

Myostatin inhibits myosatellite cell proliferation and consequently activates differentiation: evidence for endocrine-regulated transcript processing

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Abstract

Myostatin is a potent negative regulator of muscle growth in mammals. Despite high structural conservation, functional conservation in nonmammalian species is only assumed. This is particularly true for fish due to the presence of several myostatin paralogs: two in most species and four in salmonids (MSTN-1a, -1b, -2a, and -2b). Rainbow trout are a rich source of primary myosatellite cells as hyperplastic muscle growth occurs even in adult fish. These cells were therefore used to determine myostatin's effects on proliferation whereas our earlier studies reported its effects on quiescent cells. As in mammals, recombinant myostatin suppressed proliferation with no changes in cell morphology. Expression of MSTN-1a was several fold higher than the other paralogs and was autoregulated by myostatin, which also upregulated the expression of key differentiation markers: Myf5, MyoD1,

myogenin, and myosin light chain. Thus, myostatin-stimulated cellular growth inhibition activates rather than represses differentiation. IGF-1 stimulated proliferation but had minimal and delayed effects on differentiation and its actions were suppressed by myostatin. However, IGF-1 upregulated MSTN-2a expression and the processing of its transcript, which is normally unprocessed. Myostatin therefore appears to partly mediate IGF-stimulated myosatellite differentiation in rainbow trout. This also occurs in mammals, although the IGF-stimulated processing of MSTN-2a transcripts is highly unique and is indicative of subfunctionalization within the gene family. These studies also suggest that the myokine's actions, including its antagonistic relationship with IGF-1, are conserved and that the salmonid gene family is functionally diverging.

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Introduction

Muscle growth results from the proliferation of myoblasts and their subsequent differentiation into muscle fibers (Buas & Kadesch 2010). This occurs with embryonic myoblasts and with myosatellite cells that lay just under the basal lamina of mature muscle. These quiescent stem cells are activated in response to exercise or injury and ultimately contribute to hypertrophic growth as they differentiate and fuse to mature fibers (Grounds & Yablonka-Reuveni 1993, Pavlath & Horsley 2003, Charge & Rudnicki 2004). Some of the underlying molecular mechanisms involved appear to be fairly well conserved in all vertebrates (Buckingham & Vincent 2009) as primary myosatellite cells from a variety of species spontaneously differentiate and respond similarly to growth factors (Thomas *et al.* 1994, Galvin *et al.* 2003, Castillo *et al.* 2004, Manceau *et al.* 2008, Bower & Johnston 2010a, Gabillard *et al.* 2010, Zhu *et al.* 2010). Unlike birds and mammals, however, where muscle hyperplasia stops after birth, both hyperplastic and hypertrophic growth occurs in fish muscle (Mommsen 2001). Indeed, bony fish are a rich

source of primary myosatellite cells, and, in contrast to mammals, these cells are easily obtained from rainbow trout and at comparatively much higher titers (Alfei *et al.* 1989, Koumans *et al.* 1991a,b, 1993, Fauconneau & Paboeuf 2000, 2001, Mommsen 2001, Castillo *et al.* 2002, 2004, 2006). A better understanding of this unique phenomenon will, therefore, help explain myogenesis in fish. It may even help to develop a novel biomedical model assuming that the general mechanisms of myogenesis are conserved in humans and fish.

The process of proliferation and differentiation of myoblasts in general is regulated by several factors including myostatin, a negative regulator of muscle growth in mammals (Rodgers & Garikipati 2008), and insulin-like growth factor 1 (IGF-1), a positive regulator (Duan *et al.* 2010). Most bony fish species possess at least two myostatin genes (MSTN-1 and -2; Kerr *et al.* 2005, Rodgers *et al.* 2007) as a result of early genome duplication events (Amores *et al.* 1998, Postlethwait *et al.* 1998), while rainbow trout and other salmonids have four (MSTN-1a, -1b, -2a, and -2b) due to their recent tetraploidization (Postlethwait *et al.* 1998, Kerr *et al.* 2005,

Garikipati *et al.* 2006, 2007). Although MSTN-2b is a pseudogene, functions of the other paralogs are presumably intact and each gene is differentially expressed in a tissue- and developmental-specific manner (Rodgers & Garikipati 2008). Direct evidence of myostatin function in fish, however, is seriously lacking. A few studies have overexpressed or attempted to disrupt the production and/or bioavailability of individual paralogs with mixed results (Xu *et al.* 2003, Amali *et al.* 2004, Rodgers & Garikipati 2008, Lee *et al.* 2009). This includes the generation of transgenic rainbow trout overexpressing follistatin, a myostatin binding protein, which developed hypermuscularity that resembled myostatin null phenotypes in mammals (Medeiros *et al.* 2009). These phenotypes likely result in part from myostatin inactivation, although they could also be explained by compensatory changes in paralog expression or to the functional inactivation of other factors (e.g. activin, growth, and differentiation factor 11) also recognized by myostatin-binding proteins (follistatin, the myostatin latency-associated peptide (LAP), etc.). The identity of the most 'muscle relevant' paralog is also unknown as any or all may contribute to different aspects of the myogenic process.

