa-derived MAGIC-5 cells.[23] Building on this work, Yamagashi *et al.* were able to silence *human immunodeficiency virus* messenger RNA (mRNA) transcription and viral replication for up to 1 year using a retrovirus-expressed short hairpin RNAs in a T-cell line (Molt-4).[24] Similarly, Hawkins *et al.* showed that long-term (up to 31 days) transcriptional silencing of Ubiquitin C could be achieved by 3 days of tet-induced promoter-targeted short hairpin RNAs expression.[17] Collectively, these studies suggest that long-term, targeted gene suppression by TGS may be possible in a therapeutic context. The aim of this work is to demonstrate the feasibility of silencing of Mstn expression by TGS. We show that Mstn expression is silenced by a promoter-targeted siRNA and that the silencing involves epigenetic remodeling of the Mstn promoter. This study thus opens up a new therapeutic avenue in the treatment of muscle wasting disorders.

Results

Detection of Mstn promoter-associated RNA

Previous studies have shown that TGS in mammalian cells requires the presence of promoter RNA transcripts. The database of transcription start sites[25] and University of California, Santa Cruz genome browser[26] resources were used to identify the Mstn transcription start site. To characterize transcription at the Mstn promoter, directional reverse transcription polymerase chain reaction (RT-PCR) was performed

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Keywords: epigenetic; muscle degeneration; myostatin; siRNA; transcriptional gene silencing

Received 11 December 2011; revised 28 March 2012; accepted 28 March 2012

using primers that amplify a 153 bp region upstream of the annotated transcription start site in a strand-specific manner (**Figure 1a**). Transcripts were detected in both sense and antisense orientations indicating the presence of promoter-associated RNA at the Mstn promoter. PCR amplicons were sequenced to confirm identity (data not shown). Reverse transcriptase minus (RT–) control PCR reactions failed to amplify ruling out genomic DNA contamination (**Figure 1b**).

Promoter-targeting siRNAs induce transcriptional silencing of Mstn

Four siRNAs targeting the Mstn sense promoter-associated RNA (**Figure 1a**) were synthesized by *in vitro* transcription, transfected into differentiated C2C12 myotube cultures and Mstn expression assessed by reverse transcriptase-quantitative PCR (RT-qPCR). A mRNA-targeting siRNA that induces post-transcriptional gene silencing of Mstn was used as a positive control (PTGS control). One siRNA, siMstn-P2, was found to significantly reduce Mstn mRNA levels by 50% (**Figure 1c**). This level of silencing was observed in at least 20 independent transfections under similar conditions (*i.e.* 100 nM siRNA in differentiated myotube cultures) and found to be highly reproducible (mean knockdown = 48%, SD = 11.5%).

Reduction in Mstn expression was observed relative to two unrelated nonspecific control (NS ctrl) siRNAs (including an *in vitro* transcribed siRNA (siCCR5) which targets the human C–C chemokine receptor type 5) (**Supplementary Figure S1a**). In addition, transfection with two further control siRNAs; one with the siMstn-P2 sequence scrambled (siScrambled) and the other with the central four nucleotides of siMstn-P2 inverted (siMM), did not significantly reduce Mstn expression (**Supplementary Figure S1b**). Negligible batch-to-batch variation was observed between different siRNA preparations (**Supplementary Figure S1c**) and no significant cellular toxicity was observed between any of the siRNA treatments (**Supplementary Figure S1d**). Mstn silencing was further confirmed using a chemically synthesized siMstn-P2 (**Figure 2a**).

In addition, promoter-targeted silencing was dose-dependent although significant Mstn knockdown was only observed at siRNA concentrations of 50 nM and 100 nM (**Figure 2b**). Conversely, maximal silencing by the PTGS control siRNA was observed at 10 nM (data not shown). Statistically significant knockdown of Mstn was also observed in H2K *mdx* cells (a murine myoblast cell line that carries a mutation in dystrophin exon 23)[27] indicating that the silencing effect is not restricted to the C2C12 line (**Figure 2c**). Taken together, these data suggest that Mstn is susceptible to siRNA-directed TGS.

Previous studies have suggested that delivery of siRNA to the nucleus is essential to induce TGS and that nuclear targeting peptides were required to facilitate this delivery.[18,20,28] However, in this study silencing was observed with both conventional, commercially available transfection reagents (INTERFERin and RNAiMax) and with a stearylated transportan-10 derived peptide (PepFect14) that had previously been shown to effectively deliver splice-switching oligonucleotides to the nucleus[29] (**Figure 2d**).

Given that TGS and PTGS occur via different mechanisms, we hypothesized that co-transfection of TGS and PTGS siRNAs would act in a combinatorial manner to improve maximal Mstn gene silencing. Transfection of a combination of 50 nM siMstn-P2 and 50 nM PTGS control siRNA was compared against 100 nM of each individual siRNA or a NS ctrl siRNA. The combination of the two siRNAs gave the greatest knockdown (87%) (**Figure 2e**).

