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Myostatin inhibits proliferation but not differentiation of trout myoblasts

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ABSTRACT

The muscle growth in mammals is regulated by several growth factors including myostatin (MSTN), a member of the transforming growth factor-beta (TGF-beta) superfamily. To date, it is unknown in fish whether MSTN could have any effect on proliferation or differentiation of myogenic cells. Using culture of trout satellite cells, we showed that *mstn1a* and *mstn1b* mRNA are expressed in myoblasts and that their expression decreased in differentiating myoblasts. We also demonstrated that a treatment with huMSTN decreased the proliferation of IGF1-stimulated myoblasts in a dose-dependent manner. By contrast, treatment of myoblasts with 100 nM of huMSTN for 3 days, did not affect the percentage of positive cells for myogenin neither the percentage of nuclei in myosin positive cells. Moreover, our results clearly indicated that huMSTN treatment had no effect on MyoD and Myogenin protein levels, which suggests that huMSTN did not strongly affect MyoD activity.

In conclusion, we showed that huMSTN inhibited proliferation but not differentiation of trout myoblasts, probably resulting from a lack of huMSTN effect on MyoD activity. Altogether, these results show high interspecies differences in the function of MSTN.

INTRODUCTION

By contrast to the mammals and model fish species (zebrafish, medaka), salmonids such as trout exhibit an indeterminate muscle growth pattern, with muscle mass increasing until mortality or senescence occurs (Johnston 1999; Mommsen 2001). This increase in myotomal muscle mass throughout their life cycle is allowed by a continuous production of muscle fibers (hyperplasia) in addition to an increase of fibers size (hypertrophy). However, the molecular mechanisms involved in these interspecies differences are complex and not well understood.

In mammals, muscle growth is regulated by several growth factors including myostatin (MSTN), a member of the transforming growth factor-beta (TGF-beta) superfamily. The *mstn* gene, expressed mostly in skeletal muscle, was first identified in mice by McPherron et al., (1997) and has been demonstrated to negatively regulate skeletal muscle growth in several mammalian species. The knockout of *mstn* or the overexpression of *follistatin*, its endogenous inhibitor, induces a dramatic increase of muscle mass, resulting from a combination of hyperplasia and hypertrophy (McPherron et al., 1997; Lee and McPherron 2001). MSTN acts through its binding to the activin type II receptor which in turn binds to and activates the activin receptor type I that phosphorylates the transcription factors Smad2 and Smad3 (Lee and McPherron 2001; Zhu et al., 2004). In vitro studies demonstrated that MSTN inhibits the proliferation and the differentiation of myogenic cells (Thomas et al., 2000; Ríos et al., 2001; Langley et al., 2002). The inhibition of differentiation is accomplished by decreasing the level of MyoD protein as well as its activity (Langley et al., 2002). Indeed, it has been shown that TGF-beta inhibits MyoD activity through a physical interaction between MyoD and Smad3 (Liu et al., 2001).

In fish, the *mstn* cDNA has been cloned in numerous species such as trout (Rescan et al., 2001; Roberts and Goetz 2001; Garikipati et al., 2006; Garikipati et al., 2007), zebrafish (Xu et al., 2003; Kerr et al., 2005), medaka (Sawatari et al., 2010), tilapia (Rodgers et al., 2001), white bass (Rodgers