Using primary rainbow trout myosatellite cells, we recently determined that myostatin stimulates the differentiation of quiescent cells (Garikipati & Rodgers 2012), although it is unknown whether it can activate myogenic pathways in cells that are actively proliferating. These are distinctly different questions as myostatin has in fact been demonstrated to activate cell cycle withdrawal in mammalian myoblasts and consequently to stimulate quiescence rather than differentiation or apoptosis (McCroskery *et al.* 2003, Amthor *et al.* 2006, Manceau *et al.* 2008). Seiliez *et al.* (2012) recently reported that myostatin inhibits the proliferation of rainbow trout myosatellite cells but has no effect on differentiation. Their conclusions, however, are highly questionable as proliferation assays were surprisingly performed in differentiation medium and differentiation assays in proliferation medium (Garikipati & Rodgers 2012). Our goal, therefore, was to determine whether myostatin's anti-proliferative effects in mammals were conserved in fish and whether this resulted in quiescence or differentiation. Our results suggest that myostatin indeed inhibits proliferation and as a result activates differentiation. They further suggest that myostatin and IGF-1 antagonize one another's actions, that MSTN-1a and -2a are together the 'muscle myostatins', and that IGF-stimulated differentiation is likely influenced by the alternative processing and gene expression of MSTN-2a. These studies provide further evidence that the gene family has subfunctionalized in salmonids as the partitioned expression patterns support similarly partitioned roles during myogenesis. They also suggest that the current understanding of myostatin action, based primarily on mammalian cell lines, may not be entirely applicable to other vertebrate systems, or alternatively that the mammalian model is in need of significant revision.

Materials and Methods

Animal maintenance and myosatellite cell isolation

Rainbow trout were obtained from the Washington State University hatchery, a Center of Reproductive Biology core facility. Fish were reared and used according to protocols preapproved by the Institutional Animal Care and Use Committee. Myosatellite cells were isolated using a procedure previously described (Garikipati & Rodgers 2012). Briefly, fish were collected, weighed, and killed by a sharp blow to the head and immersed in 70% ethanol for 30 s. The skin was removed and the dorsal white muscle was carefully dissected and collected in cold extraction medium, DMEM (Sigma), containing 9 mM NaHCO₃, 20 mM HEPES (pH 7.4), 15% horse serum (HS, Sigma), streptomycin (200 µg/ml), penicillin (200 U/ml), fungizone (25 µg/ml), and gentamicin (7 µg/ml). Muscle was weighed and cut into small pieces with scissors and centrifuged at 300 g for 15 min to remove floating pieces. It was then resuspended in 0.25% collagenase for 70 min at 18 °C before centrifuging for 15 min. The cell pellet was washed and incubated with 0.1% trypsin for 20 min at 18 °C and the resulting cell suspension was centrifuged for 1 min. The supernatant was diluted with four volumes of DMEM containing 15% HS before a second trypsin digestion for 20 min. Trypsin was removed by diluting cells with four volumes of extraction medium, centrifuging for 20 min, and resuspending in basal medium (DMEM with 10% fetal bovine serum (FBS), antibiotics, and fungizone). The suspension was then sequentially filtered through 70 and 40 µm nylon cell strainers, centrifuged, resuspended, and counted before plating. Cells isolated using this procedure have 90% purity for myosatellite cells (Koumans *et al.* 1990, Fauconneau & Paboeuf 2001, Gabillard *et al.* 2010).

Cell culture conditions

Culture dishes and plates were pretreated with 100 µg/ml poly-L-lysine (Sigma) for at least 3 h at 18 °C. Cells were then washed with sterile water and treated with 5 µg/ml laminin (Sigma) diluted in PBS overnight at 18 °C. The laminin solution was aspirated and cells were plated the following day. In all experiments, cells were incubated at 18 °C and the culture plates were sealed to prevent evaporation of the medium.

Proliferation assays

We previously determined that serum concentration can be altered to influence the proliferative capacity and cellular differentiation of primary rainbow trout myosatellite cells (Garikipati & Rodgers 2012). Compared to cells cultured in 5% FBS, differentiation is substantially delayed in cells cultured in 1% or 2% FBS, although 2% allows cells to proliferate while 1% produces quiescence. This is in contrast to immortalized muscle cell lines as primary myosatellites

from a variety of vertebrates differentiate in response to high serum concentrations (Rando & Blau 1994, Thomas *et al.* 1994, Manceau *et al.* 2008, Beermann *et al.* 2010, Bower & Johnston 2010a, Gabillard *et al.* 2010, Zhu *et al.* 2010). In addition, rainbow trout cells spontaneously differentiate at low cell densities and high serum levels (Matschak & Stickland 1995, Castillo *et al.* 2004, Montserrat *et al.* 2007, Diaz *et al.* 2009, Bower & Johnston 2010a,b), which also differs from immortalized cell lines. Our experiments were therefore performed with 2% FBS as this allows cells to proliferate but not readily differentiate (Garikipati & Rodgers 2012).