Mstn silencing is independent of interferon induction

In contrast with chemically synthesized siRNA molecules, small RNAs generated by *in vitro* transcription from T7 promoters are tri-phosphorylated at the 5′ terminus and therefore have the potential to induce nonspecific knockdown by activating the interferon response.[30] In order to investigate this possibility, C2C12 myotube cultures were transfected with all relevant siRNAs. After 48 hours total RNA samples were reverse transcribed and levels of the interferon-induced genes 2′,5′ oligoadenylate synthase

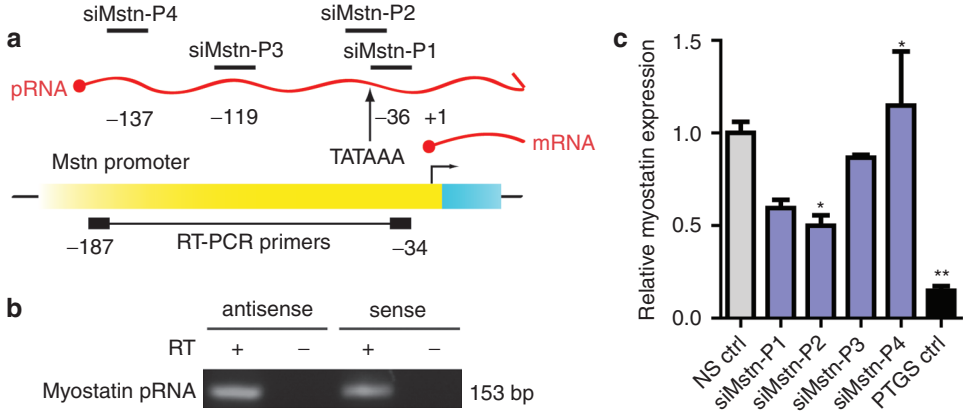

Figure 1 Screen of myostatin (Mstn) promoter-targeting siRNAs. (**a**) Schematic of Mstn promoter showing annotated transcription start site and hypothetical promoter-associated RNA transcript pRNA). Position of TATA box, location of reverse transcription polymerase chain reaction (RT-PCR) amplicon and small interfering RNA target sites are indicated. (**b**) Detection of pRNA at the Mstn promoter in sense and antisense orientations by directional RT-PCR. RT– controls do not amplify indicating no genomic DNA contamination. (**c**) Promoter-targeting siRNAs (blue bars) were transfected in differentiated C2C12 myotubes and Mstn expression assessed by RT-quantitative PCR (RT-qPCR). Results were normalized to a nontargeting control siRNA (nonspecific control, grey bar) and a coding sequence-targeting siRNA that silences Mstn by post-transcriptional gene silencing control (black bar) was used as a positive control for transfection. Values are mean + SEM, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$.

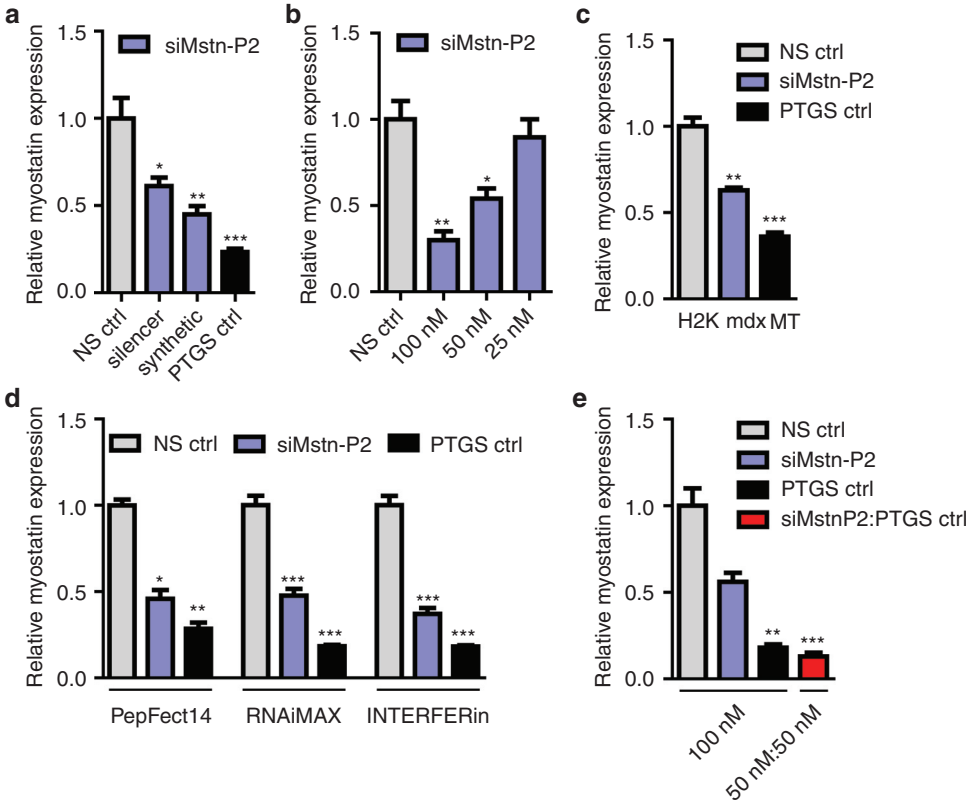

Figure 2 Promoter-targeting siRNAs silence myostatin expression. (**a**) C2C12 myotube (MT) cultures were transfected with *in vitro* transcribed (silencer) and chemically synthesized (synthetic) promoter-targeting small interfering RNA (siRNAs) (siMstn-P2). (**b**) Dose response of siMstn-P2 over the range of 25-100 nM, (**c**) H2K *mdx* MT were transfected with siMstn-P2. (**d**) Similar results are obtained in C2C12 MT cultures independent of transfection reagent used. (**e**) Transfection of a mixture of 50 nM siMstn-P2 and 50 nM post-transcriptional gene silencing control results in higher levels of silencing than either siRNA alone at 100 nM. All values are mean + SEM, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

1b and interleukin-6 were determined by RT-qPCR. Treatment with 15 μg/ml lipopolysaccharide was used as a positive control for interferon induction. Statistically significant induction of oligoadenylate synthase 1b and interleukin-6 was observed with the *in vitro* transcribed siRNAs (siMstn-P2 and siCCR5) but not with chemically synthesized siRNAs (**Figure 3a,b**). The stability of the reference gene transcript (β-Actin) was unaffected by transfection with *in vitro* transcribed siRNAs (**Figure 3c**). These results indicate that *in vitro* transcribed Silencer constructed siRNAs induce expression of interferon-stimulated genes whereas chemically synthesized siRNAs do not.

