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1 **Myostatin inhibits proliferation but not differentiation of trout myoblasts**
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24 # ABSTRACT

25

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

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

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40 # INTRODUCTION

41

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

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

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

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

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

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90 # MATERIALS AND METHODS

91

92 *Animals*

93 Rainbow trout were maintained at the "Station Commune de Recherches en
94 Ichtyophysiologie, Biodiversité et Environnement" (SCRIBE, Rennes, France) in 0.6-m^3
95 tanks in a re-circulated system at 18°C. All experiments were carried out in accordance with
96 legislation governing the ethical treatment of animals (Decret N° 2001-464, May 29, 2001),
97 and investigators were certified by the French Government to carry out animal experiments
98 (N° agrément 35-47). All animal work was approved by the Ministère de l'Enseignement
99 Supérieur et de le Recherche (Autorisation N°A352386).

100

101 *Chemicals*

102 Dulbecco's modified Eagle's medium (DMEM#D7777) and F10 (#N6635) were purchased
103 from SIGMA. Anti-MyoD (#NB100-80899) antibody was purchased from Novus Biological,
104 anti-Myogenin (#SC-567) from Santa-Cruz Biotechnology, anti-P-Smad3 ($Ser^{423/425}$) from
105 Cell Signalling (#9520), anti-Smad7 from Abgent (#AP6753b). For immunofluorescence
106 analysis, the secondary antibody anti-mouse Alexa488 (#A21441) and anti-rabbit Alexa594
107 (#A11001) were purchased from Invitrogen. Recombinant trout IGF1 (#WU100) was
108 purchased from GroPep and recombinant human MSTN (CYT-418) was purchased from
109 ProSpec and the specific inhibitor of Activin Receptor-Like kinases SB431542 (Inman et al.,
110 2002) from SIGMA (#S4317).

111

112 *Satellite cells isolation and culture*

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

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115 immersed for 30 s in 70% ethanol to sterilize external surfaces. Cells were isolated, pooled,
116 and cultured following previously described protocols (Gabillard et al., 2010). Briefly, after
117 removal of the skin, dorsal white muscle was isolated under sterile conditions and collected in
118 Dulbecco's modified Eagle's medium (DMEM) containing 9 mM $NaHCO_3$, 20 mM HEPES,
119 15% horse serum, and antibiotic-antimycotic cocktail (100 U/ml penicillin, 100 µg/ml
120 streptomycin, 0.25 g/ml fungizone) at pH 7.4. After mechanical dissociation of the muscle in
121 small pieces, the tissue was enzymatically digested with a 0.2% collagenase solution in
122 DMEM for 1 h at 18°C and gentle shaking. The suspension was centrifuged (300 *g* for 5 min
123 at 15°C) and the resulting pellet was subjected to two rounds of enzymatic digestion with a
124 0.1% trypsin solution in DMEM for 20 min at 18°C with gentle agitation. After each round of
125 trypsinization the suspension was centrifuged and the supernatant was diluted in 2 volumes of
126 cold DMEM supplemented with 15% horse serum and the same antibiotic-antimycotic
127 cocktail mentioned above. After two washes with DMEM, the cellular suspension was filtered
128 through 100- and 40-µm nylon filters. All experiments were conducted with cells seeded at a
129 density of 160 000/cm², in six-wells or 24-wells plastic plates (Nunc, Roskilde, Denmark) and
130 left for 30 min before medium change. Plates and coverslips were previously treated with
131 poly-L-lysine and laminin to facilitate satellite cells adhesion. Cells were incubated at 18°C,
132 the optimal temperature for culture, with DMEM or F10 containing 9 mM $NaHCO_3$, 20 mM
133 HEPES, 10% fetal bovine serum, and antibiotic-antimycotic cocktail under an air atmosphere.
134 According to the experiments, the cells were cultivated in proliferating medium
135 (F10+10%FCS) or differentiating medium (DMEM+2%FCS). The medium was renewed
136 every 2 days and observations of morphology were regularly made to control the state of the
137 cells.
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139 *Gene expression analysis*