Cells were seeded on laminin/poly-L-lysine 96-well plates at a density of 450 cells/well and were preincubated overnight. The medium was changed and cells were then cultured for 72 h in DMEM/2% FBS supplemented with different combinations of recombinant mouse myostatin (0, 25, or 50 nM; R&D Systems, Minneapolis, MN, USA) and/or human IGF-1 (0, 25, 50, or 100 nM; Diagnostic System Laboratories, Webster, TX, USA). The C-terminal mature myostatin peptides in mammals and fish, including each rainbow trout homolog, are highly conserved (88–100% identical) (Rodgers & Weber 2001, Rodgers & Garikipati 2008). The rainbow trout myostatin homologs also share receptor-binding domains that are conserved between mammalian myostatin and activin proteins (Harrison *et al.* 2003, 2004). We therefore used mouse myostatin as fish recombinants are not commercially available. Human IGF-1 is also well known to possess conserved bioactivity in rainbow trout (Castillo *et al.* 2002, 2004, 2006). Cultures were terminated and total cell number was determined using Cell Titer 96 (Promega) according to the manufacturer's protocol. Each experiment was repeated three times and data were compiled for analysis. For assessment of gene expression, cells were seeded on coated 12-well plates and treated with 50 nM MSTN and/or 50 nM IGF-1. They were then grown

for 3 and 7 days, although culture medium and growth factors were replaced after 3 days in the latter group.

Gene expression assays

Total RNA was extracted from duplicate wells of three separate cell cultures using Trizol reagent (Invitrogen). RNA was treated with DNase (Promega) and its integrity was assessed by agarose gel electrophoresis. Total RNA (2 µg) was then reverse transcribed using 10 µM Oligo DT primers and 200 U Superscript transcriptase III (Invitrogen) in a 20 µl reaction volume. Subsequent quantitative RT-PCR assays were performed using the iCycler iQ Real-Time PCR detection system (Bio-Rad) and specific primer sets (Table 1). For each sample, 1 µl cDNA was combined with 7.5 µl 2× SYBR Green PCR master mix (Bio-Rad). For each reaction, 6 µl of this mixture were added to 9 µl primer mixture containing 450 nM each primer. The reactions were carried out as follows: 95 °C for 10 min, then 50 cycles of 95 °C for 15 s, 60 °C for 1 min, and 72 °C for 30 s, although typical threshold cycle (Ct) values were between 20 and 30 cycles. The cycling reaction was followed by a dissociation curve to verify amplification of a single product and the relative standard curve method was employed to quantify gene expression using the Q-gene method (Muller *et al.* 2002). For each primer set, a serial dilution of a mixed tissue cDNA was used to construct a standard curve for each assay plate. The standard curve was constructed by plotting the Ct vs the natural log of input RNA (ng). This curve was then used to calculate the relative abundance of each transcript. Sample values were then normalized to those of 18s to control for differences in RNA and cDNA loading. Each sample was run in duplicate and each plate was run in duplicate. Assays were repeated with different samples and all data are presented as normalized gene expression.

Table 1 Real-time quantitative RT-PCR primer sequences. All primers were used at 60 °C

Primer name	Sequence (5'–3')	Reference
Myf5	F: CCG ACA GCA TGG TTG ACT GT R: TGC CAT AGC TTG TGT TCA TCT GA	AY751283.1 ^a
MyoD1a	F: GTG CTT GGA TAA AAA CGT GCGC R: CAA AGA AAT GCA TGT CGC	Levesque <i>et al.</i> (2008)
Myogenin	F: GTG GAG ATC CTG AGG AGT GC R: GGT CCT CGT TGC TGT AGC TC	Levesque <i>et al.</i> (2008)
MLC	F: GGC CCC ATC AAC TTC AC R: CTC CTC CTT TCC TCT CCG TG	Vegusdal <i>et al.</i> (2004)
18s	F: TGC GGC TTA ATT TGA CTC AAC A R: CAA CTA AGA ACG GCC ATG CA	Garikipati <i>et al.</i> (2007)
MSTN-1a	F: CTT CAC ATA TGC CAA TAC ATA TTA R: GCA ACC ATG AAA CTG AGA TAA A	Garikipati <i>et al.</i> (2006)
MSTN-1b	F: TTC ACG CAA ATA CGT ATT CAC R: GAT AAA TTA GAA CCT GCA TCA GAT TC	Garikipati <i>et al.</i> (2006)
MSTN-2a	F: AAT CTC CCC GCA TAA AAG CAA CCA C R: CAC CAG AAG CCA CAT CGA TCT T	Garikipati <i>et al.</i> (2007)

^aNCBI Accession number. MyoD1a amplicons were verified by sequencing.

MSTN-2a processing

For qualitative assessment of MSTN-2a transcript processing, total RNA from skeletal muscle and brain cells treated for 3 days with 50 nM MSTN, 50 nM IGF-1, or both and cells treated with 10, 20, 50, 100, and 200 nM IGF-1 for 3 and 7 days was extracted and reverse transcribed as described earlier. cDNA was then amplified using primers and conditions previously validated (Garikipati *et al.* 2007). The primers annealed to regions in the first and second exon and were therefore capable of detecting both spliced and unspliced transcripts.

Actin and nuclear staining

Myosatellite cells were grown on glass coverslips treated with poly-L-lysine and laminin as described earlier. Cells were washed twice with PBS, fixed in 2% paraformaldehyde for 15 min on ice, washed three times with PBS, permeabilized with 0.2% triton X-100, and washed three more times before staining. Rhodamine-conjugated phalloidin was then added at a concentration of 70 nM. Nuclei were stained with 5 μ M Hoechst stain and cells were imaged using a Zeiss fluorescent microscope.