et al., 2001), striped bass (Rodgers and Weber 2001), white perch (Rodgers and Weber 2001), sea bass (Terova et al., 2006), Orange spotted grouper (Ko et al., 2007) and sea bream (Funkenstein and Rebhan 2007). As a result of the recent genome duplication in salmonids (Brunelli et al., 2001), four distinct *mstn* genes (*mstn1a*, *mstn1b*, *mstn2a*, *mstn2b*) were found in trout (Garikipati et al., 2006; Garikipati et al., 2007). Nevertheless, only *mstn1a* and *mstn1b* are readily detectable in white muscle since *mstn2a* muscle expression is 20-fold lower. In contrast to what is observed in mammals, *mstn* genes are expressed in most teleost fish tissues (Garikipati et al., 2006). Comparison of MSTN sequences indicates that bioactive MSTN sequence is extremely well conserved throughout evolution. In fact, trout MSTN sequence is 90% identical to human MSTN (Garikipati et al., 2006). Several attempts have been made to elucidate the function of MSTN in fish by decreasing MSTN activity but the results are somewhat divergent between species and even within species. Injection of morpholinos in zebrafish embryos increases the number and size of somites (Amali et al., 2004) as well as the expression of the regulatory factor MyoD and myogenin. The overexpression of the prodomain of MSTN (a negative regulator of the active protein) fails to increase the muscle mass of zebrafish and induces a moderate increase (10%) of the fiber number (Xu et al., 2003). Similarly, the overexpression of a dominant negative form of MSTN does not have any strong consequences for medaka muscle mass (Sawatari et al., 2010). However, a recent study in zebrafish carrying a stable heritable myostatin knockdown genotype, reports a "double-muscle" phenotype (Lee et al., 2009). In trout, the overexpression of follistatin, an inhibitor of the TGF-beta family member, induces an increase of muscle mass (Medeiros et al., 2009), but it remains to be established whether the effect is due to solely myostatin inhibition or to another TGF-beta inhibition. Despite these in vivo data, the function of MSTN in fish remains elusive.

Therefore, in order to gain deeper insight into the function of MSTN in fish, we aimed at determining whether MSTN inhibits the proliferation and the differentiation of myogenic cells, using an in vitro system of trout satellite cells culture.

MATERIALS AND METHODS

Animals

Rainbow trout were maintained at the “Station Commune de Recherches en Ichtyophysiologie, Biodiversité et Environnement” (SCRIBE, Rennes, France) in 0.6-m³ tanks in a re-circulated system at 18°C. All experiments were carried out in accordance with legislation governing the ethical treatment of animals (Decret N° 2001-464, May 29, 2001), and investigators were certified by the French Government to carry out animal experiments (N° agrément 35-47). All animal work was approved by the Ministère de l’Enseignement Supérieur et de la Recherche (Autorisation N°A352386).

Chemicals

Dulbecco's modified Eagle's medium (DMEM#D7777) and F10 (#N6635) were purchased from SIGMA. Anti-MyoD (#NB100-80899) antibody was purchased from Novus Biological, anti-Myogenin (#SC-567) from Santa-Cruz Biotechnology, anti-P-Smad3 (Ser^{423/425}) from Cell Signalling (#9520), anti-Smad7 from Abgent (#AP6753b). For immunofluorescence analysis, the secondary antibody anti-mouse Alexa488 (#A21441) and anti-rabbit Alexa594 (#A11001) were purchased from Invitrogen. Recombinant trout IGF1 (#WU100) was purchased from GroPep and recombinant human MSTN (CYT-418) was purchased from ProSpec and the specific inhibitor of Activin Receptor-Like kinases SB431542 (Inman et al., 2002) from SIGMA (#S4317).

Satellite cells isolation and culture

Primary cultures of skeletal muscle cells were carried out as follows: for each culture, 30 to 60 animals, each weighing approximately 5 g, were killed by a blow to the head and then