Epigenetic Mstn silencing

Acetylation of histone H3 lysine residues 9 and 27 is associated with transcriptionally active chromatin. Consequently, deacetylation of these residues is a necessary first step in the process of silent state chromatin formation. In order to determine whether siMstn-P2 induces epigenetic gene silencing, transfections were performed in the presence of the histone deacetylase inhibitor trichostatin A (TSA). siRNA transfected cultures were treated with a range of TSA concentrations (50 nM to 5 μM) and for each experimental condition Mstn expression was normalized to the NS ctrl. Silencing by siMstn-P2 was found to be sensitive to TSA concentrations above 500 nM whereas silencing by the PTGS control siRNA was largely unaffected (**Figure 4a**). Treatment with TSA was found to activate basal Mstn expression at low concentrations and was toxic at high concentrations, which is consistent with other reports[31] (**Figure 4b,c**). The observation that Mstn silencing by siMstn-P2 was abrogated by treatment with TSA at concentrations that activate Mstn expression in one case (500 nM) and are highly toxic in another (5 μM) is evidence that these factors are not confounding the results.

To investigate whether changes in chromatin structure are involved in Mstn silencing we performed chromatin immunoprecipitation analysis (ChIP) using antibodies against the silent state histone modifications; dimethyl-histone H3 lysine9 (H3K9me2) and trimethyl-histone H3 lysine27 (H3K27me3). Enrichment of H3K9me2 was detected at the Mstn promoter following treatment with siMstn-P2 although no change in H3K27me3 was detected (**Figure 4d**). Sensitivity of silencing to TSA treatment and changes in H3K9 methylation suggest that epigenetic remodelling at the Mstn promoter underlies the observed silencing effect.

Discussion

Here we have demonstrated that siRNAs complementary to the Mstn promoter induce silencing of Mstn mRNA expression. We have detected promoter-associated RNAs at the

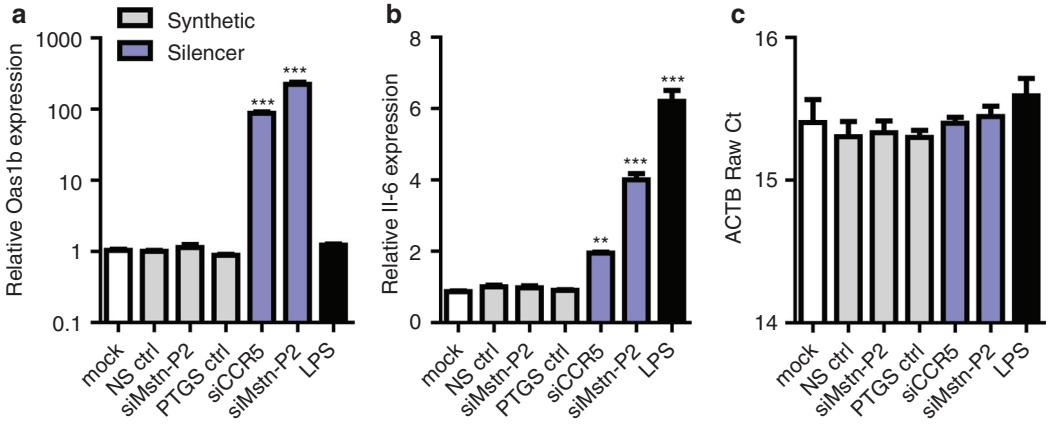

Figure 3 Myostatin silencing is independent of interferon induction. Treatment of C2C12 myotube cultures with *in vitro* transcribed, Silencer small interfering RNA (siRNAs) (blue bars), induces expression of (**a**) 2′,5′ oligoadenylate synthase 1b and (**b**) interleukin-6, whereas chemically synthesized siRNAs (grey bars) do not. Values are mean + SEM, $n = 3$, $^{**}P < 0.01$, $^{***}P < 0.001$. (**c**) Raw cycle threshold (Ct) values for β-Actin (ACTB) for the experiment depicted in (**a**) and (**b**) indicating high reference gene stability. Values are mean Ct + SD, $n = 3$. LPS, lipopolysaccharide.

