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Transgenic salmon overexpressing growth hormone exhibit decreased myostatin transcript and protein expression

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Summary

To characterize the role of myostatin (MSTN) in fast growing animals and to examine the relationship between MSTN and growth hormone (GH), MSTN transcript and protein expression were measured in coho salmon overexpressing GH and in wild-type coho salmon. Quantitative real-time RT-PCR and western analysis were used to measure RNA expression of the two salmonid *MSTN* genes (1 and 2) and levels of MSTN immunoreactive protein (MIP) in developing embryos and adult coho salmon tissues.

In transgenic and control coho embryos, *MSTN1* and *MSTN2* RNA expression were initially observed at about the time of eying, and a 42 kDa MIP was just detected prior to hatching. Expression of the *MSTN1* transcript in transgenic salmon was not different from that in wild-type adult coho salmon muscle and brain tissue. However, expression of the *MSTN2* transcript was less in white muscle, and greater in red muscle, from transgenic fish compared to wild-type salmon of the same size. Northern analysis revealed that expression of the *MSTN2* transcript was less in white muscle from wild-type, age-matched salmon than in transgenic fish. In addition, there was less presumed bioactive MIP in muscle taken from adult transgenic fish compared to controls and evidence of differential protein processing. Decreased *MSTN* expression in faster growing fish suggests that MSTN does act as a negative regulator of muscle growth in fish, as it does in mammals. The results of this study also suggest that the anabolic effects of GH could be mediated through MSTN.

Key words: myostatin, growth hormone, muscle, salmon, transgenic.

Introduction

Muscle development and growth are dynamic processes controlled by a number of factors including growth hormone (GH), insulin-like growth factors (IGFs), anabolic steroids, thyroid hormone, cytokines and cyclins. The general importance of GH in the regulation of growth has been illustrated by transgenic animals that overexpress GH, and in disorders that are a result of an underproduction (dwarfism) or over production (acromegaly) of GH. In mammals, the actions of GH on muscle growth are primarily mediated by IGF. There is also evidence that GH can affect skeletal muscle growth through other pathways (Florini et al., 1996; LeRoith et al., 2001). Regardless of the mechanism responsible for the anabolic actions of GH, the resulting effect is an increase in muscle mass. In general, an increase in muscle mass is the consequence of an increase in muscle fiber size (hypertrophy) and/or number (hyperplasia). Little is known concerning the specific role of GH in regulating hypertrophy and hyperplasia.

While GH has been extensively investigated for decades, myostatin (MSTN), a transforming growth factor β (TGF-β) family member and negative regulator of muscle growth, has only recently been identified (McPherron et al., 1997). Just as for other proteins in the TGF-β family, the MSTN precursor protein is synthesized and proteolytically cleaved, resulting in an N-terminal propeptide and a C-terminal, mature, biologically active peptide (Lee and McPherron, 2001; McPherron et al., 1997; Zimmers et al., 2002). Skeletal muscle mass in *MSTN* gene knockout mice is 2–3 times larger than in wild-type counterparts, and this increase is a result of hyperplasia and hypertrophy (McPherron et al., 1997). Naturally occurring mutations in the *MSTN* gene have been attributed to a ‘double muscle’ phenotype, observed in cattle (Grobet et al., 1997; Kambadur et al., 1997; McPherron and Lee, 1997). Without the ability to knock-out genes in fish, a clear biological action of MSTN has not been demonstrated in these vertebrates. However, recent research using transgenic

zebrafish overexpressing the MSTN prodomain suggests that MSTN does inhibit muscle growth in fish (Xu et al., 2003).

Even though GH and MSTN have been shown to have significant roles in the control of muscle growth, there has been limited research on the interaction of these hormones. Most research has focused on isolated human clinical studies, with varying results. GH administration to human subjects did not affect MSTN expression in male subjects suffering from hypogonadism (Hayes et al., 2001) or healthy older men (Brill et al., 2002). This is similar to the results observed in pigs (Ji et al., 1998) and GH-deficient rats (Kirk et al., 2000), where GH administration did not alter MSTN expression. In contrast, Liu et al. (2003) demonstrated that GH treatment suppresses MSTN expression in GH-deficient hypopituitary adult subjects and in skeletal muscle cell culture. Additionally, in a study of healthy elderly men, a significant negative relationship between skeletal muscle myostatin and GH receptor gene expression was observed (Marcell et al., 2001).