Statistical analysis

Three independent experiments were performed for proliferation and quantitative expression analysis. Differences between means were determined by a one-way ANOVA coupled to Tukey's *post hoc* test for mean comparisons when appropriate ($P \leq 0.05$).

Results

Myostatin and IGF-1 coregulation of myosatellite cell proliferation

IGF-1 is a potent regulator of skeletal muscle growth (Duan *et al.* 2010) and stimulates the proliferation of primary myosatellite cells from a variety of vertebrate species including rainbow trout (Adams 2002, Castillo *et al.* 2004, Codina *et al.* 2008, Duan *et al.* 2010). Our study verified these findings as IGF-1-stimulated proliferation in a dose-dependent manner (Fig. 1A) as total cell number increased 5–15% after 72 h, depending on the dose. These cells are cultured at 18 °C and grow considerably slower than mammalian muscle cell lines (e.g. rat L6 or mouse C2C12) (Castillo *et al.* 2002), which often doubles in <24 h. We therefore cocultured cells with myostatin and IGF-1 in order to determine whether myostatin inhibited myosatellite proliferation. As expected, basal proliferation was not significantly affected by myostatin as the cells proliferated slowly under these specific culture conditions. By contrast, 50 nM myostatin completely attenuated the proliferative effects of 50 nM IGF-1 (Fig. 1A). Furthermore, cells were inspected daily and no

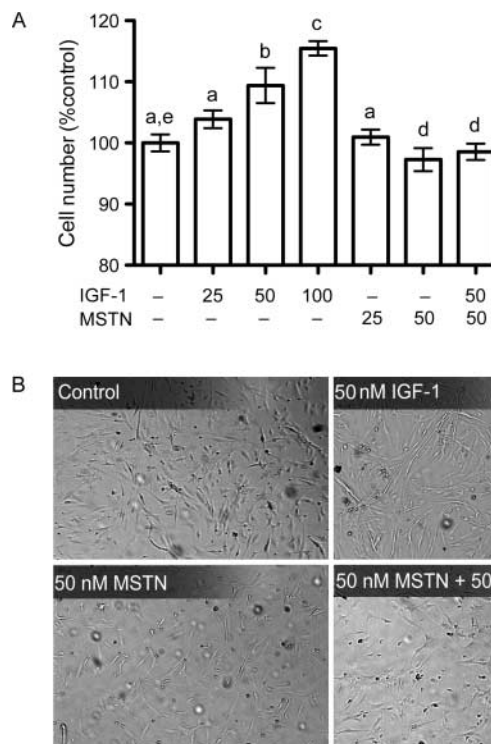


Figure 1 Myostatin and IGF-1 differentially regulate myosatellite cell proliferation. Primary myosatellite cells were cultured for 72 h in DMEM/2% FBS and with the indicated combinations and concentrations (nM) of recombinant mouse myostatin (MSTN) and IGF-1. (A) Proliferation was quantified by measuring total cell number ($n=24$, mean \pm s.e.m.; Different letters signify statistical differences) as described in Materials and Methods section and (B) cellular morphology was observed after 72 h at 100 \times magnification.

phenotypic evidence of cell death or apoptosis (e.g. rounded or floating cells) was detected. Fibroblast contamination is always a concern when culturing primary myosatellite cells and is consistently estimated to be 10% or less using our protocol. The changes described, however, are not due to contamination as myostatin stimulates rather than inhibits fibroblast proliferation (Zhu *et al.* 2007, Li *et al.* 2008).

Cell morphology

A subjective assessment of myosatellite cells treated with myostatin or IGF-1 suggested that both groups were similar in appearance despite the fact that there were more cells in the IGF-treated group (Fig. 1B). To investigate further, cells were cultured on coverslips and treated with 50 nM myostatin and/or 50 nM IGF-1 for 3 and 7 days before staining for actin, a marker of myosatellite differentiation (Fig. 2). No actin staining was observed after 3 days in control and IGF-treated cells (Fig. 2A) and only light staining was detected in cells treated with myostatin alone or in combination with IGF-1. After 7 days, however, actin staining was detected in control cells and in cells treated with myostatin alone or with

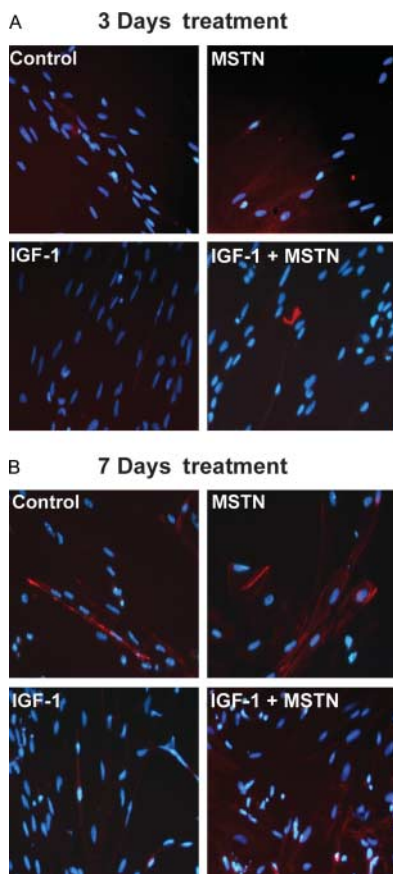


Figure 2 Myostatin and IGF-1 regulation of terminal differentiation in proliferating myosatellite cells. Primary myosatellite cells were cultured for 72 h in DMEM/2% FBS and with the indicated combinations of 50 nM recombinant mouse myostatin (MSTN) and IGF-1. Cells were fixed and filamentous actin (red) was labeled with rhodamine-conjugated phalloidin and nuclei (blue) with Hoechst DNA stain after 3 (A) or 7 days (B). Representative images are shown. Note positive actin staining of muscle debris from isolation procedure, which serves as a positive staining control in light of faint or no staining in panel A.