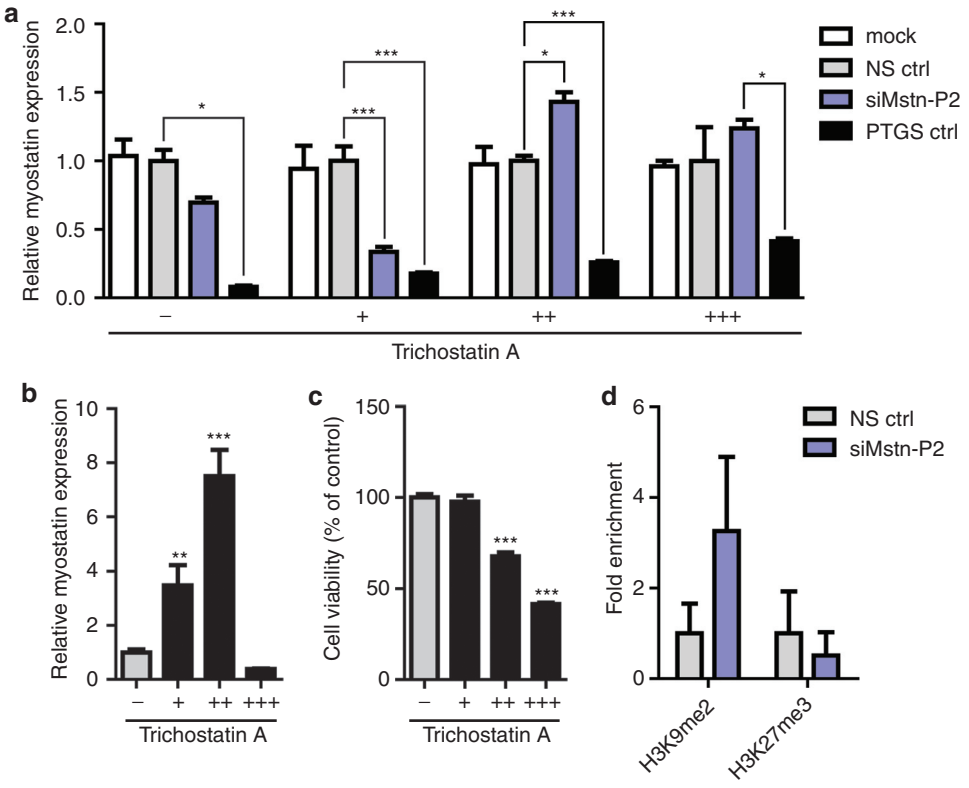

Figure 4 Epigenetic effects are involved in myostatin (Mstn) transcriptional gene silencing. (**a**) C2C12 myotubes were transfected with small interfering RNAs (siRNAs) in the presence or absence of trichostatin A and Mstn expression measured by reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR). Effects of increasing trichostatin A concentration on (**b**) basal Mstn transcription as measured by RT-qPCR and (**c**) cell viability as measured by MTS assay. (**d**) Chromatin immunoprecipitation with antibodies against H3K9me2 and H3K27me3 of C2C12 myotube cultures transfected with siMstn-P2 or nonspecific control siRNA. All values are mean + SEM, $n = 3$ for (**a**), (**b**), and (**d**) and $n = 6$ for (c). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. +, 50 nM; ++, 500 nM; +++, 5 μM.

Mstn locus and the sense-orientation transcript is the predicted target for the siRNA (although we have not shown a direct association between these two RNA species). Silencing was abrogated by treatment with the histone deacetylase inhibitor, TSA. Conversely, the PTGS control siRNA, which silences Mstn by acting upon the mature mRNA, was unaffected by TSA treatment. These results suggest that silencing with a promoter-targeting siRNA occurs by a different gene silencing pathway from conventional RNA interference. The results are consistent with other studies of TGS although we have not shown direct evidence of silencing at the level of transcription.

We also observed that the combination of TGS and PTGS siRNAs resulted in higher levels of silencing than either strategy alone suggesting that the two silencing pathways can operate in a combinatorial manner. We and others have shown that, following transient transfection of TGS effectors, target genes are typically silenced by ~50% which is considerably less than with conventional RNAi. However, the level of silencing is expected to increase over time as the chromatin at the target locus becomes progressively more compacted and the promoter DNA methylated. These silencing kinetics were observed in studies that looked at long-term knockdown by TGS.[17,24,32] However, in the case of Mstn, even relatively low levels of silencing (27% protein reduction) have been shown to result in significant functional changes in muscle mass (10% increase) suggesting that high levels of silencing are not required for a therapeutically relevant effect.[16] The demonstration of protein level silencing will be required to advance TGS as a therapy although, in this study, we were unable to consistently detect Mstn protein in cultured cells by western blot.

Several of the early studies to report TGS in mammalian cells used siRNAs produced by *in vitro* transcription.[18,20,28] siRNAs produced in this manner have the potential to elicit off-target gene silencing via induction of the interferon response.[30] These nonspecific events are either due to a gene being directly regulated by interferons, or as a result of global mRNA down-regulation, following activation of RNase L. We investigated this phenomenon by measuring expression of two interferon-induced genes (2′,5′ oligoadenylate synthase 1b and interleukin-6) following siRNA transfections in order to ensure specific Mstn silencing by siMstn-P2. We have shown that *in vitro* transcribed siRNAs induce highly statistically significant increases in expression of interferon-stimulated genes. However, the findings that; (i) silencing is observed with a synthetic siMstn-P2 in the absence of induction of the interferon-stimulated genes, (ii) silencing occurs relative to an *in vitro* transcribed control siRNA (siCCR5) and (iii) the stability of the housekeeping transcript (β-Actin) is unaffected by transfection with *in vitro* transcribed siRNAs, suggests that Mstn inhibition is not the result of off-target silencing because of interferon induction and activation of RNase L, but is rather a target-specific silencing effect.

Promoter-specific silencing was reversed in the presence of the histone deacetylase inhibitor, TSA, consistent with previous studies that have shown that histone deacetylase 1 is required for TGS.[17,32] Similarly, we have detected a modest enrichment of the silent state chromatin modification, H3K9me2, following transfection with a Mstn promoter-targeting siRNA as reported previously.[17,20,24,28,32] These results are indicative of an epigenetic silencing process. Interestingly, enrichment of H3K27me3 was not detected. Hawkins *et al.* have shown by RNAi depletion experiments that Enhancer of zeste homologue 2, a histone methyltransferase that methylates histone H3 lysine27, is not essential for TGS. This suggests that epigenetic silencing can occur in the absence of H3K27 methylation and that there is a degree of redundancy between silent state histone modifications.[17]

The detection of a promoter transcript involved in epigenetic regulation of the Mstn locus suggests that this may constitute part of an endogenous mechanism of gene regulation. Several studies have identified endogenous small RNAs (microRNAs) with complementarity to target gene promoters that induce TGS.[33–35] Long antisense RNAs have also been linked to epigenetic control of gene expression[36,37] and have been found to bind chromatin-remodeling factors.[38] Recently, a long noncoding RNA has also been implicated in myogenesis by acting as a competitive inhibitor for microRNAs involved in muscle differentiation.[39] However, the role of long noncoding RNAs, microRNAs, and pRNAs in epigenetic silencing is still not well understood.