Given that the relationship between MSTN and GH has not been clearly defined, the present study used transgenic coho salmon overexpressing GH to better characterize the role of MSTN in muscle growth. The strain of salmon used in this study was produced using the all-salmon GH/metallothionen (MT) gene construct (OnMTGH1; Devlin et al., 1994). These fish grow at a significantly higher rate relative to controls, and attain a size more than 11-fold larger than the controls by 15 months (Devlin et al., 1994). Compared to the controls, the transgenic coho salmon have significantly higher numbers of small-diameter muscle fibers, thus suggesting that their larger size is a result of increased muscle hyperplasia (Hill et al., 2000). In the present study, levels of MSTN in muscle, brain and whole-body embryos from transgenic coho salmon were compared to those in wild-type controls to determine if they were significantly altered in fish overexpressing GH. In addition, this model provided a system in which the potential interaction of GH and MSTN in the regulation of muscle development could be examined.

Materials and methods

Animal and sample collection

Transgenic coho salmon (*Oncorhynchus kisutch* Walbaum 1972) were produced with the GH gene construct OnMTGH1, using a strain from the Chehalis River in southwestern British Columbia. Previous research has shown that these transgenic salmon grow at a significantly greater rate (Devlin et al., 1994). Transgenic and non-transgenic fish were reared according to Devlin et al. (1995). Briefly, fish were held in an indoor facility in 200 l tanks supplied with flow-through fresh wellwater (10±1°C) under a natural light cycle. Fish were fed with a standard commercial feed (Moore-Clarke Ltd., Vancouver, BC, Canada). At the time of sampling, fish were overanesthetized with MS222 and tissue samples were dissected and frozen at –80°C. Fish were selected for analysis at a time when the natural fast growth rate of transgenic animals allowed them to match the size of nontransgenic animals from the previous year's brood.

Total RNA was extracted from red muscle, white muscle and brain tissue from eight transgenic and eight size-matched fish using Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) as previously described (Chomczynski, 1993; Chomczynski and Sacchi, 1987). Transgenic (0.9 years) and wild-type (2 years) fish had mean masses of 59.1 and 57.0 g, respectively. In addition, total RNA was extracted from white muscle tissue from five age-matched fish (0.9 years old, mean mass 5.8 g) for northern analysis. Unfertilized eggs and three embryos were also taken from transgenic and control fish at nine different sampling ages throughout development and processed for total RNA. Protein was extracted from 20 red and 20 white muscle samples (10 fish/transgenic fish and size-control), unfertilized eggs and three embryos from transgenic and control fish, corresponding to each of the nine sampling ages above. Adult fish harvested for protein samples were not the same as those used for RNA analysis, but were the same age and size. Protein was extracted using a tissue lysis/extraction reagent (CelLytic, Sigma, St Louis, MO, USA) containing protease inhibitors (Protease Inhibitor Cocktail, Sigma) and was used at a ratio of 1:20 (1 g tissue/20 ml reagent). The protein quantity of each sample was determined by a Coomassie dye-based assay (Coomassie Plus, Pierce, Rockford, IL, USA).

Cloning MSTNs

Total RNA from a coho muscle pool was reverse transcribed using AMV reverse transcriptase (Promega, Madison, WI, USA). The resulting cDNA was used to amplify two *MSTN* genes using PCR [95°C 1 min, (95°C 1 min, 60°C 2 min, 72°C 1 min 30 s) × 40 cycles, 72°C 10 min]. Primers were designed to amplify the *MSTN* ortholog of rainbow trout *MSTN1* (F1, 1R1) and the *MSTN* ortholog of rainbow trout *MSTN2* (F1, 2R1) (Table 1). It should be noted that the *MSTN* genes, isolated from three different salmonid species, have been named using different nomenclature, as a result of three research groups working independently. In the present paper, we have used the same nomenclature as for rainbow trout *MSTN* (1 and 2; Rescan et al., 2001). Therefore, the two *MSTN* genes in coho salmon will be referred to as *MSTN1* and *MSTN2*. Polymerase chain reactions were separated on agarose