140 The medium was removed, and wells were washed twice with PBS. The cells were lysed with
141 the lysis buffer of the Nucleospin RNA XS kit (Macherey-Nagel, #N0740-902-50) and total
142 RNA were extracted according to the manufacturer's recommendations. The total amount of
143 RNA was determined as a function of absorbance at 260 nm (Nanodrop ND-1000
144 spectrophotometer). The cDNA was generated with 0.5 µg total RNA using a commercial kit
145 (Applied Biosystems, #4368813). Briefly, 0.5 µg of total RNA was incubated in a 25 µl
146 mixture (10x RT buffer, 25x dNTPs, 10x random primers, 50UI/µl MultiScribe™ Reverse
147 Transcriptase, nuclease-free water) at 25°C for 10 min and then at 37°C for 120 min. The
148 reaction was set at 200 µl by the addition of nuclease-free water. Target gene expression levels
149 were determined by quantitative RT-PCR using a StepOnePlus system (Applied Biosystems).
150 Analyses were carried out using a real-time PCR kit (Fast SyberGreen Master mix, #4385612
151 – Applied Biosystems) with 300 nM of each primer. The primer sequences (Table 1) for
152 *mstn1a*, *mstn1b* and *elongation factor 1α (eF1α)* were already published and validated
153 (Seiliez et al., 2011). All the primers span exon-bondaries to prevent genomic DNA
154 amplification. Amplification was then performed using the following cycle: 95°C for 3 s,
155 60°C for 15 s, 40 times for all primers. The melting curves systematically monitored at the
156 end of the last amplification cycle, show one peak which confirm the specificity of the
157 amplification reaction. Each PCR run included replicate samples (duplicate of reverse
158 transcription and PCR amplification). Relative quantity (Arbitrary unit, A.U.) was determined
159 from a standard curve consisting of a serial dilutions of a pool of cDNA. The efficiencies
160 obtained with the primers pairs ranged from 85 to 100%. EF1α mRNA abundance was not
161 significantly affected by the treatments and was thus used for the normalization of qPCR data.
162

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163 *Protein extraction and Western blotting*

164 After two washes with cold PBS, proteins were extracted with RIPA Buffer (50 mM Tris pH8,
165 1 mM EDTA, 0.5 mM EGTA, 1% NP-40, 0.5% Sodium deoxyclolate, 0.1% SDS, 150 mM
166 NaCl) supplemented with 5mM NaF, 1 mM NaVO4 and protease inhibitors cocktail (Roche).
167 Laemmli Buffer was added to the sample and heated at 90°C for 5 min. Cell lysates were
168 subjected to SDS-PAGE and Western blotting using the appropriate antibody. Antibody
169 against MyoD and myogenin have been previously validated in trout (Gabillard et al., 2010).
170 The antibody against P-Smad3 (Ser$^{423/425}$) recognize the human amino acid sequence around
171 Ser$^{423/425}$ that is very well conserved in trout (MGSPS I/V RCSS V/M S). Similarly, the antibody
172 against Smad7 recognize the sequence between 362~392 aa that is very well conserved in
173 trout (PGFSIKAFD Y/F EKA Y/D SLQRPNDHEF M/T QQP W/R T). Then, preliminary western
174 blot with the anti-P-Smad3 and Anti-Smad7 antibodies were performed with lysates from
175 murine C2C12 cell line and from trout. With trout satellite cells lysate, we obtained a single
176 band for each antibody (P-Smad3 : 55-60 kDa; Smad7 : 45-50 kDa) with the same size as that
177 of C2C12 (data not shown). After washing, the membrane was incubated 1 h with secondary
178 antibody (1/15000) linked to HRP (Jackson Immunoresearch). Immunoreactive bands were
179 visualized by ECL and images were obtained with an image acquisition system (Fusion FX7,
180 Vilbert Lourmat).
181

182 *Immunofluorescence analysis*

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

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187 and anti-myogenin diluted in blocking buffer. The secondary antibodies were diluted in PBST
188 and applied for 1 h. Cells were mounted with Mowiol 4-88 (#475904, Calbiochem)
189 containing Hoescht (0.5 µg/ml). Cells were photographed using a Canon digital camera
190 coupled to a Canon 90i microscope.
191
192 *Analysis of cell proliferation*
193 The mature myostatin protein is extremely well conserved between fish and mammals and
194 trout MSTN is 90% identical to human MSTN (Rescan et al., 2001; Garikipati et al., 2006).
195 Thus, we used recombinant human MSTN (#CYT-418, ProSpec) in our experiments. From
196 the day of extraction, the cells were incubated in F10 (+10% FCS) medium. At day 1, cells
197 were cultivated in F10 alone, and at day 2 cells were cultivated in F10+2%FCS and
198 recombinant trout IGF1 (50 nM) and human MSTN (0.1, 1 or 10 nM) were added. At day 3,
199 BrdU was added for the following 24 h. The cells were fixed with ethanol/glycine buffer
200 (70% ethanol, 50 mM glycine, pH 2). The proliferation of the cells was measured by using the
201 BrdU labeling and detection kit (Roche Diagnostics, no. 1 296 736). Briefly, the cells were
202 incubated for 30 min at 37°C with mouse anti-BrdU, washed, and then incubated with the
203 secondary antibody anti-mouse FITC. Cells were mounted in Mowiol containing 0.5 µg/ml
204 DAPI.
205 *Statistical analysis*
206 Results are presented as means ± SD. Treatment effect was assessed using the non-parametric
207 Kruskall-Wallis ANOVA (Statistica 7.0, StatSoft). When ANOVA revealed significant effect
208 Mann-Whitney test was performed. All the experiments were performed at least 2 times.