This study adds to the growing literature that suggests small RNAs can direct TGS in mammalian cells and confirms that TGS occurs in mouse cells consistent with previous reports.[40] Several studies have demonstrated TGS in the absence of epigenetic changes, so-called antigene TGS.[41,42] These studies generally use oligonucleotides targeting transcriptional start sites and are speculated to involve direct interaction between the oligonucleotide and chromosomal DNA. Our data are more consistent with studies which have found a role for epigenetic changes involved in silencing and therefore lend credence to the idea that there are at least two distinct TGS mechanisms (epigenetic-TGS and antigene-TGS).

The use of TGS in animal models has so far been limited to three studies. Turunen *et al.* demonstrated that TGS of vascular endothelial growth factor is possible in mouse muscle following lentiviral transduction.[40] Similarly, local injection of promoter-targeting siRNAs against the human papillomavirus E6/E7 and thioredoxin-interacting protein promoters results in TGS in mouse xenograft tumors[43] and rat retina,[44] respectively. Although these studies are highly promising, the general application of TGS *in vivo* has not yet been fully explored and may present its own unique obstacles. For instance, the relative abundance of promoter transcripts is likely to differ between cell culture models and live animals and may affect the efficacy of a TGS approach. Despite the paucity of *in vivo* studies the therapeutic application of TGS is a promising alternative to RNAi, which is dependent on the presence of RNAi effectors that are gradually degraded within the cell or dispersed between daughter cells following cell division (effectively halving their concentration). Repeat administration is therefore required to elicit long-term silencing by siRNAs. Conversely, TGS effectors induce long-term silencing that is inherited following cell division and maintenance of silencing is not dependent on the presence of effector molecules.[17] A single treatment (or short course of treatments) may be sufficient to induce long-term gene silencing, hence repeat administration of TGS effectors is not required.[17,23] Consequently, TGS-based therapies may require a small number of high doses to elicit an effect. The overall dose will thus be lower with TGS-based therapies as opposed to conventional RNAi, meaning lower toxicity of treatments and greatly reduced material costs. In addition, it has been shown that saturation of endogenous RNA processing pathways can lead to acute toxicity *in vivo*.[45] As TGS does not require repeat administration this risk is greatly reduced. Epigenetic silencing of Mstn by TGS is therefore a promising novel therapeutic strategy in the treatment of muscle wasting disorders.

Materials and Methods

Directional RT-PCR. Total RNA was extracted from C2C12 myotubes using TRIzol reagent (Invitrogen, Paisely, UK) as according to the manufacturer's protocol and DNase treated using the TURBO DNase-*free* kit (Ambion/Applied Biosystems, Warrington, UK). 1 μg of total RNA was then reverse transcribed using SuperScript III RT (Invitrogen). In order to differentiate between sense and antisense transcripts complementary DNA (cDNA) synthesis was primed with either MstnPro-Rev (5′-AGCTTGCCCTCGACTGTAAC) or MstnPro-Fwd (5′-TCCAAGTGGCTTTTTATATTCCA), respectively. Following reverse transcription, PCR was performed with both primers and the amplification products analyzed by agarose gel electrophoresis. Mock reverse transcription controls (RT–) were performed to demonstrate that product amplification was not because of genomic DNA contamination. PCR products were gel extracted and sequenced to confirm identity.

siRNA. siRNAs were produced by *in vitro* transcription using the Silencer siRNA construction kit (Ambion) as according to manufacturer's instructions or by chemical synthesis (Eurogentec, Seraing, Belgium). Chemically synthesized siRNAs had 3′ dTdT overhangs. siRNAs designed to target the Mstn sense promoter-associated RNA all conform to the $AA(N_{19})$ pattern. All siRNA sequences used in this study are listed in **Supplementary Table S1**. Unless otherwise stated NS ctrl refers to "control siRNA duplex negative control" (Eurogentec, catalogue #SR-CL000-005). In addition, an siRNA which targets human C–C chemokine receptor type 5 (CCR5) mRNA was used as an additional NS ctrl produced by *in vitro* transcription. The sequence of the PTGS control siRNA was a kind gift from Luis Garcia.

Cell culture. C2C12 mouse myoblasts were maintained in growth media; Dulbecco's modified Eagle's media supplemented with 15% fetal bovine serum and antibiotic/antimycotic (all Invitrogen) and 5% CO_2. For differentiation, 24 well cell culture plates were coated with 0.02% gelatin (Sigma-Aldrich, Dorset, UK) and UV sterilized. C2C12 myoblasts were seeded (5×10^4 cells/ml) in growth medium and grown overnight. When confluent, the cells were switched to differentiation media; Dulbecco's modified Eagle's media supplemented with 5% horse serum (Invitrogen) and antibiotic/antimycotic. Cells were cultured in differentiation media for seven days in order to induce differentiation to myotubes.
H2K *mdx* tsA58 mouse myoblasts[27] were cultured in Dulbecco's modified Eagle's media supplemented with 20% fetal bovine serum), 2% chick embryo extract (PAA Laboratories Ltd, Yeovil, UK) and 20 U/ml γ-interferon (Invitrogen) at 33 °C and 10% CO_2 When confluent, the H2K *mdx* cells were switched to differentiation medium and switched to 37 °C and 5% CO_2 for 7 days.