myostatin and IGF-1, but not in those treated with IGF-1 alone (Fig. 2B). These results suggest that IGF-1 stimulates proliferation and thereby prevents differentiation, at least under these culture conditions, and that myostatin has the opposite effect while again attenuating IGF-1.

Expression of differentiation markers

To quantify the effects of myostatin and IGF-1 on the spontaneous differentiation of proliferating myosatellite cells, which is known to occur in fish primary cells (Matschak & Stickland 1995, Rescan *et al.* 1995, Vegusdal *et al.* 2004, Montserrat *et al.* 2007, Diaz *et al.* 2009, Bower & Johnston 2010a, Bower *et al.* 2010, Gabillard *et al.* 2010), the expression of markers for early (Myf5 and MyoD1), mid (myogenin), and late (MLC) differentiation was measured

(Perry & Rudnick 2000, Rudnicki *et al.* 2008). Differences in individual marker expression were noted, although for the most part, the patterns were similar for all markers. For example, myostatin significantly increased the expression of Myf5, MyoD, and myogenin after 3 and 7 days of treatment (Fig. 3A, B and C). By contrast, IGF-1 had only minimal effects as their expression was elevated after 7, but not 3 days. This suggests that although myostatin and IGF-1 both stimulate differentiation, the effects of myostatin are more significant, which was also reflected in phalloidin staining of actin (Fig. 2B). In fact, MLC expression was only elevated in cells stimulated by myostatin and this occurred at both time points (Fig. 3D). Myostatin and IGF-1 antagonism was probably best illustrated by the fact that their independent effects were compromised when cells were cultured with both hormones. Early marker expression was only elevated after 7 days, as with IGF-treated cells, and MLC expression was elevated, albeit slightly, after 3 days, as with myostatin-treated cells.

Autoregulation of myostatin gene expression

Although MSTN-2b is a pseudogene, the other three paralogs are expressed in several tissues, including skeletal muscle, to varying degrees (Rodgers & Garikipati 2008). It is therefore unknown whether any of the paralogs individually or together regulate myogenesis. Of the three active paralogs, MSTN-1a is the most highly expressed in skeletal muscle (Garikipati *et al.* 2006) and in myosatellite cells (Fig. 4A and D). It is therefore the most likely candidate 'muscle myostatin'. Myostatin increased MSTN-1a expression after 3 and 7 days and MSTN-2a expression after 7 days but had no effect on MSTN-1b (Fig. 4A, B and C). Changes in MSTN-2a expression were somewhat unexpected, as although we previously demonstrated MSTN-2a expression in these cells, it occurs primarily in the brain and its transcript is rarely processed to maturity (see below) in any other tissue (Garikipati *et al.* 2007). The effects of IGF-1 were equally surprising as it reduced MSTN-1a (day 7) and -1b (day 3) and increased MSTN-2a expression (days 3 and 7; Fig. 4B and C). Assuming its transcript is properly processed (see below), these results suggest that MSTN-2a specifically mediates IGF-1 action. These data also indicate that myostatin and IGF-1 differentially regulate expression of the individual paralogs, although both hormones upregulate at least one (Fig. 4D). Co-stimulating cells with both myostatin and IGF-1 produced patterns similar to those of IGF-1, except for MSTN-1a where its 7-day expression value was between those of the myostatin- and IGF-stimulated values (Fig. 4A). This once again illustrates the antagonistic relationship between myostatin and IGF-1 as both can attenuate each other's actions.

Spliced variation of MSTN-2a transcripts

We previously reported that the alternative and tissue-specific processing of MSTN-2a transcripts contributes to the gene's

