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## FoxO1 is not a key transcription factor in the regulation of *myostatin* (*mstn-1a* and *mstn-1b*) gene expression in trout myotubes

Iban Seiliez,<sup>1\*</sup> Nathalie Sabin,<sup>2</sup> and Jean-Charles Gabillard<sup>2\*</sup>

<sup>1</sup>Institut National de la Recherche Agronomique, UMR 1067 Nutrition Aquaculture et Génomique, Pôle d'hydrobiologie, St-Pée-sur-Nivelle; and <sup>2</sup>Institut National de la Recherche Agronomique, UR 1037 Station Commune de Recherches en Ichtyophysiologie, Biodiversité et Environnement, Equipe Croissance et Qualité de la Chair de Poisson, Campus de Beaulieu, Rennes, France

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**Seiliez I, Sabin N, Gabillard JC.** FoxO1 is not a key transcription factor in the regulation of *myostatin* (*mstn-1a* and *mstn-1b*) gene expression in trout myotubes. *Am J Physiol Regul Integr Comp Physiol* 301: R97–R104, 2011. First published April 13, 2011; doi:10.1152/ajpregu.00828.2010.—In mammals, much evidence has demonstrated the important role of myostatin (MSTN) in regulating muscle mass and identified the transcription factor forkhead box O (FoxO) 1 as a key regulator of its gene expression during atrophy. However, in trout, food deprivation leads to muscle atrophy without an increase of the expression of *mstn* genes in the muscle. We therefore studied the relationship between FoxO1 activity and the expression of both *mstn* genes (*mstn1a* and *mstn1b*) in primary culture of trout myotubes. To this aim, two complementary studies were undertaken. In the former, FoxO1 protein activity was modified with insulin-like growth factor-I (IGF-I) treatment, and the consequences on the expression of both *mstn* genes were monitored. In the second experiment, the expression of both studied genes was modified with growth hormone (GH) treatment, and the activation of FoxO1 protein was investigated. We found that IGF-I induced the phosphorylation of FoxO1 and FoxO4. Moreover, under IGF-I stimulation, FoxO1 was no longer localized in the nucleus, indicating that this growth factor inhibited FoxO1 activity. However, IGF-I treatment had no effect on *mstn1a* and *mstn1b* expression, suggesting that FoxO1 would not regulate the expression of *mstn* genes in trout myotubes. Furthermore, the treatment of myotubes with GH decreased the expression of both *mstn* genes but has no effect on the phosphorylation of FoxO1, FoxO3, and FoxO4 nor on the nuclear translocation of FoxO1. Altogether, our results showed that *mstn1a* and *mstn1b* expressions were not associated with FoxO activity, indicating that FoxO1 is likely not a key regulator of *mstn* genes in trout myotubes.

forkhead box O; myostatin; fish; muscle; atrophy; growth hormone; insulin-like growth factor-I

IN THE LAST DECADE, MYOSTATIN (MSTN), a member of the transforming growth factor- $\beta$  superfamily, has emerged as a key factor in muscle growth regulation (43). The importance of the *mstn* gene in muscle growth comes from the phenotype of MSTN-deficient cattle (natural mutation or deletion) called double-muscling bovines, like the Belgium Blue breed (29, 44). In these bovines, muscle overgrowth is due to both hyperplasia (increased number of muscle fibers) and hypertrophy (increased size of individual muscle fibers). Similarly, inactivation of the *mstn* gene or MSTN function is reported to cause

increased muscle mass in a variety of species, including mice (*Mus musculus*) (43), dogs (*Canis familiaris*) (47), sheep (*Ovis aries*) (8), humans (*Homo sapiens*) (56), zebrafish (*Danio rerio*) (35), and trout (36, 45). Conversely, overexpression of MSTN in transgenic mice has been shown to induce muscle atrophy *in vivo* (50). These data demonstrate the predominant role of MSTN in regulating muscle size in both lower and higher vertebrates.

In agreement with its role as a negative regulator of skeletal muscle mass, expression data from a wide variety of mammalian models show that *mstn* is upregulated during muscle atrophy induced by hindlimb unloading (9), thermal injury (31), and food deprivation (2). However, other findings show some differences in the response of the *mstn* gene to environmental changes depending on the species or the conditions studied (20, 25, 26, 63). To make inroads in the understanding of *mstn* regulation, several groups investigate the regulatory elements controlling *mstn* expression. Conserved sequences in the *mstn* promoter from several species have thus been identified that share many binding sites for forkhead box O (FoxO) transcription factors (3, 4, 13). Furthermore, some of these FoxO-binding sites were shown to be critical for FoxO1 binding and *mstn* gene expression (3, 4). FoxO1, which belongs to a subfamily of transcription factors consisting of FoxO1, FoxO3, FoxO4, and FoxO6 (39), is also known as the main coordinator of the two main proteolytic pathways (the ubiquitin proteasome and the autophagy lysosome) by inducing several autophagy-related genes as well as the two muscle-specific ubiquitin ligases *atrogen-1* and *murfl* (40, 64). Nuclear localization and transcriptional activity of the FoxO transcription factors are inhibited via phosphorylation by the phosphatidylinositol 3-kinase (PI 3-kinase)/protein kinase B (Akt) signaling pathway, which in turn is activated by insulin-like growth factor-I (IGF-I) binding to its cell surface receptor (55, 61). Thus the regulation of FoxO function may play a central role in mediating effects on gene expression in response to atrophic and/or hypertrophic signaling.

