Myostatin-induced inhibition of the long noncoding RNA Malat1 is associated with decreased myogenesis

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Watts R, Johnsen VL, Shearer J, Hittel DS. Myostatin-induced inhibition of the long noncoding RNA Malat1 is associated with decreased myogenesis. Am J Physiol Cell Physiol 304: C995–C1001, 2013. First published March 13, 2013; doi:10.1152/ajpcell.00392.2012.—Myostatin, a member of the transforming growth factor-β (TGF-β) superfamily of secreted proteins, is a potent negative regulator of myogenesis. Free myostatin induces the phosphorylation of the Smad family of transcription factors, which, in turn, regulates gene expression, via the canonical TGF-β signaling pathway. There is, however, emerging evidence that myostatin can regulate gene expression independent of Smad signaling. As such, we acquired global gene expression data from the gastrocnemius muscle of C57BL/6 mice following a 6-day treatment with recombinant myostatin compared with vehicle-treated animals. Of the many differentially expressed genes, the myostatin-associated decrease (−11.20-fold; P < 0.05) in the noncoding metastasis-associated lung adenocarcinoma transcript 1 (Malat1) was the most significant and the most intriguing because of numerous reports describing its novel role in regulating cell growth. We therefore sought to further characterize the role of Malat1 expression in skeletal muscle myogenesis. RT-PCR-based quantification of C2C12 and primary human skeletal muscle cells revealed a significant and persistent upregulation (4- to 7-fold; P < 0.05) of Malat1 mRNA during the differentiation of myoblasts into myotubes. Conversely, targeted knockdown of Malat1 using siRNA suppressed myoblast proliferation by arresting cell growth in the G0/G1 phase. These results reveal Malat1 as novel downstream target of myostatin with a considerable ability to regulate myogenesis. The identification of new targets of myostatin will have important repercussions for regenerative biology through inhibition and/or reversal of muscle atrophy and wasting diseases.

myostatin; myogenesis; Malat1; microarray

Myostatin, a member of the transforming growth factor-β (TGF-β) superfamily of secreted proteins, is a potent negative regulator of muscle development and size. Myostatin inhibits myoblast proliferation and differentiation and attenuates postnatal muscle fiber protein accretion, resulting in decreased skeletal muscle mass (1). This is why the pharmacological or genetic inhibition of myostatin produces a hyper-muscular phenotype in rodents and humans (9, 21). Moreover, the inhibition of myostatin is protective against skeletal muscle atrophy in a wide range of animal and cell culture models (6). This has particular clinical relevance given the important role of myostatin in muscle wasting conditions such as HIV, cachexia, cancer, and diabetes (12, 29). There is also mounting evidence that myostatin affects the growth and metabolic state of other tissues, including the adipose and the liver (1).

Genome-wide transcriptional profiling has contributed significantly to our understanding of how myostatin inhibits the growth development of skeletal muscle (myogenesis). For instance, we have learned that that the antianabolic effects of myostatin are exerted, in part, through the differential regulation of genes involved in cell cycle control, protein degradation, and myogenic differentiation (26). However, the relative contributions of myostatin-regulated genes to skeletal muscle growth and development needs to be further elucidated. We therefore sought to identify novel myostatin-regulated genes using microarray data acquired from skeletal muscle of mice treated with recombinant myostatin vs. sham-treated controls (11). In this study we identify the long noncoding metastasis-associated lung adenocarcinoma transcript 1 (Malat1) as a novel myostatin-regulated gene and describe its role in the regulation of skeletal muscle growth and development.

METHODS AND MATERIALS

Mouse maintenance and experimentation. All procedures were approved by the University of Calgary Animal Care and Use Committee and abide by the Canadian Association for Laboratory Animal Science guidelines for animal experimentation. Animals were maintained in a humidity-controlled room with a 12-h light-dark cycle. Following weaning at 3 wk of age, male C57BL/6J littermates were randomly segregated into two groups (n = 6 each) and maintained in microisolator cages for 1 wk. Following this acclimation period, treated animals received 15 μg kg−1 day−1 of recombinant myostatin (R&D Systems, Minneapolis, MN), while the control group received 100 μl PBS as described previously (11, 32). The recombinant myostatin was diluted in 100 μl of PBS and administered daily by subcutaneous injection. Food and water were provided ad libitum throughout the experiment. Mice were euthanized on the sixth day using pentobarbital and cervical dislocation. Gastrocnemius muscles were collected, immediately weighed and frozen in liquid nitrogen, and stored at −80°C for subsequent analysis.