Cells were transfected with RNAiMax (Invitrogen) and INTERFERin (PolyPlus Transfection, Strasbourg, France) were performed as according to manufacturer's instructions. Peptide transfections with PepFect 14 (CePep, Stockholm, Sweden) were performed as follows. Separate peptide and siRNA solutions were prepared in sterile water. The mixes were combined in a ratio of 30:1 (peptide:siRNA), mixed gently and incubated for 30 minutes at 37 °C to allow transfection complexes to form. After incubation, the transfection mixtures were diluted in optiMEM (Invitrogen) to a total volume of 0.45 ml per well (24 well plate). The media was removed from the C2C12 cultures and the cells washed with phosphate buffered saline (Invitrogen). Transfection complexes in optiMEM were then added to each well as appropriate and the cultures returned to the incubator. After 1 hour the cultures were supplemented with 50 μl fetal bovine serum and after 24 hours the transfection mixture was removed and replaced with fresh media. For all experiments mock transfections received transfection reagent/peptide only. Trichostatin A and lipopolysaccharide (both Sigma-Aldrich) were added to cell culture medium as appropriate. Cell viability was determined using the MTS assay (Promega, Southampton, UK) as according to manufacturer's instructions.

RT-qPCR. RT-qPCR was performed as follows. The planning, implementation and reporting of RT-qPCR experiments were designed to comply with the Minimum Information for publication of Quantitative real-time PCR Experiments guidelines[46,47] as much as was possible or appropriate. To generate cDNA 2 μg of high quality total RNA was reverse transcribed using the High-Capacity cDNA Synthesis kit (Applied Biosystems) as according to manufacturer's instructions. 20 μl RT reactions were primed using random primers. Samples were incubated at 25 °C for 10 minutes to allow primer extension to occur and then cDNA was reverse transcribed for 2 hours at 37 °C. The reaction was terminated at 80 °C for 15 minutes. Typically 200 ng cDNA was added to each reaction such that a maximum of 2 μl of RT reaction was added per well. RT-qPCR was performed on a StepOne Plus Real-Time PCR System (Applied Biosystems) using TaqMan Gene Expression Mastermix (Applied Biosystems). Relative quantities of target mRNA were determined using the relative standard curve method. Data were analyzed using the StepOne Software v2.1 (Applied Biosystems). Standard curves were produced from serial dilutions of cDNA reverse transcribed from untreated experimental samples. Universal cycling conditions were used (95°C for 10 minutes (hotstart) and then 40 cycles of 95°C for 15 seconds, 60°C for 1 minute). Typically, gene of interest expression was normalized to β-Actin expression. TaqMan assays were purchased from Applied Biosystems and are listed in **Supplementary Table S2**. Validation of RT-qPCR assays is described in **Supplementary Materials and Methods** and **Supplementary Figures S2 and S3**.

ChIP. ChIP was performed as described previously with several modifications.[17] A full protocol is described in the **Supplementary Materials and methods**. Briefly, C2C12 myotubes were cultured in 10 cm dishes and siRNAs transfected with RNAiMax. After 48 hours cells were harvested for ChIP assays. Chromatin samples were formaldehyde cross-linked, sonicated to shear the chromatin DNA and histone complexes and immunoprecipiated with antibodies against H3K9me2 (#07-441, Upstate/Millipore, Billerica, MA) and H3K27me3 (#97565, Cell Signalling Technologies, Danvers, MA). DNA was recovered by phenol chloroform extraction and resuspended in 30 μl of nuclease free water. Quantitative PCR was performed on a Mastercycler Realplex real-time thermal cycler (Eppendorf, Hauppauge,

NY) using KAPA SYBR Fast SYBR mastermix (KAPA Biosystems, Cambridge, MA) and the primers; MstnChIP-Fwd (5′-AGATTCATTGTGGAGCAGGAG) and MstnChIP-Rev (5′-ATATTAGTGCATGTACCGTCCG). The relative standard curve method was used to compare samples and a five fold dilution of input chromatin DNA used to prepare the standard curve (**Supplementary Figure S4a**). Dissociation curve analysis confirmed that only a single amplification product was generated by the PCR reaction (**Supplementary Figure S4b**). Sample values were calculated as background subtracted, fraction of input for each experimental group and then normalized to the NS ctrl siRNA group.

Statistical analysis. All statistical analysis was performed in SPSS v11.5 (IBM, Armonk, NY, USA). Data were assessed for normalcy using the Shapiro–Wilk test and equality of variance by the Levene's test. Significance within a data set was determined by one-way ANOVA (analysis of variance) and significant differences between treatment groups were determined *by post hoc* analysis. The Bonferroni correction or the Games–Howell test were used in the instances of equality and inequality of variance respectively. *P* values less than 0.05 were considered significant. Unless otherwise indicated all statistical comparisons are made against NS ctrl siRNA treated cultures (grey bars).

Acknowledgments. T.C.R. is supported by a MRC UK doctoral training award. S.E.A. is supported by a postdoctoral research fellowship from the Swedish Society of Medical Research (SSMF). This work was supported by grants awarded to M.J.A.W. from the AFM and ISIS Innovation. We acknowledge the kind gift of the PepFect14 peptide from Ülo Langel (Stockholm University and CePep, Stockholm). The authors declare no conflict of interest.

Supplementary Material

Figure S1. Validation of specific myostatin silencing.
Figure S2. Standard curves for (**a**) myostatin, and (**b**) β-Actin (ACTB) reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) assays.
Figure S3. Reverse transcriptase minus controls for myostatin (Mstn) and β-Actin (ACTB) reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) assays.
Figure S4. Validation of quantitative polymerase chain reaction (qPCR) for chromatin immunoprecipitation analysis.
Table S1. Sequences of small interfering RNAs used in this study.
Table S2. Reverse transcriptase-quantitative polymerase chain reaction TaqMan Primer/Probe assays used in this study.
Materials and Methods.