In fish, less is known on the regulation of the expression of *mstn* genes (52). A recent phylogenetic analysis of the entire *mstn* subfamily (30) indicates that fish possess multiple *mstn* genes and that a gene duplication event during early fish radiation (5, 49) produced two distinct *mstn* clades: *mstn-1* and *mstn-2*. A second duplication event within salmonids, likely resulting from tetraploidization, produced two subsequent divisions, one in each clade. This suggests that most, if not all, salmonids possess four distinct *mstn* genes: two within the first clade (1a and 1b) and two in the second (2a and 2b). In rainbow trout, from these four *mstn* genes only two (*mstn1a* and

\* I. Seiliez and J.-C. Gabillard contributed equally to this work.

Address for reprint requests and other correspondence: J.-C. Gabillard, INRA, UR1037 Station Commune de Recherches en Ichtyophysiologie Biodiversité et Environnement (SCRIBE), Equipe Croissance et Qualité de la Chair de Poisson, Campus de Beaulieu, 35000 Rennes, France (e-mail: Jean-Charles.Gabillard@rennes.inra.fr).

*mstn1b*) are expressed in the muscle and are downregulated during starvation (28). In addition, they are differentially regulated under different conditions. Indeed, muscle atrophy during the reproductive stage is associated with a decrease of *mstn1b* expression, whereas *mstn1a* expression is unaffected (51). Injection of growth hormone (GH) upregulates *mstn1a* and downregulates *mstn1b* gene expression in muscle (7, 16). Overall, these data suggest that, in rainbow trout, regulation of *mstn* gene expression is complex and likely different from that observed in mammals.

Recent *in vitro* studies show that the hormonal (insulin and/or IGF-I) regulation of the Akt-FoxO signaling in rainbow trout is well conserved (10, 12, 32, 48, 57, 58). Furthermore, similarly to what is observed in mammalian and birds, this pathway has been shown to be associated with muscle atrophy in this species (59). However, we know little about the molecular mechanisms regulating the expression of the *mstn* genes in any fish species. The purpose of the present work was therefore to determine the role of FoxO1 in regulating the expression of the muscle antigrowth factors *mstn1a* and *mstn1b* in rainbow trout. To establish a causal link between FoxO activity and the expression of both *mstn* genes, two complementary experiments were performed in primary cultures of trout muscle cells. In the former, FoxO1 protein activity was modified with IGF-I treatment, and the consequences on the expression of both *mstn* genes were monitored. In the second experiment, the expression of both studied genes was modified with GH treatment, and the activation of FoxO1 protein was investigated.

## MATERIALS AND METHODS

**Animals.** Rainbow trout were maintained at the “Station Commune de Recherches en Ichtyophysiologie, Biodiversité et Environnement” (Rennes, France) in 0.6-m<sup>3</sup> tanks in a recirculated system at 18°C. All experiments were carried out in accordance with legislation governing the ethical treatment of animals (Decret No. 2001-464, May 29, 2001), and investigators were certified by the French Government to carry out animal experiments (No. agrément 35–47). All animal work was approved by the Ministère de l'Enseignement Supérieur et de la Recherche (Autorisation No. A352386).

**Myosatellite cell isolation and culture.** Primary cultures of skeletal muscle cells were carried out as follows: for each culture, 30–60 animals, each weighing ~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 (15, 58). 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 NaHCO<sub>3</sub>, 20 mM HEPES, 15% horse serum, and antibiotic-antimycotic cocktail (100 U/ml penicillin, 100 µg/ml streptomycin, and 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 two 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/cm<sup>2</sup>, in 6-well or 24-well plastic plates (Nunc, Roskilde, Denmark), and left for 30 min before medium

change. Plates and cover slips were previously treated with poly-L-lysine and laminin to facilitate satellite cell adhesion. Cells were incubated at 18°C, the optimal temperature for culture, with DMEM (no. D7777; Sigma) containing 9 mM NaHCO<sub>3</sub>, 20 mM HEPES, 10% FBS, and antibiotic-antimycotic cocktail under an air atmosphere. The medium was renewed every 2 days, and observations of morphology were regularly made to control the state of the cells. They were cultured for 7 days to obtain myotubes.

**Treatment conditions.** The experiments of the present study were performed on myotubes that, compared with myoblasts, are closer to a myofiber and thus more relevant at a physiological point of view. On the day of the experiment, cells were deprived of serum for 24 h and subsequently incubated in the presence or absence of 100 nM salmon/trout IGF-I (WU100 GroPep) or 0.5 and 5 nM of trout GH [homemade recombinant GH (34)] for 15 min, 30 min, 1 h, 2 h, 5 h, or 24 h. Each experiment was performed at least two times.

**Gene expression analysis.** Treatment medium was removed, and wells were washed two times with PBS. Total RNA was extracted with a Nucleospin RNA XS kit (no. N0740–902-50; Macherey-Nagel) 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). cDNA was generated with 0.5 µg total RNA using a commercial kit (no. 4368813; Applied Biosystems). Briefly, 0.5 µg of total RNA was incubated in a 25-µl mixture (10× RT buffer, 25× dNTPs, 10× random primers, 50 IU/µl MultiScribe Reverse Transcriptase, and 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, no. 4385612; Applied Biosystems) with 300 nM of each primer. The primer sequences (Table 1) were already published and validated (28). Amplification was then performed using the following cycle: 95°C for 3 s and 60°C for 15 s, 40 times for all primers. Real-time PCR data were normalized according to elongation factor 1α (EF1α) mRNA abundance in each sample. Melting curves were systematically monitored (temperature gradient at 0.5°C/10 s from 55 to 94°C) at the end of the last amplification cycle to confirm the specificity of the amplification reaction. Each PCR run included replicate samples (duplicate of reverse transcription and PCR amplification) and negative controls (reverse transcriptase-free and RNA-free samples).