Microarray quality control. Expression profiling was performed as described previously (13), using 5 μg of total RNA extracted from gastrocnemius muscle of six control and six myostatin-treated animals described in Mouse maintenance and experimentation (11). Biotinylated cRNA was synthesized from cDNA and fragmentation as detailed in the manufacturer’s protocol (Affymetrix, Santa Clara, CA) for Affymetrix 430 2.0 microarrays. Stringent quality control methods were employed (14). Each array fulfilled the following quality control measures: average cRNA fold changes of 15.6, average scaling factor of 3.9 to a target intensity of 500, average “present” calls 43%, showed >80% present calls, consistent values across samples, average 5.3/3 ratios of housekeeping genes, and internal probe set controls was 0.81 (15).

Statistical analysis of gene expression profiles. Expression values for probe sets were generated from the probe logarithmetic intensity error (PLIER) algorithm in Expression Console (Affymetrix) and imported directly into the Partek Genomics Suite, version 6.2 (Partek, St. Louis, MO) for statistical processing. Microarray metadata complied with MIAME standards, and all samples have been submitted to the NCBI GEO database for public access. All outputs were filtered with a P < 0.01 cutoff. Following these analyses we applied a
Bonferroni multiple testing correction to reduce the false positive rate. With the exception of data sets uploaded into pathway generation software, a 1.2-fold change filter was also applied. Lists of probe sets passing the P-value filter for differential expression between genotype groups were imported into Ingenuity Pathway Analysis software, version 7.5 (Ingenuity Systems, Redwood City, CA), for network and pathway generation.

**Cell culture.** Human vastus lateralis muscle samples weighing ~50 mg were obtained from three healthy female subjects. Satellite cells were cultured as previously described to produce primary cultures of human myoblasts (12). To prevent fibroblast contamination of the primary culture, cells were seeded on to an uncoated flask and incubated for 30 min to induce fibroblast attachment, leaving myoblasts suspended in the medium. This process was repeated, and myoblast-containing medium was aspirated and transferred to an extracellular matrix-coated (Sigma, St. Louis, MO) flask. Experiments were performed at a low passage to limit the contribution of fibroblasts to the final result. Myoblasts were maintained in high-glucose DMEM (Life Technologies, Carlsbad, CA) supplemented with 20% FBS (Life Technologies) and 1% antibiotic antimycotic solution (Sigma). C2C12 mouse myoblasts were obtained from the American Type Culture Collection (Manassas, VA) and maintained in high-glucose DMEM supplemented with 10% FBS and 1% antibiotic antimycotic solution. Cells were cultured in T-75 culture flasks and maintained at 37°C in a humidified incubator in 5% CO2. Culture medium was changed every 2 or 3 days, and cells were subcultured when ~80% confluent.

**Myostatin treatment.** Primary skeletal myoblasts were passaged upon reaching 70% confluency. Media were aspirated, and cells were rinsed twice with PBS. Cells were harvested using trypsin (Life Technologies) and equilibrated in proliferation media for a period of 24 h before commencement of a 48-h treatment with 4 μg/ml recombinant myostatin.