Table 1. *Primers used in PCR or quantitative RT–PCR*

Primers	Sequence (5′–3′)
F1	AGGTCCGACAGCAGATC
1R1	AGAGCTCTGCCGGTGAGG
2R1	AGAGTTCTGCTGGGGAAG
1F2	TGGCCACTGAACCCGAAT
1R2	AAAAGGTGCACCCATAACTGCGCAT
2F2	CGCGCCCAATATTAGCCGAG
2R2	TGAAGATGCACCCATAGCTGTGCCC
18s F	GCTTTGGTGACTCTAGATAAC
18s R	GTCGGGAGTGGGTAATTTGC

gels, visualized under UV light, and the appropriate size band cut, gel-purified and cloned in pCR 2.1-Topo (Invitrogen, Carlsbad, CA, USA). Positive clones were grown for plasmid preparation and DNA template was prepared in a Rev Prep Orbit (GeneMachines, Ann Arbor, MI, USA). The resulting cDNAs were sequenced using a modified dideoxy chain termination method using Big Dye Terminator (Applied Biosystems, Foster City, CA, USA). Sequencing reactions were precipitated and pellets resuspended in Hi-Di Formamide with EDTA (Applied Biosystems) and analyzed using the 3730 Sequencer (Applied Biosystems).

Quantitative real time reverse transcription–polymerase chain reaction

To examine the two forms of MSTN in coho salmon tissues and embryos, total RNA was analyzed quantitatively using real time RT-PCR (Brilliant SYBR Green QRT-PCR Master Mix Kit, 1-Step, Stratagene, La Jolla, CA, USA) in the Opticon Continuous Fluorescence Detection System (MJ Research, Waltham, MA, USA). Only transgenic and size-matched wild-type coho salmon were used for the quantitative RT-PCR analysis because the small size of age-matched wild-type salmon made it difficult to accurately obtain sufficient quantities of the red muscle and brain tissue required for precise quantitative analysis. However, white muscle RNA from age-matched fish was analyzed by northern blotting. Reverse transcription (RT) and polymerase chain reaction (PCR) were performed consecutively in the same reaction wells as follows: 30 min RT at 50°C, 10 min initial denaturation at 95°C, 40 cycles of 30 s denaturation at 95°C, 1 min annealing at 63–65°C, and 30 s extension at 72°C, with fluorescence measured at the end of every annealing and extension step. Primers were designed to amplify *MSTN1* (1F2 and 1R2), *MSTN2* (2F2 and 2R2), or 18s RNA (18s F and 18s R) (Table 1). Both *MSTN1* and *MSTN2* primer pairs were designed across an intron to ensure that contaminating DNA (if present) was not amplified. *MSTN1* and *MSTN2* primers were also designed to amplify regions of different lengths to ensure that melting curve temperatures were easily differentiated. Each reaction was performed in a separate well of a 96-well plate with a final volume of 25 μl. Immediately after each PCR, a melting curve analysis was performed to determine if the desired product was amplified by increasing the temperature from 55°C to 95°C at a rate of 0.2°C s^{-1}, and measuring fluorescence at every 0.5°C step.

Complementary DNA plasmid preparations of *MSTN1* and *MSTN2* were run to confirm that primers were specific and there was no cross reactivity between *MSTN1* and *MSTN2* primers. Each total RNA sample was run in duplicate with all three primer pairs. Serially diluted standard curves of *MSTN1* and *MSTN2* cDNA plasmid preparations were assayed so that data could be quantified. Additionally, a coho muscle RNA pool was assayed using 18S primers. Reactions with each primer pair were run on an agarose gel to verify size.

For all real-time assays, melting curves were analyzed to verify that no primer dimers were formed and that C_T values represented the desired amplicon. C_T values were then converted to relative MSTN abundance levels based on their respective standard curves and were normalized to the corresponding 18S RNA values.