The relative expression ratio of a target gene was calculated on the basis of real-time PCR efficiency and the cycle threshold (C<sub>T</sub>) deviation (ΔC<sub>T</sub>) of the unknown sample vs. a control sample and expressed compared with the EF1α reference gene. PCR efficiency was measured by the slope of a standard curve using serial dilutions of cDNA. PCR efficiency values ranged between 1.9 and 2.

**Protein extraction and western blotting.** After two washes with cold PBS, proteins were extracted with RIPA buffer (50 mM Tris, pH 8, 1 mM EDTA, 0.5 mM EGTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 150 mM NaCl) supplemented with 5 mM NaF, 1 mM NaVO<sub>4</sub>, and protease inhibitor 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

Table 1. Sequences of the primer pairs used for real-time quantitative RT-PCR

Gene	5'-3' Forward Primer	5'-3' Reverse Primer
<i>mstn1a</i>	CCGCCTTCACATATGCCAA	CAGAACCTGCGTCAGATGCA
<i>mstn1b</i>	AGTCGGCCTTCACGAAA	ACCGAAAGCAACCATAAACTCA
<i>Atrogin-1</i>	TGCGATCAAATGGATTCAAA	GATTGCATCATTTCCCCACT
<i>EF1α</i>	TCCTCTGGTCTTTCCGCTG	ACCGAGGGACATCCTGTG

GenBank accession no.: myostatin (*mstn*) 1a, AF273035; *mstn1b*, AF273036; *atrogin-1*, CX026010; *elongation factor 1α* (*EF1α*), AF498320.



Table 2. Location and sequences of sites matching the consensus for FoxO binding (C/G A/T A A C/T A) in *mstn1a* and *mstn1b* promoters

Location*	Sequence
<i>mstn1a</i>	
–837 to –843	gtaaata
–862 to –868	ctaaata
–1153 to –1159	gaaaata
<i>mstn1b</i>	
–793 to –799	gtaaata
–883 to –889	gaaaata
–1121 to –1127	gtaaata
–1765 to –1771	ctaaata
–2247 to –2253	gtaaata

\*The table shows the location of the putative forkhead box O (FoxO)-binding sites within the trout *mstn1a* and *mstn1b* promoter sequences previously published by Garikipati et al. (17). Nos. represent the position of these elements relative to the transcription start site.

the appropriate antibody. Anti-phospho Akt (Ser<sup>473</sup>) (no. 9271), anti-Akt (no. 9272), anti-phospho-FoxO1 (Thr<sup>24</sup>)/FoxO3 (Thr<sup>32</sup>) (no. 9464), and anti-phospho-FoxO1 (Ser<sup>319</sup>)/FoxO4 (Ser<sup>262</sup>) (no. 2487) were purchased from Cell Signaling Technologies (Ozyme, Saint Quentin Yvelines, France). Anti-FoxO1 (no. 1874–1) was purchased from Epitomics, and anti- $\beta$ -actin (no. sc-47778) was from Santa Cruz Biotechnology. Anti-Akt, anti-phospho-Akt, anti-phospho-FoxO1 (Thr<sup>24</sup>)/FoxO3 (Thr<sup>32</sup>), and anti-phospho-FoxO1 (Ser<sup>319</sup>)/FoxO4 (Ser<sup>262</sup>) antibodies have been previously validated in trout (12, 58). For anti-FoxO1 antibody, the amino acid sequences of FoxO1 were monitored in the SIGENAE database (60) to check for well-conservation of the antigen sequence. Next, preliminary Western blots with the anti-FoxO1 antibody were performed with lysates from the murine C<sub>2</sub>C<sub>12</sub> cell line and from trout. With satellite cell lysate, we obtained a single band (75 kDa) with the same size as that of C<sub>2</sub>C<sub>12</sub> (data not shown). After being washed, the membrane was incubated for 1 h with secondary antibody (1:15,000) linked to horseradish peroxidase (Jackson ImmunoResearch). Immunoreactive bands were visualized by enhanced chemiluminescence, and images were obtained with an image acquisition system (Fusion FX7; Vilbert Lourmat).

**Immunofluorescence analysis.** Cells on glass cover slips were briefly washed two times by PBS and fixed for 10 min with 4% paraformaldehyde. For permeabilization, cells were incubated for 3 min in 0.1% Triton X-100/PBS. After three washes, cells were saturated for 1 h with 3% BSA and 0.1% Tween 20 in PBS (PBST). Cells were incubated for 3 h with the first antibody anti-FoxO1 (Cell Signaling Technologies) diluted in blocking buffer. The secondary antibody, anti-rabbit Alexa594 (Invitrogen), was diluted in PBST and applied for 1 h. Cells were mounted with Mowiol 4–88 (no. 475904; Calbiochem) containing Hoescht (0.5  $\mu$ g/ml). Cells were photographed using a Canon digital camera coupled to a Canon 90i microscope.