**Malat1 gene silencing.** A siRNA targeting murine Malat1 was purchased from Life Technologies. Strand sequences (with dTdT overhang) are listed in the 5’- to 3’-direction as follows: sense, CUUAUCAUUCCACAAGGATT and antisense, UCCUUGGUAUAUGGAATA. A universal control siRNA was purchased from Sigma as a scrambled sequence with no homology within the mouse or human genomes. siRNA duplexes were transfected using 2 μl/ml Lipofectamine siRNAMAX (Life Technologies) according to the manufacturer’s instructions. Briefly, proliferating C2C12 and primary human myoblasts were transfected with either 0.1 or 0.5 μM siRNA, respectively, upon propagation onto plastic ware, where they were cultured as previously described to produce primary cultures of human myoblasts (12). To prevent fibroblast contamination of the primary culture, cells were seeded on to an uncoated flask and incubated for 30 min to induce fibroblast attachment, leaving myoblasts suspended in the medium. This process was repeated, and myoblast-containing medium was aspirated and transferred to an extracellular matrix-coated (Sigma, St. Louis, MO) flask. Experiments were performed at a low passage to limit the contribution of fibroblasts to the final result. Myoblasts were maintained in high-glucose DMEM (Life Technologies, Carlsbad, CA) supplemented with 20% FBS (Life Technologies) and 1% antibiotic antimycotic solution (Sigma). C2C12 mouse myoblasts were obtained from the American Type Culture Collection (Manassas, VA) and maintained in high-glucose DMEM supplemented with 10% FBS and 1% antibiotic antimycotic solution. Cells were cultured in T-75 culture flasks and maintained at 37°C in a humidified incubator in 5% CO2. Culture medium was changed every 2 or 3 days, and cells were subcultured when ~80% confluent.

**Cell proliferation assay.** Cell proliferation was measured using the Quick Cell Proliferation Assay Kit (Biovision, Mountain View, CA). Cells were cultured at a density of 2.5 × 10^5 cells per well in a flat-bottomed 96-well plate and incubated in transfection media. Following 24 and 48 h treatment, WST1/ECs solution was added to each well according to manufacturer’s instructions. Cell viability was determined by measuring the absorbance at 450 nm using a Spectra MAX 190 spectrophotometer (Molecular Devices, Sunnyvale, CA). All assays were performed in triplicate and independently repeated twice. As a blank control, 100 μl DMEM was used. Cell proliferation was validated using a Cell Signaling (Beverly, MA) bromodeoxyuridine (BrdU) cell proliferation assay following the manufacturer’s protocol. Briefly, 10× BrdU was added to 24-h-treated cells for a final 1× concentration, and cells were incubated for an additional 24 h. After the medium was removed, cells were fixed/denatured and then incubated with a detection antibody and then an horseradish peroxidase-conjugated secondary antibody. After incubation with TMB substrate, BrdU incorporation was quantified using a Spectra MAX 190 spectrophotometer (Molecular Devices). Densitometric analysis was performed using ImageJ software (National Institute of Health, CA) using 10.220.32.247 on July 27, 2017 http://ajpcell.physiology.org/ Downloaded from

**RESULTS**

**Malat1 expression in myostatin-treated skeletal muscle.** To examine the extent to which myostatin regulates gene expression in skeletal muscle we performed microarray analysis on gastrocnemius muscle from mice injected with saline or myostatin as described previously (11). The myostatin-treated animals used to obtain microarray data were used in a study published in our laboratory exploring the relationship between myostatin and insulin resistance (11). In that study no significant difference in total animal or skeletal muscle mass with the described myostatin treatment. Using stringent analysis condi-
expression is suppressed in skeletal muscle by myostatin and siRNA in vivo and in vitro. Suppression of Malat1 in gastrocnemius (Gastroc) muscle was confirmed by quantitative RT-PCR in 6-day myostatin- vs. vehicle-treated mice, and primary human myoblasts (HuSkMc) following a 48-h treatment with 4 µg/ml myostatin. Proliferating murine C2C12 (siRNA-M) and primary human myoblasts (siRNA-H) were reverse transfected with 0.1 µM Malat1 siRNA (Treated) or universal control (Control). Columns represent the means ± SE of n = 10 male mice per treatment and the means ± SE of n = 3 from 2 independent experiments for cell culture studies. Statistically significant differences (*P < 0.05) between control and treated columns were analyzed by Student’s t-test.