immersed for 30 s in 70% ethanol to sterilize external surfaces. Cells were isolated, pooled, and cultured following previously described protocols (Gabillard et al., 2010). Briefly, after removal of the skin, dorsal white muscle was isolated under sterile conditions and collected in Dulbecco's modified Eagle's medium (DMEM) containing 9 mM NaHCO₃, 20 mM HEPES, 15% horse serum, and antibiotic-antimycotic cocktail (100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 g/ml fungizone) at pH 7.4. After mechanical dissociation of the muscle in small pieces, the tissue was enzymatically digested with a 0.2% collagenase solution in DMEM for 1 h at 18°C and gentle shaking. The suspension was centrifuged (300 g for 5 min at 15°C) and the resulting pellet was subjected to two rounds of enzymatic digestion with a 0.1% trypsin solution in DMEM for 20 min at 18°C with gentle agitation. After each round of trypsinization the suspension was centrifuged and the supernatant was diluted in 2 volumes of cold DMEM supplemented with 15% horse serum and the same antibiotic-antimycotic cocktail mentioned above. After two washes with DMEM, the cellular suspension was filtered through 100- and 40-µm nylon filters. All experiments were conducted with cells seeded at a density of 160 000/cm², in six-wells or 24-wells plastic plates (Nunc, Roskilde, Denmark) and left for 30 min before medium change. Plates and coverslips were previously treated with poly-L-lysine and laminin to facilitate satellite cells adhesion. Cells were incubated at 18°C, the optimal temperature for culture, with DMEM or F10 containing 9 mM NaHCO₃, 20 mM HEPES, 10% fetal bovine serum, and antibiotic-antimycotic cocktail under an air atmosphere. According to the experiments, the cells were cultivated in proliferating medium (F10+10%FCS) or differentiating medium (DMEM+2%FCS). The medium was renewed every 2 days and observations of morphology were regularly made to control the state of the cells.

Gene expression analysis

The medium was removed, and wells were washed twice with PBS. The cells were lysed with the lysis buffer of the Nucleospin RNA XS kit (Macherey-Nagel, #N0740-902-50) and total RNA were extracted according to the manufacturer's recommendations. The total amount of RNA was determined as a function of absorbance at 260 nm (Nanodrop ND-1000 spectrophotometer). The cDNA was generated with 0.5 µg total RNA using a commercial kit (Applied Biosystems, #4368813). Briefly, 0.5 µg of total RNA was incubated in a 25 µl mixture (10x RT buffer, 25x dNTPs, 10x random primers, 50UI/µl MultiScribe™ Reverse Transcriptase, nuclease-free water) at 25°C for 10 min and then at 37°C for 120 min. The reaction was set at 200 µl by the addition of nuclease-free water. Target gene expression levels were determined by quantitative RT-PCR using a StepOnePlus system (Applied Biosystems). Analyses were carried out using a real-time PCR kit (Fast SyberGreen Master mix, #4385612 – Applied Biosystems) with 300 nM of each primer. The primer sequences (Table 1) for *mstn1a*, *mstn1b* and *elongation factor 1α (eF1α)* were already published and validated (Seilliez et al., 2011). All the primers span exon-bondaries to prevent genomic DNA amplification. Amplification was then performed using the following cycle: 95°C for 3 s, 60°C for 15 s, 40 times for all primers. The melting curves systematically monitored at the end of the last amplification cycle, show one peak which confirm the specificity of the amplification reaction. Each PCR run included replicate samples (duplicate of reverse transcription and PCR amplification). Relative quantity (Arbitrary unit, A.U.) was determined from a standard curve consisting of a serial dilutions of a pool of cDNA. The efficiencies obtained with the primers pairs ranged from 85 to 100%. EF1α mRNA abundance was not significantly affected by the treatments and was thus used for the normalization of qPCR data.

Protein extraction and Western blotting

After two washes with cold PBS, proteins were extracted with RIPA Buffer (50 mM Tris pH8, 1 mM EDTA, 0.5 mM EGTA, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, 150 mM NaCl) supplemented with 5mM NaF, 1 mM NaVO₄ and protease inhibitors cocktail (Roche). Laemmli Buffer was added to the sample and heated at 90°C for 5 min. Cell lysates were subjected to SDS-PAGE and Western blotting using the appropriate antibody. Antibody against MyoD and myogenin have been previously validated in trout (Gabillard et al., 2010). The antibody against P-Smad3 (Ser^{423/425}) recognize the human amino acid sequence around

Ser^{423/425} that is very well conserved in trout (MGSPS / RCSS / S). Similarly, the antibody

against Smad7 recognize the sequence between 362~392 aa that is very well conserved in

trout (PGFSIKAFD / EKA / SLQRPNDHEF / QQP / T). Then, preliminary western

blot with the anti-P-Smad3 and Anti-Smad7 antibodies were performed with lysates from murine C2C12 cell line and from trout. With trout satellite cells lysate, we obtained a single band for each antibody (P-Smad3 : 55-60 kDa; Smad7 : 45-50 kDa) with the same size as that of C2C12 (data not shown). After washing, the membrane was incubated 1 h with secondary antibody (1/15000) linked to HRP (Jackson Immunoresearch). Immunoreactive bands were visualized by ECL and images were obtained with an image acquisition system (Fusion FX7, Vilbert Lourmat).