**Statistical analysis.** Data on gene expression analysis are expressed as means  $\pm$  SD ( $n = 6$ ) and were analyzed by one-way ANOVA followed by the Student-Newman-Keuls test. For all statistical analyses, the level of significance was set at  $P < 0.05$ .

## RESULTS

**FoxO-binding sites are present in the promoter of both *mstn* genes.** To assess the possibility that FoxO transcription factors could bind to the trout *mstn* promoters, we searched for the presence of putative binding sites for FoxO within the sequences previously published by Garikipati et al. (17). Sequence analysis based on a consensus sequence for FoxO-

binding sites ([C/G][A/T]AAA[C/T]A) derived from a previous study (21) identified the presence of three putative FoxO-binding sites in the *mstn1a* promoter and five in the *mstn1b* promoter (Table 2).

**IGF-I inhibits FoxO1 activity but has no effect on *mstn1a* and *mstn1b* gene expression.** To study the involvement of the Akt-FoxO signaling pathway in the regulation of *mstn1a* and *mstn1b* gene expression, we first investigated the specific effect of IGF-I on the Akt-FoxO signaling pathway and the expression of both *mstn* genes in primary cultures of trout muscle cells. Trout cultured myotubes (day 7 of culture) were serum-deprived for 24 h to enhance the nonphosphorylated nuclear form of FoxO1 and subsequently incubated in the absence or presence of 100 nM salmon IGF-I during different periods of time. As shown in Fig. 1, IGF-I stimulated the phosphorylation of Akt at Ser<sup>473</sup>, FoxO1 at Ser<sup>319</sup>, and Foxo4 at Ser<sup>262</sup> as early as 15 min after treatment, and this stimulatory effect was observed for up to 5 h.

We then monitored by immunofluorescence the localization of FoxO1 in serum-deprived cells for 24 h and incubated or not with 100 nM of IGF-I for 1–5 h. As shown in Fig. 2, immunofluorescence of serum-starved cells (control) revealed that FoxO1 colocalizes with Hoescht staining, indicating a nuclear localization of the studied protein. In contrast, IGF-I-treated cells showed the loss of nuclear staining for FoxO1, indicating that IGF-I treatment prevents nuclear translocation of FoxO1.

To test the effect of IGF-I on *mstn1a* and *mstn1b* gene expression, cells were then stimulated for 24 h with 100 nM IGF-I. The expression of *atrogen-1* has been previously shown to be downregulated by IGF-I treatment of trout muscle cells (12, 58) and was therefore monitored as a control. As expected, *atrogen-1* was downregulated ( $\sim$ 2-fold) after 24 h of stimulation with IGF-I (Fig. 3). In contrast, the hormonal treatment had no significant effect on either *mstn1a* or *mstn1b* gene

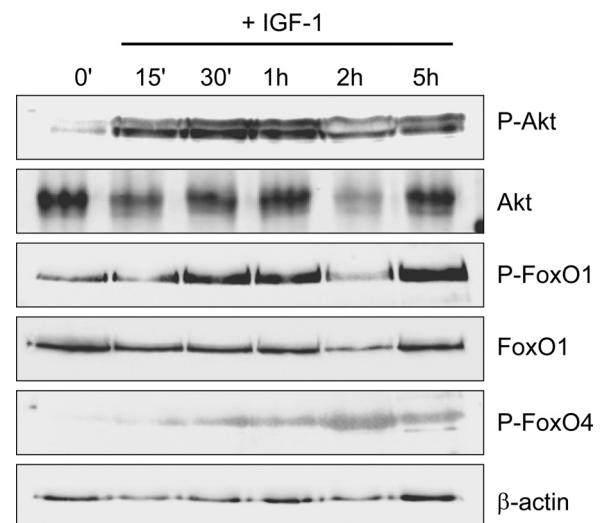


Fig. 1. Effect of insulin-like growth factor-I (IGF-I) on the protein kinase B (Akt)-forkhead box O (FoxO) signaling pathway in primary culture of trout muscle cells. Seven-day-old cells were serum starved for 24 h and then stimulated or not with 100 nM of trout IGF-I for 15 min, 30 min, 1 h, 2 h, or 5 h before harvest. Cell lysates were analyzed by Western blot with the indicated antibodies.  $\beta$ -Actin was used as a loading control. P, phosphorylated. Each treatment was performed in triplicate, and similar results were obtained. This figure shows a representative blot.