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209 **RESULTS**

210

211 *Mstn1a and mstn1b gene expression decreases during in vitro differentiation of trout*
212 *myoblasts*

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

223

224 *HuMSTN activates Smad3 phosphorylation in myoblasts and myotubes of trout*

225 Sequence analysis showed that the sequences of trout myostatins were very similar to that of
226 human (90%) as previously reported (Rescan et al., 2001; Garikipati et al., 2006). To validate
227 the use of heterologous myostatin in our in vitro system, we monitored the activity of
228 recombinant human MSTN (HuMSTN) by measuring the phosphorylation status of Smad3 a
229 key factor in the MSTN signaling pathway (Sartori et al., 2009). As shown in figure 2A and
230 2B, the stimulation of both trout myoblasts and myotubes with 100 nM of huMSTN for 1h
231 induced the phosphorylation of Smad3. Furthermore, to demonstrate the specificity of this
232 hormonal response, we used a chemical inhibitor (SB431542) specific to the activin receptor
233 type I (ActRI) known to transduce MSTN signal (Lee et al., 2005; Miyake et al., 2010). The

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

239

240 *HuMSTN inhibits proliferation of myoblasts in vitro*

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

253

254 *HuMSTN has no effect on the differentiation of trout myoblasts*

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

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259 not have an effect (data not shown). Therefore, a higher dose of huMSTN was tested (100
260 nM) that is within the range previously reported (Langley et al., 2002). Our results (Figure 4A
261 and 4B) showed that after 3 days in the differentiation medium (DMEM+2%FCS), myoblasts
262 were clearly differentiated into myotubes. Indeed after 3 days in differentiating medium, the
263 percentage of nuclei in myosin positive cells (1% versus 23%) and the percentage of
264 myogenin positive nuclei increased (20% versus 39%). Moreover, treatment of the cells with
265 100 nM huMSTN (Figure 4A and 4B) did not significantly affect the differentiation of
266 myogenic cells (23% versus 20% of nuclei in myosin positive cells) and (39% versus 37% of
267 myogenin positive nuclei). Altogether, these results showed that huMSTN treatment did not
268 inhibit differentiation of trout myoblasts in vitro.

269

270 *HuMSTN has no effect on the level of MyoD protein*

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

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282 **DISCUSSION**

283 In mammals, MSTN belongs to the TGF-beta familly and is well known as a potent inhibitor
284 of the muscle growth (McPherron et al., 1997; Lee and McPherron 2001). Indeed, it has been
285 shown that MSTN inhibits both proliferation and differentiation of mammalian myogenic
286 cells (Thomas et al., 2000; Langley et al., 2002; Joulia et al., 2003; Trendelenburg et al.,
287 2009). In fish, several works report an increase of muscle mass after decreasing MSTN
288 activity (Lee et al., 2009; Medeiros et al., 2009), while others failed to observe a strong (if
289 any) effect on muscle mass (Amali et al., 2004; Acosta et al., 2005; Sawatari et al., 2010). To
290 date, it is unknown in fish whether MSTN could have any effect on proliferation or
291 differentiation of myogenic cells. Therefore, the purpose of the present work was to determine
292 whether MSTN can affect the proliferation and the differentiation using an in vitro system of
293 trout satellite cells culture.

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

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

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

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

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

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

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

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

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

364 Another interesting result is the absence of inhibitory effect of huMSTN on the differentiation
365 of trout myoblasts by contrast to what is observed in mammals (Langley et al., 2002; Joulia et
366 al., 2003; Trendelenburg et al., 2009). This result strongly suggests that the function of
367 endogenous MSTN in trout is partly different from that of mammals. In this context it is
368 relevant to point out that the fish *mstn* genes are widely expressed and that numerous non-
369 muscle tissues express *mstn* as much as in the muscle (Rescan et al., 2001; Roberts and Goetz
370 2001; Amali et al., 2004; Garikipati et al., 2006; Ko et al., 2007). Moreover, we recently
371 showed that the mechanisms involved in *mstn* expression in trout are different from that of
372 mammals (Seiliez et al., 2011). Finally, it is surprising that upon physiological conditions, no
373 changes of *mstn* expression in accordance with a strong inhibitory effect of MSTN on muscle
374 growth, has been reported in trout (Rescan et al., 2001; Chauvigné et al., 2003; Martin and
375 Johnston 2005) .