1. McPherron, AC, Lawler, AM and Lee, SJ (1997). Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature* **387**: 83–90.
2. Grobet, L, Martin, LJ, Poncelet, D, Pirottin, D, Brouwers, B, Riquet, J *et al.* (1997). A deletion in the bovine myostatin gene causes the double-muscled phenotype in cattle. *Nat Genet* **17**: 71–74.
3. Zhu, X, Hadhazy, M, Wehling, M, Tidball, JG and McNally, EM (2000). Dominant negative myostatin produces hypertrophy without hyperplasia in muscle. *FEBS Lett* **474**: 71–75.
4. Schuelke, M, Wagner, KR, Stolz, LE, Hübner, C, Riebel, T, Kömen, W *et al.* (2004). Myostatin mutation associated with gross muscle hypertrophy in a child. *N Engl J Med* **350**: 2682–2688.
5. Bogdanovich, S, Perkins, KJ, Krag, TO, Whittemore, LA and Khurana, TS (2005). Myostatin propeptide-mediated amelioration of dystrophic pathophysiology. *FASEB J* **19**: 543–549.
6. Whittemore, LA, Song, K, Li, X, Aghajanian, J, Davies, M, Girgenrath, S *et al.* (2003). Inhibition of myostatin in adult mice increases skeletal muscle mass and strength. *Biochem Biophys Res Commun* **300**: 965–971.
7. Nakatani, M, Takehara, Y, Sugino, H, Matsumoto, M, Hashimoto, O, Hasegawa, Y *et al.* (2008). Transgenic expression of a myostatin inhibitor derived from follistatin increases skeletal muscle mass and ameliorates dystrophic pathology in mdx mice. *FASEB J* **22**: 477–487.
8. Qiao, C, Li, J, Jiang, J, Zhu, X, Wang, B, Li, J *et al.* (2008). Myostatin propeptide gene delivery by adeno-associated virus serotype 8 vectors enhances muscle growth and ameliorates dystrophic phenotypes in mdx mice. *Hum Gene Ther* **19**: 241–254.
9. Bogdanovich, S, McNally, EM and Khurana, TS (2008). Myostatin blockade improves function but not histopathology in a murine model of limb-girdle muscular dystrophy 2C. *Muscle Nerve* **37**: 308–316.
10. Bogdanovich, S, Krag, TO, Barton, ER, Morris, LD, Whittemore, LA, Ahima, RS *et al.* (2002). Functional improvement of dystrophic muscle by myostatin blockade. *Nature* **420**: 418–421.
11. Hoffman, EP, Brown, RH Jr and Kunkel, LM (1987). Dystrophin: the protein product of the Duchenne muscular dystrophy locus. *Cell* **51**: 919–928.
12. Dumonceaux, J, Marie, S, Beley, C, Trollet, C, Vignaud, A, Ferry, A *et al.* (2010). Combination of myostatin pathway interference and dystrophin rescue enhances tetanic and specific force in dystrophic mdx mice. *Mol Ther* **18**: 881–887.
13. Wagner, KR, Fleckenstein, JL, Amato, AA, Barohn, RJ, Bushby, K, Escolar, DM *et al.* (2008). A phase I/IItrial of MYO-029 in adult subjects with muscular dystrophy. *Ann Neurol* **63**: 561–571.
14. Lee, SJ and McPherron, AC (2001). Regulation of myostatin activity and muscle growth. *Proc Natl Acad Sci USA* **98**: 9306–9311.
15. Kang, JK, Malerba, A, Popplewell, L, Foster, K and Dickson, G (2011). Antisense-induced myostatin exon skipping leads to muscle hypertrophy in mice following octa-guanidine morpholino oligomer treatment. *Mol Ther* **19**: 159–164.
16. Magee, TR, Artaza, JN, Ferrini, MG, Vernet, D, Zuniga, FI, Cantini, L *et al.* (2006). Myostatin short interfering hairpin RNA gene transfer increases skeletal muscle mass. *J Gene Med* **8**: 1171–1181.
17. Hawkins, PG, Santoso, S, Adams, C, Anest, V and Morris, KV (2009). Promoter targeted small RNAs induce long-term transcriptional gene silencing in human cells. *Nucleic Acids Res* **37**: 2984–2995.
18. Morris, KV, Chan, SW, Jacobsen, SE and Looney, DJ (2004). Small interfering RNA-induced transcriptional gene silencing in human cells. *Science* **305**: 1289–1292.
19. Castanotto, D, Tommasi, S, Li, M, Li, H, Yanow, S, Pfeifer, GP *et al.* (2005). Short hairpin RNA-directed cytosine (CpG) methylation of the RASSF1A gene promoter in HeLa cells. *Mol Ther* **12**: 179–183.
20. Weinberg, MS, Villeneuve, LM, Ehsani, A, Amarzguioui, M, Aagaard, L, Chen, ZX *et al.* (2006). The antisense strand of small interfering RNAs directs histone methylation and transcriptional gene silencing in human cells. *RNA* **12**: 256–262.
21. Turner, AM, De La Cruz, J and Morris, KV (2009). Mobilization-competent lentiviral vector-mediated sustained transcriptional modulation of HIV-1 expression. *Mol Ther* **17**: 360–368.
22. Han, J, Kim, D and Morris, KV (2007). Promoter-associated RNA is required for RNA-directed transcriptional gene silencing in human cells. *Proc Natl Acad Sci USA* **104**: 12422–12427.
23. Suzuki, K, Shijuuku, T, Fukamachi, T, Zaunders, J, Guillemin, G, Cooper, D *et al.* (2005). Prolonged transcriptional silencing and CpG methylation induced by siRNAs targeted to the HIV-1 promoter region. *J RNAi Gene Silencing* **1**: 66–78.
24. Yamagishi, M, Ishida, T, Miyake, A, Cooper, DA, Kelleher, AD, Suzuki, K *et al.* (2009). Retroviral delivery of promoter-targeted shRNA induces long-term silencing of HIV-1 transcription. *Microbes Infect* **11**: 500–508.
25. Wakaguri, H, Yamashita, R, Suzuki, Y, Sugano, S and Nakai, K (2008). DBTSS: database of transcription start sites, progress report 2008. *Nucleic Acids Res* **36**(Database issue): D97–101.
26. Karolchik, D, Hinrichs, AS and Kent, WJ (2011). The UCSC Genome Browser. *Curr Protoc Hum Genet Chapter 18*: Unit18.6.
27. Morgan, JE, Beauchamp, JR, Pagel, CN, Peckham, M, Ataliotis, P, Jat, PS *et al.* (1994). Myogenic cell lines derived from transgenic mice carrying a thermolabile T antigen: a model system for the derivation of tissue-specific and mutation-specific cell lines. *Dev Biol* **162**: 486–498.
28. Kim, DH, Villeneuve, LM, Morris, KV and Rossi, JJ (2006). Argonaute-1 directs siRNA-mediated transcriptional gene silencing in human cells. *Nat Struct Mol Biol* **13**: 793–797.
29. Ezzat, K, Andaloussi, SE, Zaghloul, EM, Lehto, T, Lindberg, S, Moreno, PM *et al.* (2011). PepFect 14, a novel cell-penetrating peptide for oligonucleotide delivery in solution and as solid formulation. *Nucleic Acids Res* **39**: 5284–5298.