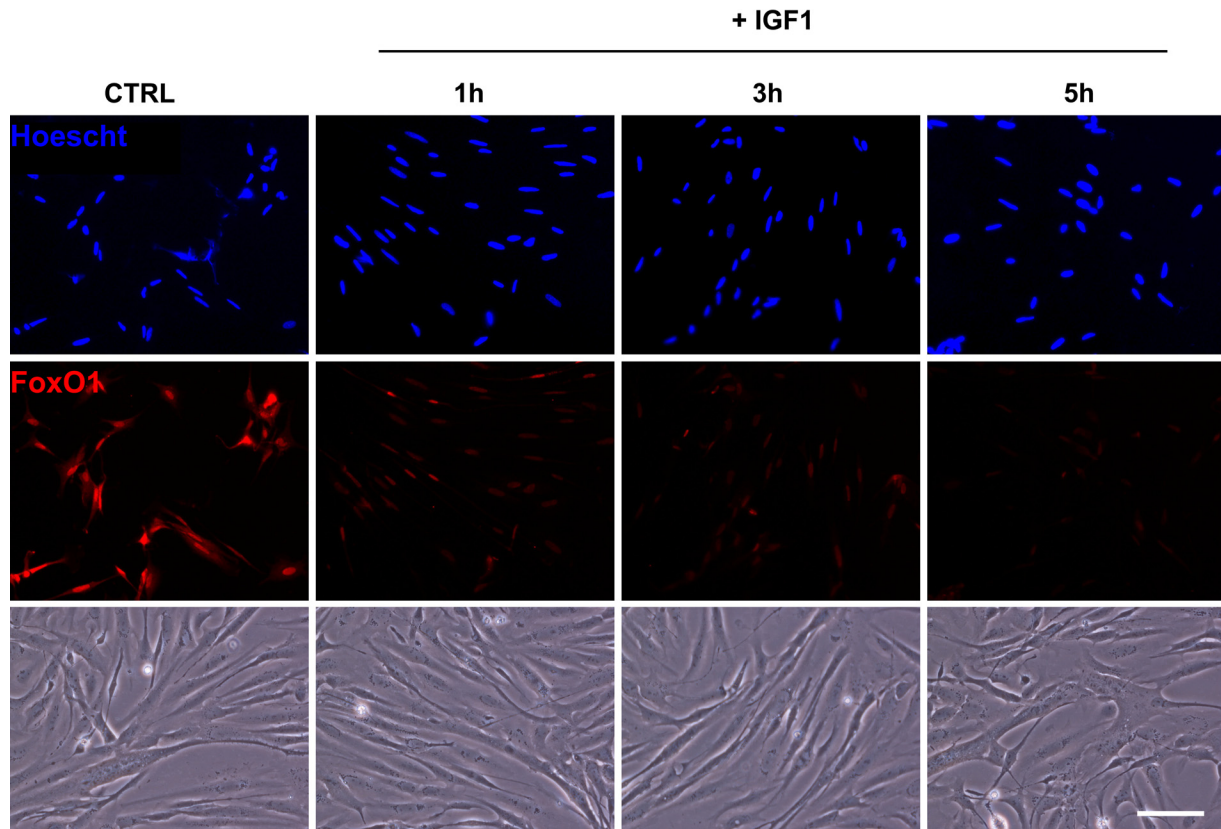


Fig. 2. Effect of IGF-I on FoxO1 localization in primary culture of trout muscle cells. Seven-day-old cells were serum starved for 24 h [control (CTRL)] and then stimulated or not with 100 nM of trout IGF-I for 1, 3, or 5 h. Immunolocalization of FoxO1 (red) was performed as described in MATERIALS AND METHODS. Nuclei were stained with Hoescht (blue). *Bottom*: representative bright-field images of control and IGF-I-treated myotubes. The scale represents 50  $\mu$ m.

expression. Overall, these results indicate that IGF-I inhibits FoxO1 activity without changing *mstn1a* and *mstn1b* gene expression.

*GH downregulates mstn1a and mstn1b gene expression but has no effect on the Akt-FoxO signaling pathway.* Another way to study the involvement of FoxO transcription factors in the regulation of *mstn1a* and *mstn1b* gene expression was to analyze the Akt-FoxO signaling pathway in cells over- or underexpressing the studied genes. GH has been previously

shown to regulate the expression of both *mstn1a* and *mstn1b* in rainbow trout (7, 16). Therefore, we first investigated the effect of GH on *mstn1a* and *mstn1b* gene expression in our cell culture model. Trout cultured myotubes (*day 7* of culture) were serum-deprived for 24 h and subsequently incubated in the absence or presence of trout GH (0.5 or 5 nM) for 24 h. As shown in Fig. 4, both *mstn1a* and *mstn1b* genes were downregulated ( $\sim 7$ - and 20-fold, respectively) after 24 h of stimulation with 5 nM of GH. Thus these GH-treated cells may serve as a relevant model to characterize the factors involved in the transcriptional regulation of *mstn* genes.

We then monitored the activity of the Akt-FoxO signaling pathway in these GH-treated cells. Previous studies had identified signal transducer and activator of transcription 5 (STAT5) as a key transcription factor in GH signaling (62), and we therefore monitored its phosphorylation status as a positive control. As expected, the phosphorylation of STAT5 was highly induced after 1 h of stimulation with GH (Fig. 5). In contrast, the hormonal treatment has no effect on the phosphorylation of Akt at Ser<sup>473</sup>, FoxO1 at Ser<sup>319</sup>, FoxO3 at Thr<sup>32</sup>, and FoxO4 at Ser<sup>262</sup>.