Immunofluorescence analysis

Cells on glass coverslips were briefly washed twice with PBS and fixed 10 min with paraformaldehyde 4%. For permeabilization, cells were incubated 3 min in 0.1% TritonX100/PBS. After three washes, cells were saturated for 1 h with 3% BSA, 0.1% Tween20 in PBS (PBST). Cells were incubated 3 h with the primary antibodies anti-myosin

and anti-myogenin diluted in blocking buffer. The secondary antibodies were diluted in PBST and applied for 1 h. Cells were mounted with Mowiol 4-88 (#475904, Calbiochem) containing Hoescht (0.5 µg/ml). Cells were photographed using a Canon digital camera coupled to a Canon 90i microscope.

Analysis of cell proliferation

The mature myostatin protein is extremely well conserved between fish and mammals and trout MSTN is 90% identical to human MSTN (Rescan et al., 2001; Garikipati et al., 2006). Thus, we used recombinant human MSTN (#CYT-418, ProSpec) in our experiments. From the day of extraction, the cells were incubated in F10 (+10% FCS) medium. At day 1, cells were cultivated in F10 alone, and at day 2 cells were cultivated in F10+2%FCS and recombinant trout IGF1 (50 nM) and human MSTN (0.1, 1 or 10 nM) were added. At day 3, BrdU was added for the following 24 h. The cells were fixed with ethanol/glycine buffer (70% ethanol, 50 mM glycine, pH 2). The proliferation of the cells was measured by using the BrdU labeling and detection kit (Roche Diagnostics, no. 1 296 736). Briefly, the cells were incubated for 30 min at 37°C with mouse anti-BrdU, washed, and then incubated with the secondary antibody anti-mouse FITC. Cells were mounted in Mowiol containing 0.5 µg/ml DAPI.

Statistical analysis

Results are presented as means ± SD. Treatment effect was assessed using the non-parametric Kruskal-Wallis ANOVA (Statistica 7.0, StatSoft). When ANOVA revealed significant effect Mann-Whitney test was performed. All the experiments were performed at least 2 times.

RESULTS

Mstn1a and mstn1b gene expression decreases during in vitro differentiation of trout

myoblasts

We monitored the expression of *mstn1a* and *mstn1b* genes by quantitative PCR during proliferation and differentiation of **myoblasts**. Our results (Figure 1) showed that *mstn1a* and *mstn1b* were expressed in **myoblasts**. During the first week of culture in proliferation medium, no significant change of *mstn1a* and *mstn1b* mRNA levels was observed. By contrast, as soon as 2 days after induction of differentiation (DM2), *mstn1a* and *mstn1b* mRNA levels significantly decreased (~ 4-fold). After 4 days in differentiation medium (DM4), the levels of *mstn1a* and *mstn1b* were similar to that of observed at DM2. These results indicated that both *mstn* genes were regulated during the proliferation and the differentiation phases suggesting a role of MSTN on trout **myoblast** proliferation and (or) differentiation.