**Malat1 expression during myogenesis.** Given the important role of myostatin in regulating myoblast proliferation and differentiation, transcript levels Malat1 were assessed using RT-PCR in actively proliferating murine (C2C12) and primary human myoblasts in 90–100% confluent myoblasts and at 2, 4, and 6 days postinduction of differentiation. This analysis revealed a significant increase in Malat1 expression at the initiation of differentiation in confluent myoblasts (60% increase; P < 0.05) that was sustained at 2, 4, and 6 days postdifferentiation induction (4.6-, 4.3-, and 4.8-fold all P < 0.01, respectively; Fig. 2). Similarly pronounced and significant increases in Malat1 were measured in primary human myoblasts upon becoming confluent and throughout differentiation.

**Myostatin regulates Malat1 in proliferating primary human myoblasts.** Malat1 gene expression level (relative to β-actin) was determined by RT-PCR in proliferating C2C12 and primary human myoblasts following 48-h treatment with or without 4 µg/ml of recombinant myostatin. We found that treatment of proliferating human primary myoblasts with myostatin leads to a 22% (P < 0.001) reduction in Malat1 transcript levels (Fig. 1). This is consistent with data obtained from the gastrocnemius tissue of myostatin-treated mice where we found a 28% reduction in Malat1 (Fig. 1). Surprisingly, there was no effect of myostatin treatment on Malat1 expression in proliferating murine C2C12 myoblasts.

**Partial silencing of Malat1 suppresses myoblast proliferation.** Because myostatin has been previously shown to suppress the proliferation of myoblasts in culture (34), we examined the effect of Malat1 silencing on C2C12 myoblasts. A two-way ANOVA was conducted to examine this and duration of treatment. While the magnitude of the siRNA-mediated silencing was lower than expected, it was conveniently in the same range of myostatin-induced suppression in murine skeletal muscle (Fig. 1). Furthermore, our findings are consistent with previous efforts to silence Malat1 in cancer cell lines (28). Interestingly, the high average CT values of Malat1 (data not shown) suggest that it may be difficult to completely silence because it is quite highly expressed. While transfection with siRNA Malat1 produced no significant changes in proliferation during the first 24 h, we found significantly reduced myoblast proliferation compared with myoblasts transfected with control siRNA following the 48 h incubation (Fig. 3A; 37% reduction; P < 0.001). Regardless of treatment effects, proliferation increased 255 and 199% (P < 0.001) during treatment with control siRNA or Malat1 siRNA, respectively, over from 24 to 48 h. For comparison, myoblasts incubated for 24 and 48 h with 4 µg/ml myostatin also exhibited significantly impaired growth following 48 h treatment (Fig. 3B; a 70% reduction; P < 0.001). As one would expect, proliferation of vehicle-treated (control) myoblasts significantly increased (93%; P < 0.001) from 24 to 48 h. Myostatin abolished such growth.

**Fig. 1. Metastasis associated lung adenocarcinoma transcript 1 (Malat1) expression is suppressed in skeletal muscle by myostatin and siRNA in vivo and in vitro.** Suppression of Malat1 in gastrocnemius (Gastroc) muscle was confirmed by quantitative RT-PCR in 6-day myostatin- vs. vehicle-treated mice, and primary human myoblasts (HuSkMc) following a 48-h treatment with 4 µg/ml myostatin. Proliferating murine C2C12 (siRNA-M) and primary human myoblasts (siRNA-H) were reverse transfected with 0.1 µM Malat1 siRNA (Treated) or universal control (Control). Columns represent the means ± SE of n = 10 male mice per treatment and the means ± SE of n = 3 from 2 independent experiments for cell culture studies. Statistically significant differences (*P < 0.05) between control and treated columns were analyzed by Student’s t-test.

**Fig. 2. Malat1 is up-regulated during murine (M) and primary human muscle (H) differentiation.** Transcript level of Malat1 relative to β-actin was determined by RT-PCR during active proliferation (prolif), when confluent (CMB), and 2, 4, and 6 days postinduction of differentiation. Each time point represents the means ± SE obtained from 3 independent experiments performed in duplicate. Statistically significant differences (†P < 0.001) between proliferating myoblasts and subsequent time points were determined using Student’s t-test.
To confirm the absence of cell death due to toxicity from siRNAs, quantitative assessment of both viable and nonviable cells present in sample preparations from control and Malat1 siRNA-treated proliferating C2C12 myoblasts was performed using flow cytometry. Using this assay we confirmed a 38% drop in the total number of 48-h Malat1-silenced cells relative to cells transfected with the scrambled control. Furthermore, there was no change in the relative number of dead or apoptotic cells with Malat1 silencing (data not shown). For a direct indication of cell proliferation we also used an ELISA-based BrdU incorporation assay (data not shown) and found a 40% drop ($P < 0.001$) in the total number of 48-h Malat1-silenced cells relative to cells transfected with the scrambled control.