Finally, we monitored by immunofluorescence the localization of FoxO1 in serum-deprived cells for 24 h and incubated or not with 0.5 nM of GH or 100 nM of IGF-I for 1–5 h. As shown in Fig. 6, immunofluorescence of serum-starved and GH-treated cells revealed that FoxO1 remained colocalized with Hoescht staining, indicating a nuclear localization of the studied protein. In contrast, IGF-I-treated cells showed a loss

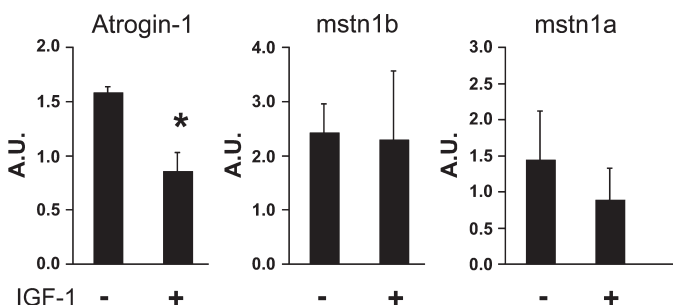


Fig. 3. Effect of IGF-I on the expression of *atrogen-1*, myostatin gene (*mstn1a*), and *mstn1b* in primary culture of trout muscle cells. Seven-day-old cells were serum starved for 24 h and then stimulated or not with 100 nM of trout IGF-I for 24 h before harvest. *Atrogen-1*, *mstn1a*, and *mstn1b* mRNA levels were estimated using real-time RT-PCR. For each treatment, six replicates were performed. Expression values [arbitrary units (AU)] were normalized with that of elongation factor 1 $\alpha$  (EF1 $\alpha$ ) transcripts. Results are expressed as means  $\pm$  SD ( $n = 6$  experiments). \*Significant difference from the serum-deprived group ( $P < 0.05$ , Student's *t*-test).

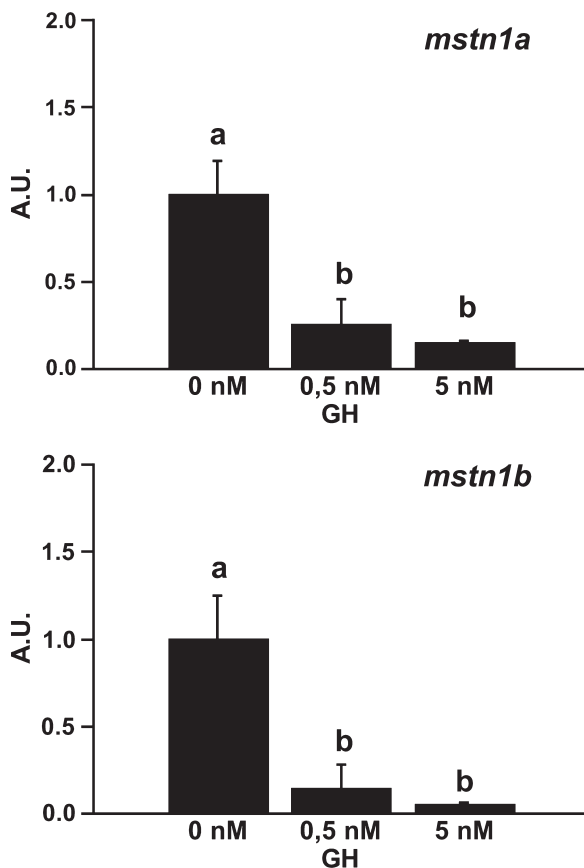


Fig. 4. Effect of growth hormone (GH) on the expression of *mstn1a* and *mstn1b* in primary culture of trout muscle cells. Seven-day-old cells were serum starved for 24 h and then stimulated or not with 0.5 or 5 nM of trout GH for 24 h before harvest. *mstn1a* and *mstn1b* mRNA levels were estimated using real-time RT-PCR. For each treatment, six replicates were performed. Expression values (AU) were normalized with that of EF1 $\alpha$  transcripts. Results are means  $\pm$  SD ( $n = 6$ ) and were analyzed using the one-way ANOVA followed by the Student-Newman-Keuls test for multiple comparisons. The different letters indicate significantly ( $P < 0.05$ ) different means.

of nuclear localization of FoxO1. Taken together, these results show that GH downregulates *mstn1a* and *mstn1b* gene expression in a FoxO-independent manner.

#### DISCUSSION

In mammals, there is accumulating evidence on the important role of the MSTN in regulating muscle mass and on the role of transcription factor FoxO1 as a key regulator of its gene expression in response to atrophic signaling (3, 4). In this regard, expression data from several mammalian models show that muscle atrophy is often associated with an increase of *mstn* expression (2, 9, 31). However, in rainbow trout, food deprivation leads to muscle atrophy without an increase of *mstn* expression in muscle (11, 28, 46). Therefore, the purpose of the present work was to determine the role of FoxO1 in regulating the expression of the muscle anti-growth factors *mstn1a* and *mstn1b* in rainbow trout.

To address this issue, we first conducted an *in silico* analysis aiming at identifying the presence of putative FoxO-binding sites within trout *mstn1a* and *mstn1b* promoter sequences previously published (17). The very well conservation of the DNA-binding domain of FoxO proteins along the evolution (6)

lets us hypothesize that the DNA sequence to which FoxO binds is the same between lower and higher vertebrates. Sequence analysis based on the consensus sequence for FoxO-binding sites ([C/G][A/T]AAA[C/T]A) derived from a previous study (21) identified the presence of several putative FoxO-binding sites in both trout *mstn* promoters. This suggested the possible involvement of FoxO transcription factors on the control of *mstn1a* and *mstn1b* expression.