Northern analysis

Northern blot analysis was used to examine RNA expression of *MSTN2* in muscle tissue. Total RNA (10 μg/lane) from red and white muscle tissue was separated on formaldehyde–agarose gels [1.6% agarose, 2.2 mol l^{-1} formaldehyde, 1× MOPS (3-(*n*morpholino)propanesulfonic acid)] and transferred to nylon membranes (Magna Charge Nylon Transfer Membranes, Micron Separations Inc., Westboro, MA, USA) by downward capillary elution using 20× SSC (3 mol l^{-1} NaCl, 0.3 mol l^{-1} sodium citrate, pH 7.2). Nylon membranes were pre-hybridized for at least 2 h in roller tubes at 42°C in a buffer containing 5× SSPE (0.75 mol l^{-1} NaCl, 0.05 mol l^{-1} sodium phosphate monobasic, 5 mmol l^{-1} EDTA, pH 7.4), 0.1% SDS, 5× Denhardt's solution, 50% formamide and 150 mg ml^{-1} calf thymus DNA. Northern blots were probed with radiolabeled ([α-^{32}P]dATP; 3000 Ci $mmol^{-1}$; ICN Biomedicals, Irvine, CA, USA) double-stranded partial MSTN2 (714 bp). The cDNA was labeled using Klenow (Prime-It II, Stratagene) and the denatured probe was added directly to the pre-hybridization buffer and incubated with the northern mixture at 42°C overnight. Following hybridization, the blots were washed twice (15 min each) under medium stringency (1× SSPE, 0.1% SDS, 45°C) and twice (15 min each) under high stringency (0.1% SSPE, 0.5% SDS, 65°C). Northern blots were dried, exposed to phosphorimaging screens, and visualized using a Storm 840 phosphorimager (Molecular Dynamics, Amersham, Piscataway, NJ, USA).

Protein quantification

The levels of MSTN in coho salmon muscle and eggs/embryos were determined by western analysis using a primary antibody produced against brook trout recombinant MSTN. This antibody has been characterized previously with brook trout muscle and embryos and recognizes the precursor and C-terminal mature peptide (Roberts and Goetz, 2003). For western analysis, 6 μg of muscle or whole egg/embryo protein extract were electrophoresed on NuPage 4–12% Bis-Tris gels (Invitrogen). Gels were transferred to nitrocellulose membranes, blocked and incubated with the diluted (1:1000) primary MSTN antibody. After rinsing in 1× TBS-T, the membranes were incubated with a horseradish peroxidase-labeled secondary antibody. For detection, the ChemiGlow detection kit (Alpha Innotech, San Leandro, CA, USA) was used in conjunction with a SuperChemiNova 12-bit CCD camera (ChemiImager, Alpha Innotech). A 16 bit per pixel image was captured with a 1 min exposure time and detection level linearity was verified by image saturation analysis software (ChemiImager 5500 v3.1, Alpha Innotech). Integrated density values were calculated, the background

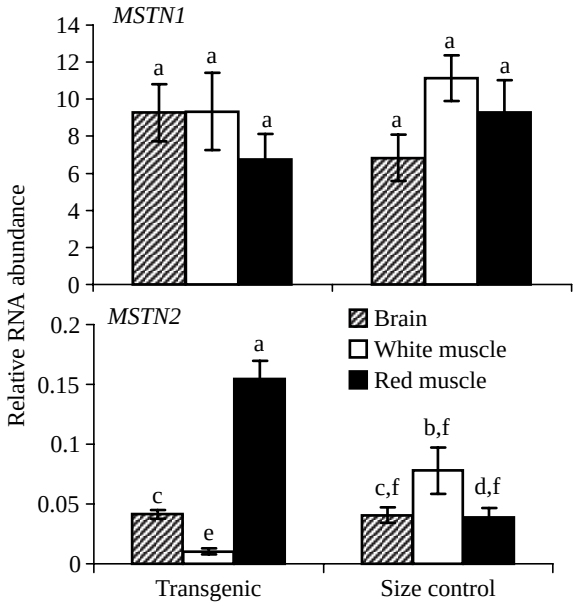

Fig. 1. Relative RNA abundance of *MSTN1* and *MSTN2* in brain, white muscle and red muscle. Values are means ± S.E.M. (*N*=8) for each fish group (transgenic, size control). For each graph, bars that do not share identical lowercase letters are significantly different when compared within fish groups (transgenic, size control) or tissue type (brain, white muscle, red muscle) at $P \leqslant 0.05$.

subtracted, and all data normalized with a pooled muscle sample run in duplicate on each gel.

Statistical analysis

All data are given in terms of relative abundance levels and expressed as means ± standard errors (S.E.M.). One-way analyses of variance (ANOVAs) were performed for adult tissue and two-way ANOVAs for developing coho embryo samples. For statistical analysis, RNA samples from developing coho embryos in which *MSTN1* or *MSTN2* was undetected were given a value of 5% less than the lowest relative abundance level within each respective *MSTN* form, rather than a zero value. When there were significant differences within factors (i.e. tissue analysis: fish group, tissue type; developing embryo analysis: treatment, time) a *post hoc* comparison test (Holm–Sidak) was performed. All significance levels were set at $P \leqslant 0.05$.