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

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Gene	Accession Number	Forward primer	Reverse Primer
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Mstn1a	AF273035	ccgccttcacatatgccaa	cagaacctgcgtcagatgca
Mstn1b	AF273036	agtccgccttcacgcaaa	accgaaagcaaccataaaactca

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Figure1

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A

B

Figure2

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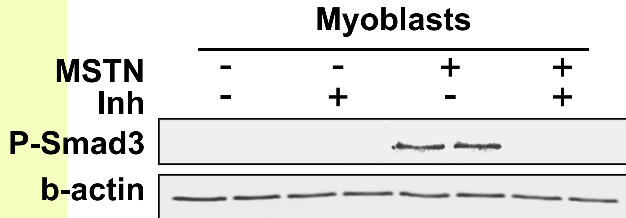

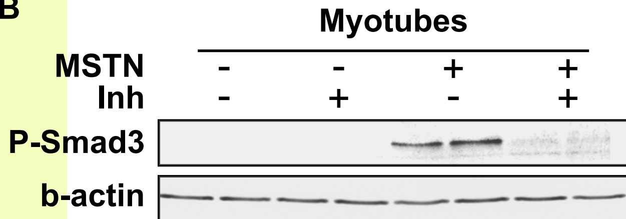

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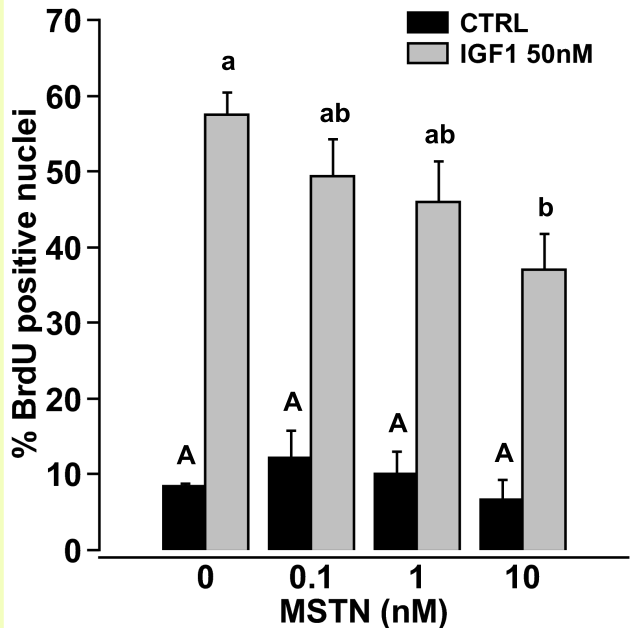
Figure3



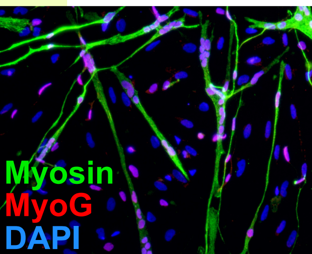
differentiation of trout myoblasts. Molecular
DOI : 10.1016/j

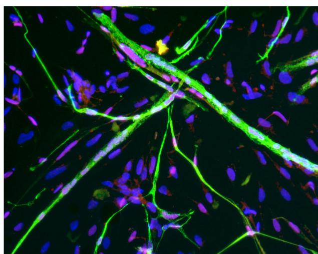
Figure4

A

CTRL

MSTN 100 nM





B

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% nuclei in positive cells

0 10 20 30 40 50

MyoG
Myosin

A a B b B b

PM

- + MSTN

DM

Comment cite
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Figure5

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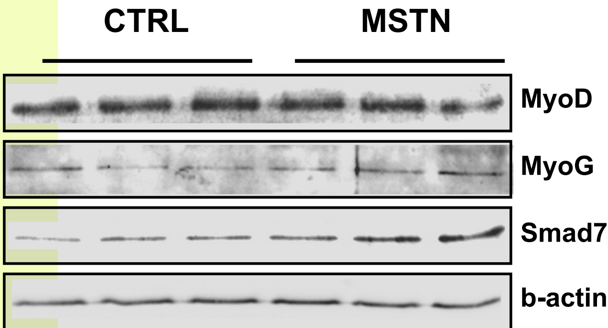
A



B