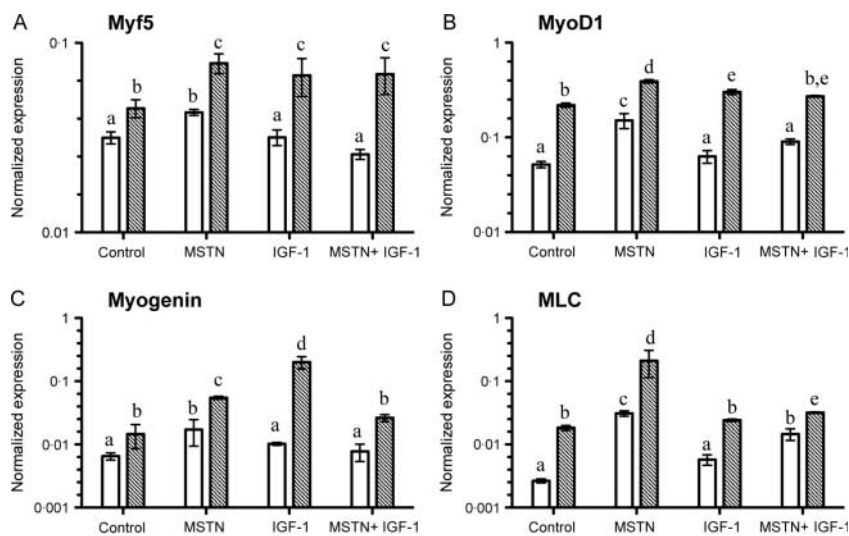


Figure 3 Myostatin and IGF-1 regulation of differentiation marker expression in proliferating myosatellite cells. Primary myosatellite cells were cultured for 72 h in DMEM/2% FBS and with the indicated combinations of 50 nM recombinant mouse myostatin (MSTN) and IGF-1. Cells were terminated after 3 (white bars) and 7 (hatched bars) days and RNA was extracted for gene expression analysis. Expression of markers for early (Myf5 and MyoD1), mid (myogenin), and late (MLC) stages of differentiation was then measured by quantitative RT-PCR. Treatments with different superscripts are statistically different ($P < 0.05$). Same letters denote no statistical difference ($n = 9$, mean \pm s.e.m.).

subfunctionalization, as full transcript processing is limited to the brain (Garikipati *et al.* 2007). Thus, the IGF-induced changes in MSTN-2a mRNA are presumably insignificant as it would not result in increased MSTN-2a protein. We therefore performed qualitative RT-PCR with intron-spanning primers to determine whether the MSTN-2a transcript was processed. As shown in Fig. 5A, MSTN-2a is expressed as an unspliced transcript in skeletal muscle as it retains both introns (band shown represents intron 1 inclusion), although it is fully processed in the brain. Basal expression of MSTN-2a was below detection limits in control cells and in those treated with myostatin. However, IGF-1 not only stimulated MSTN-2a expression (Figs 4C and 5A, B and C), but also the processing of mature transcripts. This effect was dose dependent after 3 and 7 days of culture (Fig. 5B and C) and was blocked by myostatin (Fig. 5A) but never resulted in complete processing of MSTN-2a transcripts.

Discussion

We recently demonstrated, for the first time, direct evidence for myostatin action in any fish system (Garikipati & Rodgers 2012). These studies determined that myostatin activates differentiation in quiescent rainbow trout myosatellite cells and that the expression of MSTN-1a, in particular, and that of MSTN-1b and -2a increases with differentiation (serum and myostatin stimulated), although MSTN-2a transcripts remain unprocessed. The current study indicates that myostatin

suppresses the proliferation of these cells and consequently initiates differentiation. They further suggest that although IGF-1 also stimulates proliferation and differentiation, the latter effects are likely mediated by the upregulation and endocrine-controlled alternative processing of MSTN-2a. Activating differentiation, either by serum or IGF-1, therefore appears to be independently regulated by different myostatin paralogs and by different cellular processes (i.e. gene expression and transcript processing).

Recent studies suggest that myostatin may indirectly regulate fish muscle growth as it does in mammals (Medeiros *et al.* 2009, Lee *et al.* 2010, Li *et al.* 2011). Muscle mass and fiber number were increased in transgenic rainbow trout overexpressing follistatin (Medeiros *et al.* 2009) and in transgenic medaka (Sawatari *et al.* 2010) overexpressing a MSTN-1 LAP (a.k.a. prodomain) respectively. Both follistatin and LAP are myostatin binding proteins that sequester and inactivate the myokine. However, these phenotypes could be partly due to the sequestration of activin and/or GDF11 as both also bind follistatin and possibly LAP (Rodgers & Garikipati 2008). Nevertheless, these reports and our current findings together indicate that myostatin is indeed a negative regulator of skeletal muscle growth in fish.

Myostatin's growth inhibitory actions are well documented in mammalian systems and include cell cycle arrest of myoblasts and primary myosatellite cells (Joulia *et al.* 2003, McCroskery *et al.* 2003, Rios *et al.* 2004, Rodgers *et al.* 2009). Myostatin has also been reported to downregulate MyoD, Myf5, and myogenin and thereby inhibit muscle cell