HuMSTN activates Smad3 phosphorylation in myoblasts and myotubes of trout

Sequence analysis showed that the sequences of trout myostatins were very similar to that of human (90%) as previously reported (Rescan et al., 2001; Garikipati et al., 2006). To validate the use of heterologous myostatin in our in vitro system, we monitored the activity of recombinant human MSTN (HuMSTN) by measuring the phosphorylation status of Smad3 a key factor in the MSTN signaling pathway (Sartori et al., 2009). As shown in figure 2A and 2B, the stimulation of both trout myoblasts and myotubes with 100 nM of huMSTN for 1h induced the phosphorylation of Smad3. Furthermore, to demonstrate the specificity of this hormonal response, we used a chemical inhibitor (SB431542) specific to the activin receptor type I (ActRI) known to transduce MSTN signal (Lee et al., 2005; Miyake et al., 2010). The

treatment of the cells with this chemical inhibitor completely abolished the huMSTN-induced phosphorylation of Smad3 in myoblasts as well as in myotubes (Figure 2A and 2B). Therefore the huMSTN specifically phosphorylated Smad3 through the activation of the ActRI as expected and our results showed that trout myoblasts and myotubes were able to transduce the huMSTN signal.

HuMSTN inhibits proliferation of myoblasts in vitro

To determine whether huMSTN inhibits the proliferation of myoblasts, we treated the cells with huMSTN during 48h and monitored the cell proliferation by mean of the BrdU incorporation technique (Gabillard et al., 2010). To limit the putative non-specific effect of the serum, we cultivated the cells in F10 with only 2% of fetal calf serum (FCS). Under these conditions the basal level of proliferation was low (~10%) and huMSTN treatment had no effect on proliferation whatever the doses (0.1 to 10 nM) (Figure 3). Then, to increase the proliferation, we treated the cells with 50 nM recombinant trout IGF1 (rtIGF1) given that it has been shown to stimulate the proliferation of myoblasts (Gabillard et al., 2010). Under these conditions, rtIGF1 treatment strongly stimulated proliferation (10% versus 60%) and we observed a dose-dependent decrease of proliferation in presence of increasing doses of huMSTN (Figure 3). These results clearly showed that huMSTN was able to decrease the proliferation of trout myoblasts.

HuMSTN has no effect on the differentiation of trout myoblasts

In order to determine whether huMSTN inhibited the differentiation of myoblasts, we treated the cells with huMSTN during the induction of differentiation. The differentiation index (% of nuclei in myosin or myogenin positive cells) was determined by immunofluorescence analysis. Preliminary experiments indicated that low doses of huMSTN (0.5, 5, 50 nM) did

not have an effect (data not shown). Therefore, a higher dose of huMSTN was tested (100 nM) that is within the range previously reported (Langley et al., 2002). Our results (Figure 4A and 4B) showed that after 3 days in the differentiation medium (DMEM+2%FCS), myoblasts were clearly differentiated into myotubes. Indeed after 3 days in differentiating medium, the percentage of nuclei in myosin positive cells (1% versus 23%) and the percentage of myogenin positive nuclei increased (20% versus 39%). Moreover, treatment of the cells with 100 nM huMSTN (Figure 4A and 4B) did not significantly affect the differentiation of myogenic cells (23% versus 20% of nuclei in myosin positive cells) and (39% versus 37% of myogenin positive nuclei). Altogether, these results showed that huMSTN treatment did not inhibit differentiation of trout myoblasts in vitro.

HuMSTN has no effect on the level of MyoD protein

It has been shown in mammals, that MSTN inhibits differentiation by decreasing MyoD protein level as well as MyoD activity (Langley et al., 2002). Our results (Figure 5) showed that huMSTN treatment during 48h increased the amount of Smad7, a protein known to be responsive to TGF β treatment Zhao et al., (2000). Surprisingly, huMSTN did not affect MyoD protein level in our cell culture model (Figure 5). Because MSTN inhibits also MyoD activity, we measured the protein level of myogenin which function downstream of MyoD as a key regulator of myoblast differentiation. Again western blot analysis clearly showed that huMSTN treatment had no effect on myogenin protein level (Figure 5). Altogether, these results indicated that treatment of trout muscle cells with huMSTN did not affect MyoD activity.