In mammals, FOXO proteins are known to mediate the transcriptional output of insulin/IGF-I signal transduction (33, 61). When insulin/IGF-I signaling is active, a PI 3-kinase/Akt kinase cascade phosphorylates FOXO, leading to its nuclear exclusion. When insulin/IGF-I signaling is inhibited, unphosphorylated FOXO enters the nucleus where it induces the expression of several genes coding for key mediators of skeletal muscle atrophy, including *mstn* (reviewed in Ref. 18). We therefore investigated the effect of IGF-I on the Akt-FoxO signaling pathway and the expression of *mstn1a* and *mstn1b* genes in our cell culture model. Our results showed that the treatment of cells with IGF-I enhanced the phosphorylation of Akt at Ser<sup>473</sup>, FoxO1 at Ser<sup>319</sup>, and Foxo4 at Ser<sup>262</sup>. Moreover, immunolocalization of FoxO1 clearly showed that IGF-I stimulation led to the loss of nuclear localization of FoxO1. The effect of IGF-I on the phosphorylation of Akt and FoxO1 in primary culture of trout muscle cells has already been reported (10, 12, 58). The results presented here are in good agreement with these data and provide evidence for the first time that the effect of IGF-I is also accompanied by a nuclear exclusion of FoxO1 in this cell culture model, leading to the loss of FoxO1 staining. Indeed, Akt-mediated phosphorylation of FOXO leads to their proteasomal degradation through polyubiquitination by the E3 ubiquitin ligase Skp2 (24, 42). In this study, we did not examine the effects of IGF-I on the nuclear translocation of other FoxO family members. However, according to data on mammals showing that both FoxO1 and FoxO3 are downstream targets of the insulin/IGF-I signaling (55), it is probable that IGF-I may have similar effects on the activation

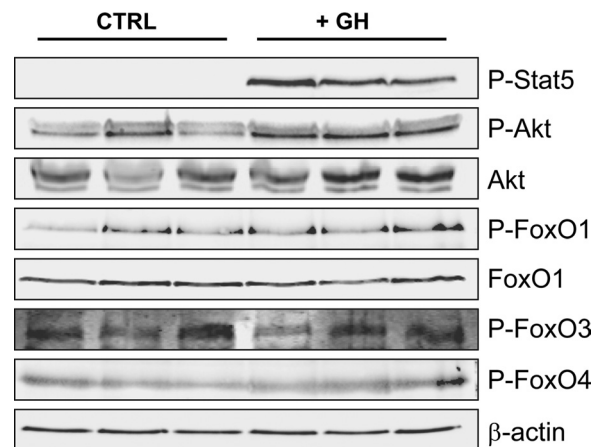


Fig. 5. Effect of GH on the Akt-FoxO signaling pathway in primary culture of trout muscle cells. Seven-day-old cells were serum starved for 24 h and then stimulated or not with 0.5 nM of trout GH for 1 h before harvest. Cell lysates were analyzed by Western blot with the indicated antibodies. The phosphorylation of signal transducer and activator of transcription 5 (STAT5) was monitored as the positive control, and  $\beta$ -actin was used as the loading control. Each treatment was performed in triplicate, and similar results were obtained. This figure shows a representative blot.



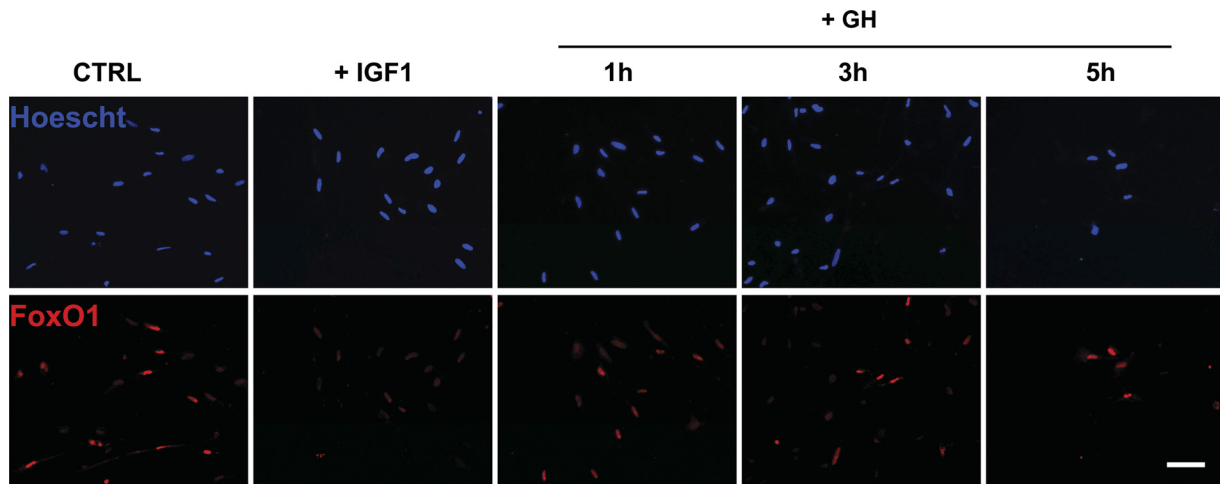


Fig. 6. Effect of GH on FoxO1 localization in primary culture of trout muscle cells. Seven-day-old cells were serum starved for 24 h (CTRL) and then stimulated or not with 100 nM of trout IGF-I for 3 h or 0.5 nM of trout GH for 1, 3, or 5 h. Immunolocalization of FoxO1 (red) was performed as described in MATERIALS AND METHODS. Nuclei were stained with Hoescht (blue). The scale bar represents 50  $\mu$ m.