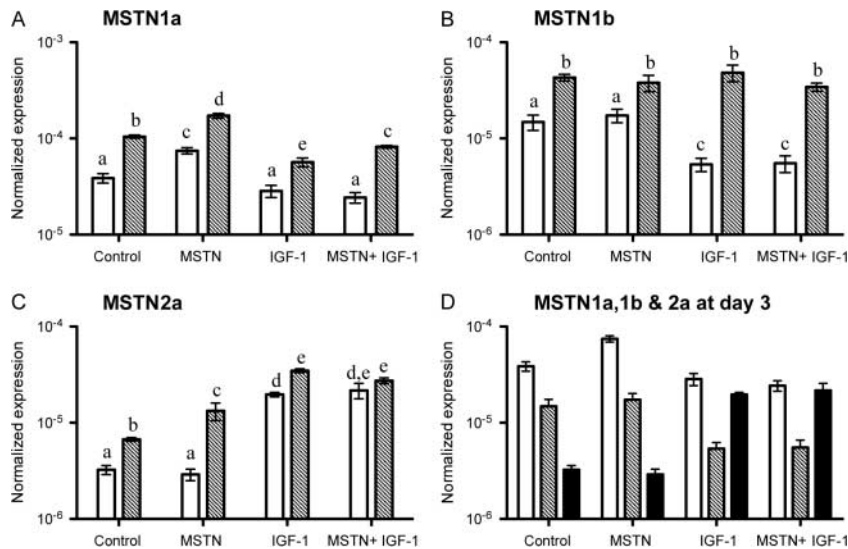


Figure 4 Myostatin and IGF-1 differentially regulate MSTN-1a, -1b, and -2a expression in proliferating myosatellite cells. Primary myosatellite cells were cultured for 72 h in DMEM/2% FBS and with the indicated combinations of 50 nM recombinant mouse myostatin (MSTN) and IGF-1. (A, B, and C) Cells were terminated after 3 (white bars) and 7 (hatched bars) days and RNA was extracted for gene expression analysis. Expression of each paralog was then measured by quantitative RT-PCR. Treatments with different superscripts are statistically different ($P < 0.05$). Same letters denote no statistical difference. (D) Day 3 values for each paralog are plotted to facilitate the relative comparison of changes between MSTN-1a (white bars), 1b (hatched bars), and 2a (black bars). ($n = 9$, mean \pm s.e.m.).

differentiation (Amthor *et al.* 2002, 2004, Langley *et al.* 2002, Rios *et al.* 2002, Jotulia *et al.* 2003), although the significance of this effect is somewhat controversial as cell cycle arrest is necessary for the initiation of differentiation (Thomas *et al.* 2000, Rios *et al.* 2001). Our data directly conflict with these reports and suggest that myostatin increases the expression of several differentiation markers and may even facilitate IGF-stimulated differentiation (see below). Immortalized mammalian cell lines were used in the previous studies and differentiation was therefore 'artificially' induced in confluent cells by serum withdrawal. Thus, myostatin did not inhibit differentiation *per se*, but progression through the already activated process. By contrast, our data indicate that myostatin activates differentiation in proliferating cells and may therefore be more physiologically relevant.

Using primary rainbow trout myosatellite cells, Seiliez *et al.* (2012) recently reported that myostatin inhibits proliferation, although they additionally reported that the differentiation of cells was unaffected by myostatin. Their culture conditions, however, were inappropriate and were based on those used with mammalian cell lines rather than primary myosatellites. Differentiation is induced in immortalized cell lines by serum withdrawal and only after cells are confluent, whereas primary myosatellite cells from a variety of vertebrates, including rainbow trout, will spontaneously differentiate when subconfluent and in response to serum (Rando & Blau 1994, Thomas *et al.* 1994, Matschak & Stickland 1995,

Castillo *et al.* 2004, Montserrat *et al.* 2007, Manceau *et al.* 2008, Diaz *et al.* 2009, Beermann *et al.* 2010, Bower & Johnston 2010a,b, Gabillard *et al.* 2010, Zhu *et al.* 2010). In fact, our recent studies clearly demonstrate this positive effect of serum on rainbow trout myosatellite cell differentiation *in vitro* (Garikipati & Rodgers 2012).

By contrast, Seiliez *et al.* performed proliferation assays in high serum, which promotes differentiation, and differentiation assays in low serum, which suppresses differentiation. They also report that MSTN-1a and -1b expression is downregulated (MSTN-2a expression and processing was not measured) as cells differentiate, which again conflicts with data presented herein and previously (Garikipati & Rodgers 2012). Keeping in mind that their culture conditions are reversed (proliferation medium is actually differentiation medium), their results are consistent with ours and support the conclusion that myostatin facilitates myosatellite cell differentiation. Myostatin also failed to increase MyoD or myogenin expression in their studies, although they cultured cells for only 48 h and our results again clearly indicate that 3–7 days are required to detect myostatin's stimulatory effects on differentiation (Fig. 3). It is important to note that our current and previous studies are consistent with Sato *et al.* (2006) who demonstrated that disrupting myostatin production in proliferating primary chick cells delays rather than stimulates differentiation. Describing myostatin's actions as purely inhibitory may therefore be overly simplistic, as it