DISCUSSION

In mammals, MSTN belongs to the TGF-beta family and is well known as a potent inhibitor of the muscle growth (McPherron et al., 1997; Lee and McPherron 2001). Indeed, it has been shown that MSTN inhibits both proliferation and differentiation of mammalian myogenic cells (Thomas et al., 2000; Langley et al., 2002; Joulia et al., 2003; Trendelenburg et al., 2009). In fish, several works report an increase of muscle mass after decreasing MSTN activity (Lee et al., 2009; Medeiros et al., 2009), while others failed to observe a strong (if any) effect on muscle mass (Amali et al., 2004; Acosta et al., 2005; Sawatari et al., 2010). To date, it is unknown in fish whether MSTN could have any effect on proliferation or differentiation of myogenic cells. Therefore, the purpose of the present work was to determine whether MSTN can affect the proliferation and the differentiation using an in vitro system of trout satellite cells culture.

As a first step, we measured the expression of both *mstn1a* and *mstn1b* during the differentiation of myoblasts. In agreement with our previous study (Seiliez et al., 2011), we showed that *mstn1a* and *mstn1b* genes were expressed in trout myoblasts and myotubes. This result is in accordance with the published data showing that *mstn1a* and *mstn1b* were expressed in muscle of rainbow trout (Rescan et al., 2001; Garikipati et al., 2006). Furthermore, our results demonstrate that the expression of *mstn1a* and *mstn1b* genes decreased during the differentiation of myoblasts. This result contrasts with the observations made in higher vertebrates where *mstn* gene expression increases during the differentiation of myogenic cells (Ríos et al., 2001; Kocamis and Killefer 2002; Artaza et al., 2005; McFarland et al., 2006; Theil et al., 2006; McFarland et al., 2007). This observation shows that the regulation of *mstn* genes in a lower vertebrate such as the rainbow trout is different than in higher vertebrates. Indeed, in mammals *mstn* is expressed nearly exclusively in skeletal muscle whereas in fish *mstn* mRNA have been detected in a wide range of tissues (Rescan et

al., 2001; Rodgers et al., 2001; Garikipati et al., 2006). Moreover, we recently demonstrated that contrary to mouse *mstn* gene, trout *mstn* genes were not regulated by FoxO1 transcription factor (Seiliez et al., 2011). Altogether, these results show that at least in trout, the MSTN protein is present in different tissues and at different cellular process than in mammals, which may suggest that endogenous MSTN protein may have a different function in fish than in mammals.

In an attempt to elucidate the function of MSTN in trout, we determined whether recombinant human MSTN can inhibit the proliferation of trout myoblasts. When the cells were cultivated with only 2% of FCS, the basal level of proliferation is too low to observe any inhibitory effect of huMSTN. However, under IGF1 stimulation, we were able to observe an inhibitory effect of huMSTN on the proliferation of myoblasts in good agreement with published data obtained with mammalian (Thomas et al., 2000; Joulia et al., 2003) and avian (McFarland et al., 2006; McFarland et al., 2007) models. Altogether, this result shows that endogenous trout MSTN could inhibit the proliferation of myoblasts in vivo.

MSTN has been shown in mammals to inhibit not only the proliferation but also the differentiation of myogenic cells in vitro (Langley et al., 2002; Joulia et al., 2003; Trendelenburg et al., 2009). Using our in vitro system of satellite cells culture, we showed for the first time in fish that huMSTN failed to inhibit the differentiation of the myogenic cells in contrast to what is observed in mammals. This may explain the decrease of *mstn1a* and *mstn1b* gene expressions observed during the differentiation of trout myoblasts, and supports the hypothesis that endogenous MSTN has no effect on trout myoblast differentiation.

The MSTN action is mediated first by its binding to the activin type II receptors (ActRIIB and ActRIIA) resulting in the phosphorylation of Smad2 and Smad3 (Lee and McPherron 2001; Zhu et al., 2004). Our results clearly showed that huMSTN stimulation induced a specific phosphorylation of Smad3 both in myotubes and myoblasts. Moreover, the phosphorylation of

Smad3 is completely abolished in presence of the ActRI inhibitor. These results show that phosphorylation of Smad3 is fully mediated by the activin type I receptor as in mammals (Lee and McPherron 2001; Zhu et al., 2004). The high sequence identity (90%) of huMSTN with trout MSTN together with the specificity of the signal validate the use of human MSTN in our *in vitro* system. Our results indicate that myotubes are well responsive to huMSTN stimulation and that an absence of functional MSTN signaling pathway can not explain the absence of inhibitory effect of huMSTN on differentiation of trout myoblasts.