Finally, using a separate flow cytometry protocol, we detected a small but significant increase in the percentage of Malat1-silenced cells arrested in the G0/G1 phase (Fig. 4, 61 vs. 58%; $P < 0.005$), which is consistent with the previously described effects of myostatin on cell cycle arrest (16).

Previously, Malat1 has been identified as being highly expressed in early stage metastasizing tumors cell lines. Subsequent studies found that partial Malat1 silencing reduced cancer and fibroblast cell migration in vitro and tumor growth in vivo (28). As such we assessed the transcript levels of several cell cycle regulators and found that partial Malat1 silencing decreased Cdk6 gene expression by 30% ($P < 0.001$) in myoblasts (Fig. 5).

Reduced myogenin expression in differentiating myotubes in response to partial Malat1 inhibition. To investigate whether Malat1 is necessary for successful differentiation of C2C12 myoblasts into myotubes, the gene expression of myogenin, an important target of myostatin and myogenic regulatory factor necessary for differentiation, was examined. Partial Malat1 depletion (25%) led to a 17% ($P < 0.01$) reduction in myogenin gene expression in cells following 4 days serum withdrawal in the absence of any obvious differences in myotube morphology or fusion index (data not shown) (Fig. 6). While this is consistent with the previously described effects of myostatin...
on myogenin expression (16), Malat1 itself may not be essential for myoblast differentiation.

DISCUSSION

Protein-coding genes constitute a surprisingly small fraction (<2%) of the human genome. However, the human and mouse transcriptomes contain many different types of expressed RNA than can be accounted for by the ~25,000 protein-coding genes (30). In recent years several classes of noncoding RNA have been classified based on size: the miRNAs (~22 nt), piRNAs (18–30 nt), short translational-regulatory RNAs (100–200 nt), and long noncoding RNAs (200–10,000 nt; Ref. 4). Although ncRNAs account for ~1.5% of the transcriptional output of the mammalian genome, the function of only a handful has been determined (20). While the smaller miRNAs are involved in the regulation of many protein-coding genes, including myostatin (2), the function of the long mRNA-like ncRNAs is less clear. There is evidence for involvement of ncRNAs in myogenesis. Two ncRNA isoforms of the multiple endocrine neoplasia 1 (Men1) locus are upregulated during bovine (18) and murine (30) muscle development. Furthermore, large-scale studies of ncRNAs have shown that many are dynamically regulated during differentiation and exhibit cell- and tissue-specific expression patterns (3, 22).

The noncoding Malat1 is a highly conserved 8.7-kb transcript that is abundantly expressed in cancer cells and a strong predictor of metastasis (28). Malat1 has been proposed to regulate alternative splicing (31), transcriptional activation, and the expression (in cis) of nearby genes (23, 33). While it appears that Malat1 is largely dispensable for normal mouse development (5, 35), the exact cellular role of Malat1 is unclear due to conflicting published results (5, 31, 33, 35). There is compelling experimental evidence supporting a functional role for Malat1 in the regulation of cell growth. For instance, partial Malat1 silencing suppresses tumor growth by inhibiting the proliferation and migration of a variety of cancer cells (10, 19). Given its important and novel role in promoting cancer cell proliferation and migration, it is surprising that Malat1 has not been well characterized in other mammalian cell types. Our finding that Malat1 is strongly responsive to myostatin in skeletal muscle supports a possible novel myogenic role for this ncRNA.