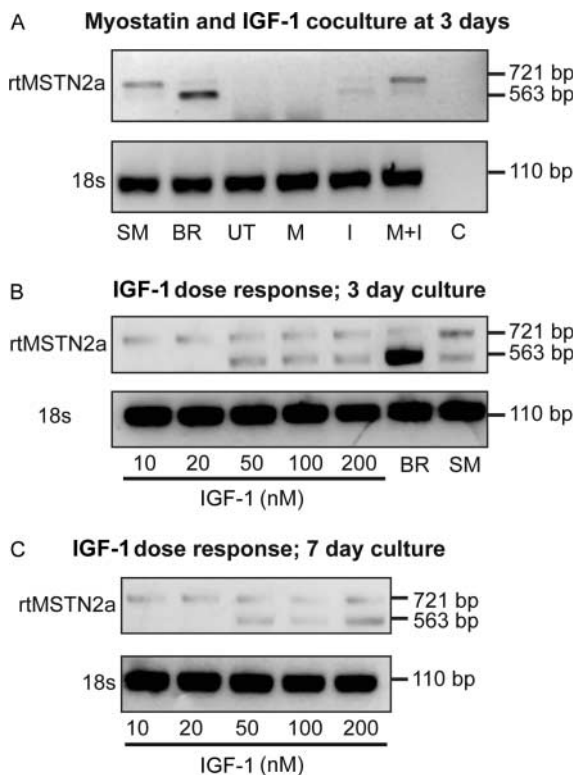


Figure 5 Endocrine regulation of MSTN-2a transcript processing in proliferating myosatellite cells. (A) MSTN-2a transcripts were amplified using a nonquantitative RT-PCR assay and RNA extracted from brain (BR), skeletal muscle (SM) or proliferating myosatellite cells. The latter were treated with IGF-1 alone (I) or with myostatin and IGF-1 (M+I, 50 nM for all doses; UT, untreated control; C, RT-control). Cells were also treated with only IGF-1 for 3 (B) or 7 days (C) using the indicated doses. Larger molecular mass bands correspond to unspliced transcripts, smaller bands to spliced.

appears to stimulate differentiation in proliferating primary myosatellite cells and in growth arrested cells. In fact, trout myosatellite cells were far more sensitive to myostatin than IGF-1, a known muscle differentiation factor, as differentiation marker expression increased at day 3 in cells treated with myostatin and at day 7 in IGF-1 treated cells (Figs 2 and 3).

Muscle growth in mammals is largely regulated by the opposing effects of IGF-1 and myostatin, which together stimulate and inhibit different myogenic processes respectively. This includes the regulation of myoblast and myosatellite cell proliferation and differentiation as well as myotube protein synthesis (Rodgers & Garikipati 2008, Duan *et al.* 2010). This yin-yang relationship extends intracellularly as myostatin attenuates IGF receptor signaling in both myoblasts and myotubes (McFarlane *et al.* 2006, Morissette *et al.* 2009, Trendelenburg *et al.* 2009). We recently demonstrated that hepatic production of IGF-1 and several IGF-binding proteins is altered in myostatin null mice in a way that increases total and free IGF-1 (Williams *et al.* 2011). The two hormones, therefore, oppose one another's actions

and optimize muscle growth under different physiological conditions, including stress (Schakman *et al.* 2008). This relationship appears extremely well conserved, as in the current study, myostatin inhibited IGF-stimulated myosatellite cell proliferation and MSTN-2a processing. Similarly, IGF-1 inhibited myostatin-stimulated differentiation of myosatellite cells and MSTN-1a expression.

Myostatin autoregulation of expression was demonstrated by its ability to increase MSTN-1a mRNA levels at 3 and 7 days (Fig. 4). This also occurs in mammals (Forbes *et al.* 2006) and may provide a potential mechanism for synchronizing cell populations through the paracrine actions of myostatin. The expression of other paralogs was unaffected by myostatin while basal expression of MSTN-1a was several fold higher, a pattern that mirrors mature rainbow trout skeletal muscle (Garikipati *et al.* 2006, 2007). This suggests that MSTN-1a is the 'muscle myostatin', although it is by no means the only paralog involved in the myogenic process as both MSTN-2a expression and transcript processing were stimulated by IGF-1 (Figs 4 and 5), a known mitogen in rainbow trout myosatellite cells (Castillo *et al.* 2004, 2006, Codina *et al.* 2008, Gabillard *et al.* 2010). This effect was dose dependent and is the first example of endocrine-regulated alternative processing of myostatin in any vertebrate. Together, these data also suggest that endocrine-regulated differentiation of proliferating myosatellite cells is uniquely regulated by different myostatin paralogs; MSTN-1a for autoregulation and MSTN-2a for IGF-stimulated differentiation.

IGF-1 also stimulates myostatin expression in mammalian skeletal and cardiac muscle and is a proposed chalone for both tissues (Shyu *et al.* 2005, Yang *et al.* 2007, Rodgers *et al.* 2009). It is not surprising, therefore, that IGF-1 similarly upregulates the expression of at least one trout MSTN paralog. However, IGF-1 also downregulated MSTN-1b expression, indicating that it differentially regulates the production of each paralog. Although the physiological significance of this is currently unknown, the high level of conservation shared between each rainbow trout homolog suggests that functional specification is determined by differences in gene expression, not protein structure, as MSTN-1a and -1b are identical and are 96% similar to MSTN-2a. Nevertheless, each paralog's role in regulating myosatellite cell proliferation and differentiation, in rainbow trout and other salmonids, has clearly subfunctionalized via mechanisms that include divergence of gene promoters and transcript processing sites.

These studies together indicate that the general understanding of myostatin action in immortalized mammalian cell lines may not reflect its actions in primary cells, especially those from fish. Specifically, they suggest that myostatin inhibition of proliferation activates, rather than inhibits, differentiation and that subfunctionalization of the salmonid myostatin gene family helped preserve these gene duplicates. The system itself is therefore similar to zebra fish, as despite widespread gene duplication, which also occurs in zebra fish, the phenotypic responses of primary trout myosatellites are similar to those from mammals, although both differ

considerably from immortalized mammalian cell lines. Future studies are needed to determine the functional significance of MSTN-2a processing in differentiating cells. Nevertheless, this represents a novel and powerful tool for investigating not only myostatin's comparative actions but also fundamental networks that control transcript processing.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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