To determine the reasons of the lack of inhibitory effect of huMSTN on differentiation, we focused on the myogenic regulatory factor MyoD given that in mammals, MSTN inhibits differentiation by decreasing MyoD protein level as well as MyoD activity (Liu et al., 2001; Langley et al., 2002). Our results show that huMSTN stimulation did not induce a decrease of MyoD protein level nor of myogenin which is dependent on MyoD activity (Edmondson et al., 1992). Therefore, our results indicate that despite an effective activation of the MSTN signaling pathway, the activity of MyoD was not modified which explains the lack of inhibitory effect of huMSTN treatment on differentiation. It has been shown that TGF-beta, belonging to the same family of MSTN, inhibits MyoD activity through a physical interaction between MyoD and Smad3 (Liu et al., 2001). Thus, we can hypothesize that at least in trout, Smad3 does not interact with MyoD preventing the inhibition of differentiation of trout myoblasts by a huMSTN treatment. Further studies are required to verify the reason for the lack of decrease of myoD protein level in huMSTN treated cells.

Overall, our work show for the first time that stimulation of the MSTN pathway inhibits the proliferation of trout myoblasts *in vitro*. This result appears in good agreement with results obtained *in vivo* in which overexpression of follistatin, an endogenous inhibitor of MSTN, induces an increase of muscle mass mainly due to hyperplasia in trout (Medeiros et al., 2009). By contrast, in zebrafish overexpressing an antisense RNA of *mstn*, the increase of muscle

mass is mainly due to hypertrophy of muscle fiber (Lee et al., 2009). The discrepancy of the results obtained between these species may come from the specificities of their muscle growth. Indeed, trout exhibits indeterminate growth with continued hyperplasia throughout much of its life cycle, whereas zebrafish exhibits determinate growth. This hypothesis is reminiscent of the very interesting data showing that *mstn* is differentially regulated in two closely related zebrafish species exhibiting either indeterminate (*Danio aequipinnatus*) or determinate (*Danio rerio*) growth (Biga and Meyer 2009).

Another interesting result is the absence of inhibitory effect of huMSTN on the differentiation of trout myoblasts by contrast to what is observed in mammals (Langley et al., 2002; Joulia et al., 2003; Trendelenburg et al., 2009). This result strongly suggests that the function of endogenous MSTN in trout is partly different from that of mammals. In this context it is relevant to point out that the fish *mstn* genes are widely expressed and that numerous non-muscle tissues express *mstn* as much as in the muscle (Rescan et al., 2001; Roberts and Goetz 2001; Amali et al., 2004; Garikipati et al., 2006; Ko et al., 2007). Moreover, we recently showed that the mechanisms involved in *mstn* expression in trout are different from that of mammals (Seiliez et al., 2011). Finally, it is surprising that upon physiological conditions, no changes of *mstn* expression in accordance with a strong inhibitory effect of MSTN on muscle growth, has been reported in trout (Rescan et al., 2001; Chauvigné et al., 2003; Martin and Johnston 2005).

Overall these data highlight interspecies differences in the function of MSTN. In this context, the trout with an indeterminate growth, is an interesting model to better understand of the mechanisms involved in muscle growth.

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Myostatin inhibits proliferation but not differentiation of trout myoblasts

Gene	Accession Number	Forward primer	Reverse Primer
eF1a	AF498320	cattgacaagagaaccattga	ccttcagcttgccagcac
Mstn1a	AF273035	ccgccttcacatatgccaa	cagaacctgcgtcagatgca
Mstn1b	AF273036	agtccgccttcacgcaaa	accgaaagcaaccataaaaactca

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