Results

Genes

Two *MSTN* cDNAs were isolated from coho salmon that correspond to the two *MSTN* genes found in other salmonids. Using a similar nomenclature as for rainbow trout, these two cDNAs are referred to as *MSTN1* (GenBank accession number AY434465) and *MSTN2* (GenBank accession number AF394687). Each sequence is 981 bp and spans 87% of the open reading frame. *MSTN1* and *MSTN2* are 93% identical to one another. When the nucleotide sequences are compared with *MSTN*s from other salmonids, *MSTN1* is most similar to rainbow trout *1* (GenBank accession number AF273035), Atlantic salmon *II* (GenBank accession number AJ344158), and brook trout *ov MSTN* (GenBank accession number AF313912). *MSTN2* is most similar to rainbow trout *2* (GenBank accession number AF273036), Atlantic salmon *I* (GenBank accession number AJ297267), and brook trout *b/m MSTN* (GenBank accession number AF247650).

RNA analysis

Total RNA levels of both *MSTN1* and *MSTN2* were measured using quantitative real-time RT–PCR in white muscle, red muscle and brain from transgenic and size-matched control coho. Among samples tested for *MSTN1* expression, there was no significant treatment or tissue effect (Fig. 1). There were however, differences observed in the expression of *MSTN2* (Fig. 1). Specifically, expression of *MSTN2* in white muscle was significantly higher in size-control fish than in transgenic fish. The opposite was seen in red muscle, where *MSTN2* expression was significantly higher in transgenic fish than in size-control fish. The same results were observed with northern analysis (Fig. 2) and with conventional RT-PCR analysis (data not shown). Northern analysis also showed that *MSTN2* expression was lower in age-control fish than in size-control and transgenic fish (Fig. 2). In the brain, expression levels between control and transgenic fish were not significantly different. In transgenic fish, expression of *MSTN2* was significantly higher in red muscle than brain and white muscle, and expression in both red muscle and brain was significantly higher than in white muscle.

In transgenic and control coho salmon embryos, expression of both *MSTN* forms (1 and 2) was measured quantitatively from total RNA throughout development (Fig. 3). In general, there was no significant difference in *MSTN* expression

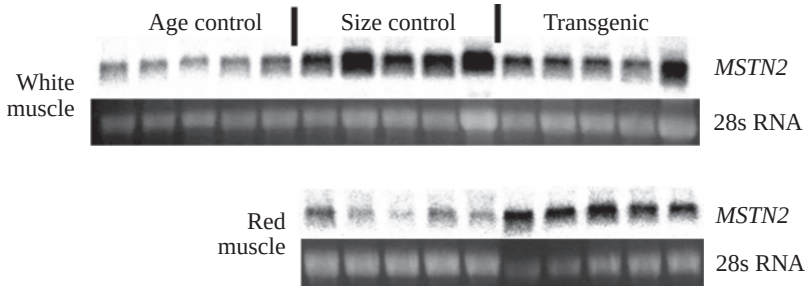

Fig. 2. Northern blot of total RNA (10 μg/lane) from white and red muscle taken from five transgenic and five size-matched coho salmon probed with *MSTN2*. Corresponding 28s RNA expression is shown for each lane.