Our characterization of Malat1 throughout mouse and human skeletal myogenesis demonstrated a sharp and sustained increase in its gene expression upon differentiation initiation. These results are likely to indicate a role for Malat1 in the transition from the proliferative phase to differentiation in skeletal myogenesis, as well as a commitment to differentiation. It is possible the downregulation of Malat1 was secondary to the inhibition of postnatal muscle maturation by myostatin; however, myostatin-treated mice exhibited no difference in total or skeletal muscle mass (12). Previous studies report a similar expression profile for the muscle-specific transcription factor myogenin (16). Interestingly, targeted knockdown of Malat1 led to a proportionate reduction in myogenin gene expression and is likely to indicate compromised differentiation. From these findings we predict that Malat1 may play functional role in regulating myogenin expression, and hence differentiation.

Both Malat1 inhibition and myostatin treatment decrease C2C12 myoblast proliferation. Because myostatin also suppresses Malat1 expression in murine skeletal muscle and primary human skeletal muscle cells, it stands to reason that Malat1 is responsible, in part, for myostatin-induced inhibition of myogenesis. Unlike in cancer cells (10), flow cytometry analysis did not indicate that Malat1-silenced C2C12 myoblasts were apoptotic. We did, however, find a small but significant increase in the percentage of Malat1 cells arrested in the G0/G1 phase, which is consistent with the previously described effects of myostatin on cell cycle arrest (16). Because Malat1 increases tumor cell proliferation through the regulation of cell cycle gene expression (28) we assessed the transcript levels of several cell cycle regulators and found that partial Malat1 silencing decreased Cdk6 gene expression. During myogenesis, progression from G1 to the S phase in mammalian cells is thought to be regulated by association of the D-type cyclins with Cdk4 and Cdk6 (25). Reduced proliferation in the absence of myostatin suggests that Malat1 may regulate cell growth independent of growth factor stimulation.

Interestingly, Cdk6 is a critical regulator of differentiation in a number of cell types, most notably in its role in promoting myogenic differentiation (8, 27). Furthermore, the Smad-signaling pathway that is shared by all TGF-β family members, including myostatin, has been implicated in transcriptional silencing of Cdk6 (24). Taken together, these observations suggest that the inhibition of myoblast proliferation by the partial silencing of Malat1 may, in turn, be mediated by the inhibition of Cdk6 expression in myoblasts. Given the role of Cdk6 in initiating and maintaining cell cycle exit during differentiation coupled with our observations of the significant and persistent upregulation of Malat1 in differentiation myotubes, it would be interesting to explore in future studies.

Based on the observation that decreased expression of Malat1 leads to reduced myoblast proliferation, it could be proposed that an increased Malat1 would enhance proliferation, suggesting a straightforward regulatory mechanism for this RNA in myogenesis. However, this simplistic hypothesis is unlikely due to increased expression of Malat1 in both human and murine myoblasts following mitogen withdrawal from cells in culture. Instead, it is likely that Malat1 exerts its effects on myogenin, both alone and in response to myostatin, at the posttranscriptional level via modification of pre-mRNA. Indeed, its abundant expression evidenced by the high average
CT values of Malat1 (data not shown) supports an important, yet largely undefined, role in the regulation of RNA processing in skeletal muscle. We can speculate that Malat1 may function at the posttranscriptional level to counteract the downstream effects of myostatin on skeletal muscle cells; however, more experiments are necessary to clarify this. Further studies will determine whether Malat1 overexpression induces the opposite influence on cell physiology from the ones reported herein.

In summary, we have identified the long noncoding Malat1 as a novel myostatin regulated gene and describe its role in the regulation of skeletal muscle myogenesis. Although Malat1 is known to be capable of regulating tumor development and metastasis, this is the first time it has been characterized in skeletal muscle, let alone implicated in skeletal myogenesis. Although the mechanisms by which myostatin regulates the expression of Malat1 and by which Malat1 regulates Cdk6 and myogenin expression still need to be elucidated, both Malat1 and myostatin contain a CAMP-response elements in their proximal promoter as do many myogenic genes (7, 17). This suggests that myostatin and Malat1 may be regulated by a common signaling pathway. This work has, however, uncovered at least one more target through which myostatin is likely to inhibit skeletal muscle growth. In the future Malat1 may become a viable therapeutic target for diseases associated with myostatin-related muscle loss.

**REFERENCES**