of FoxO3 and other FoxO family members. In this regard, we recently showed that the treatment of primary culture of trout muscle cells with IGF-I enhances the phosphorylation of FoxO3 at Thr<sup>32</sup> (58). To validate this decrease of FoxO activity, we then measured the expression of *atrogen-1*, a gene well-known to be dependent on FoxO activity (55, 61). As expected, IGF-I stimulation decreased the expression of *atrogen-1* in trout myotubes. This result confirmed that IGF-I stimulation induced a strong decrease of FoxO activity and thus validated our experimental conditions. However, under the same conditions, we were not able to observe any significant changes of *mstn1a* and *mstn1b* gene expressions. Therefore, our results showed that, despite a strong decrease of FoxO1 activity, *mstn* gene expressions remained constant. These results suggest a slight role for the Akt-FoxO signaling pathway in the regulation of the expression of *mstn* genes in our cell culture model but do not exclude that other factors may mask the requirement of this intracellular pathway in the regulation of the studied genes. For example, new findings have provided direct evidence on the role of the transcription factors SMAD and CCAAT/enhancer-binding factor in mediating the induction of the expression of *mstn* gene in muscle wasting (1, 4). Whether some of these factors are affected in our cell culture model is worth investigating.

Another way to study the involvement of FoxO transcription factors in the regulation of *mstn1a* and *mstn1b* gene expression was to analyze the activation of the Akt-FoxO signaling pathway in cells over- or underexpressing the studied genes. GH has been previously shown to regulate the expression of *mstn* in the muscle of human (38) and rainbow trout (7, 16) as well as in the murine myoblast cell line C<sub>2</sub>C<sub>12</sub> (38). The mechanism underlying GH-mediated regulation of *mstn* expression is yet unknown. However, this effect could involve the Akt-FoxO signaling, as suggested by the induction of Akt phosphorylation in many cell systems stimulated by GH (22, 27, 54). Thus GH-stimulated cells may serve as a relevant model to study the involvement of FoxO1 in regulating the expression of the muscle antiproliferation factors *mstn1a* and *mstn1b*. Our in vitro results clearly showed that GH decreased the expression of both *mstn1a* and *mstn1b* in trout myotubes. These results

contrast with previous in vivo studies in trout showing that GH injection differentially regulates both *mstn* genes (7, 16), possibly reflecting inherent differences between cells in vivo and cells in culture. To gain insight on the involvement of Akt-FoxO signaling in the GH-mediated downregulation of both trout *mstn* genes in our in vitro model, we then monitored the activity of this signaling pathway in cells stimulated with GH. Our Western blot analysis clearly showed that GH stimulation induced a strong phosphorylation of Stat5, known to be the major target of the cytokine signaling pathway (JAK/Stat) (23). However, under the same conditions, we did not observe any phosphorylation of Akt, FoxO1, FoxO3, and FoxO4. Moreover, FoxO1 protein remained in the nucleus after GH stimulation. Altogether, these results showed that GH did not change FoxO1 activity although GH strongly downregulated *mstn1a* and *mstn1b* expression in trout myotubes. In other words, the GH-induced decrease of *mstn* expression cannot be explained by a drop of FoxO1 activity in trout myotubes.

In this study, we clearly show that IGF-I inhibits FoxO1 activity without any changes of *mstn1a* and *mstn1b* expressions and that GH stimulation strongly decreases *mstn* expressions without any changes in FoxO1 activity. Altogether, these results indicate that FoxO1 is not a key transcription factor in the regulation of the expression of both trout *mstn* genes in trout myotubes. In mammals, FoxO transcription factors are recognized to play a central role in the control of the expression of several genes coding for key mediators of skeletal muscle atrophy in response to atrophic and/or hypertrophic signaling (18). Therefore, the results presented here could be related to previous in vivo studies in trout showing no induction of the expression of either *mstn1a* or *mstn1b* gene during muscle atrophy induced by starvation (11, 28, 46). Furthermore, in tilapia (*Oreochromis mossambicus*), a decrease of *mstn* expression after food deprivation has even been observed (53), whereas, in zebrafish, *mstn* expression was shown to be independent of food restriction (41). In addition, during the reproduction period, trout undergoes muscle atrophy that is associated with a decrease of *mstn1b* expression (51). Altogether, these data show that the regulation of *mstn* gene in these fish species differs from that described in mammals and that the

involvement of FoxO transcription factors in this process may account for a part in this difference. Furthermore, they indicate that, although the antigrowth properties of MSTN seem to be conserved throughout evolution (19, 37), the regulation of the *mstn* gene has dramatically evolved and remains to be explored in lower vertebrates.

### Perspectives and Significance

The highly homologous sequences of MSTN protein's COOH-terminal active region among species ranging from zebrafish to humans suggested that the functions of MSTN were extremely conserved throughout evolution (17, 19, 37). However, there were higher differences for the promoter region among animals compared with the coding region (14, 17, 19), resulting in some interspecies differences in the response of *mstn* gene to environmental changes (7, 16, 20, 26, 51, 53, 63). The search for regulatory factors of *mstn* gene expression in different (model or livestock) species would therefore help the understanding of its function in the regulation of muscle mass. In this regard, rainbow trout is a very interesting model because of its two *mstn* genes differentially regulated in several environmental conditions. The present data indicate that FoxO1 activity is not a determining factor in the regulation of the expression of both *mstn1a* and *mstn1b* genes in primary culture of trout myotubes. Further studies are warranted to follow these specific genes as affected by nutritional and hormonal factors.

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### DISCLOSURES

No conflicts of interest are declared by the authors.

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