30. Kim, DH, Longo, M, Han, Y, Lundberg, P, Cantin, E and Rossi, JJ (2004). Interferon induction by siRNAs and ssRNAs synthesized by phage polymerase. *Nat Biotechnol* **22**: 321–325.
31. Han, DS, Huang, HP, Wang, TG, Hung, MY, Ke, JY, Chang, KT *et al.* (2010). Transcription activation of myostatin by trichostatin A in differentiated C2C12 myocytes via ASK1-MKK3/4/6-JNK and p38 mitogen-activated protein kinase pathways. *J Cell Biochem* **111**: 564–573.
32. Suzuki, K, Juelich, T, Lim, H, Ishida, T, Watanebe, T, Cooper, DA *et al.* (2008). Closed chromatin architecture is induced by an RNA duplex targeting the HIV-1 promoter region. *J Biol Chem* **283**: 23353–23363.
33. Younger, ST, Pertsemlidis, A and Corey, DR (2009). Predicting potential miRNA target sites within gene promoters. *Bioorg Med Chem Lett* **19**: 3791–3794.
34. Kim, DH, Saetrom, P, Snøve, O Jr and Rossi, JJ (2008). MicroRNA-directed transcriptional gene silencing in mammalian cells. *Proc Natl Acad Sci USA* **105**: 16230–16235.
35. Tan, Y, Zhang, B, Wu, T, Skogerbø, G, Zhu, X, Guo, X *et al.* (2009). Transcriptional inhibiton of Hoxd4 expression by miRNA-10a in human breast cancer cells. *BMC Mol Biol* **10**: 12.
36. Morris, KV, Santoso, S, Turner, AM, Pastori, C and Hawkins, PG (2008). Bidirectional transcription directs both transcriptional gene activation and suppression in human cells. *PLoS Genet* **4**: e1000258.
37. Hawkins, PG and Morris, KV (2010). Transcriptional regulation of Oct4 by a long noncoding RNA antisense to Oct4-pseudogene 5. *Transcription* **1**: 165–175.
38. Khalil, AM, Guttman, M, Huarte, M, Garber, M, Raj, A, Rivea Morales, D *et al.* (2009). Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. *Proc Natl Acad Sci USA* **106**: 11667–11672.
39. Cesana, M, Cacchiarelli, D, Legnini, I, Santini, T, Sthandier, O, Chinappi, M *et al.* (2011). A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. *Cell* **147**: 358–369.
40. Turunen, MP, Lehtola, T, Heinonen, SE, Assefa, GS, Korpisalo, P, Girnary, R *et al.* (2009). Efficient regulation of VEGF expression by promoter-targeted lentiviral shRNAs based on epigenetic mechanism: a novel example of epigenetherapy. *Circ Res* **105**: 604–609.
41. Janowski, BA, Huffman, KE, Schwartz, JC, Ram, R, Hardy, D, Shames, DS *et al.* (2005). Inhibiting gene expression at transcription start sites in chromosomal DNA with antigene RNAs. *Nat Chem Biol* **1**: 216–222.
42. Napoli, S, Pastori, C, Magistri, M, Carbone, GM and Catapano, CV (2009). Promoter-specific transcriptional interference and c-myc gene silencing by siRNAs in human cells. *EMBO J* **28**: 1708–1719.
43. Zhou J, Peng C, Li B, Wang F, Zhou C, Hong D, *et al.* (2012). Transcriptional gene silencing of HPV16 E6/E7 induces growth inhibition via apoptosis in vitro and in vivo. *Gynecol Oncol* **124**: 296–302.
44. Perrone, L, Devi, TS, Hosoya, KI, Terasaki, T and Singh, LP (2010). Inhibition of TXNIP expression *in vivo* blocks early pathologies of diabetic retinopathy. *Cell Death Dis* **1**: e65.
45. Grimm, D, Streetz, KL, Jopling, CL, Storm, TA, Pandey, K, Davis, CR *et al.* (2006). Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature* **441**: 537–541.
46. Bustin, SA, Benes, V, Garson, JA, Hellemans, J, Huggett, J, Kubista, M *et al.* (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* **55**: 611–622.
47. Taylor, S, Wakem, M, Dijkman, G, Alsarraj, M and Nguyen, M (2010). A practical approach to RT-qPCR-Publishing data that conform to the MIQE guidelines. *Methods* **50**: S1–S5.

Supplementary Information accompanies this paper on the Molecular Therapy–Nucleic Acids website (http://www.nature.com/mtna)