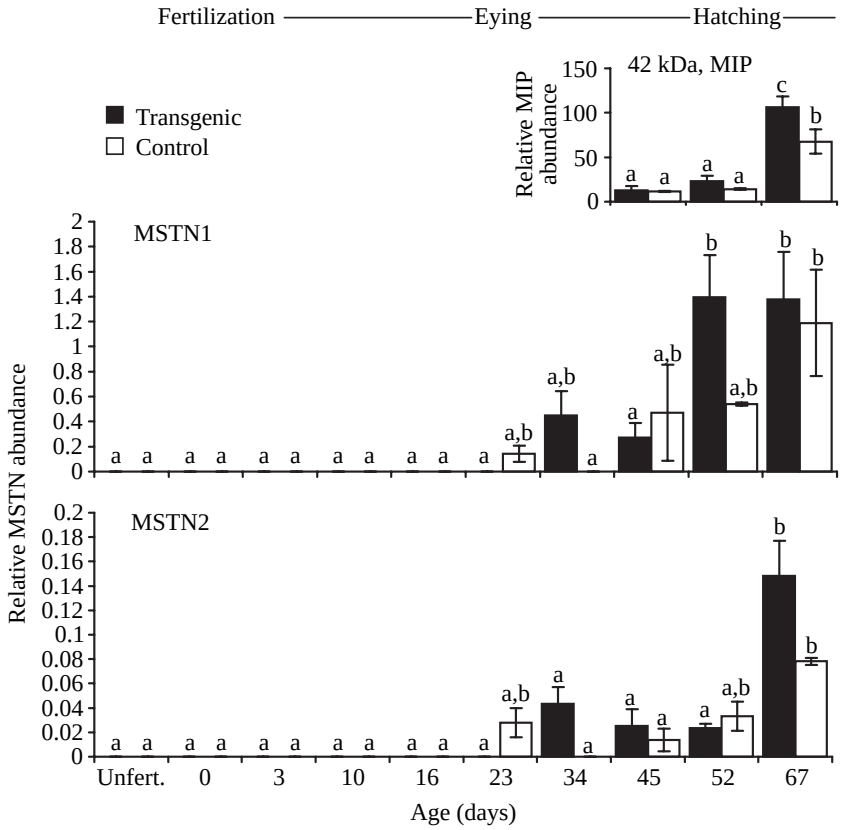

Fig. 3. Relative abundance of MSTN1 and MSTN2 RNAs and MSTN immunoreactive protein (MIP; inset) in transgenic and control coho embryos taken prior to fertilization through hatching. Values are means ± S.E.M. (*N*=3). For RNA data, bars that do not share identical lowercase letters are significantly different when compared within fish group (transgenic, size-matched.) For protein data (inset), bars that do not share identical lowercase letters are significantly different when compared within fish group or within age at $P \leqslant 0.05$. The 42 kDa MIP was only detected at these three sampling ages shown, and the 14 kDa MIP was not detected in any embryo samples examined.

between embryos from control and transgenic fish for either *MSTN1* or *MSTN2*. Therefore, only RNA levels within fish groups were further compared. *MSTN1* was first detected in embryos from control and transgenic fish on day 23 and day 34, respectively. The highest mean values (not significant) of *MSTN1* RNA in transgenic samples were observed at day 52 whereas the highest mean levels of *MSTN1* in control samples were observed at day 67. Just as with *MSTN1*, expression of *MSTN2* was not detected in embryos from control and transgenic fish until eying at day 23 and day 34, respectively. The highest levels of *MSTN2* were not observed until day 67 for both transgenic and control samples.

Protein quantification

Protein samples from red and white muscle obtained from transgenic and size-matched control coho salmon, were analyzed by western analysis using an antibody generated against recombinant brook trout MSTN (tMSTNAb) (Roberts and Goetz, 2003). In both muscle types, 42 kDa and 14 kDa protein bands were detected. Expression of the 42 kDa MSTN immunoreactive protein (MIP) was significantly higher than the 14 kDa MIP in all tissues (Fig. 4). Levels of the 42 kDa MIP were significantly higher in white muscle tissue in transgenic compared to control samples. Expression of the 14 kDa MIP was significantly higher in red muscle tissue than in white muscle tissue for both transgenic and control samples (Fig. 4). Levels of the 14 kDa MIP were significantly lower in transgenic fish than in size-controls for both red and white muscle tissue.

In developing coho salmon embryos, a 42 kDa MIP was first evident 45 days after fertilization, just after eying (Fig. 3). Expression levels increased through hatching. In contrast, there was no expression of the 14 kDa MIP throughout the entire embryo sampling period.

Discussion

The results of the present study clearly indicate a difference in the expression of both MSTN transcripts and protein in transgenic coho salmon overexpressing GH. To quantify the transcript levels of *MSTN* in this experiment using real-time PCR, it was first necessary to characterize *MSTN* cDNA from coho salmon. Isolation of two *MSTN* cDNAs from coho salmon was anticipated as two *MSTN* genes have been found in all other salmonids examined (Ostbye et al., 2001; Rescan et al., 2001; Roberts and Goetz, 2001). Quantitative RT–PCR analysis indicated that *MSTN1* RNA expression was similar in transgenic and control coho salmon and was not different across tissues. The lack of differential expression between red and white muscle tissue has also been observed in the orthologs of *MSTN1* in rainbow trout (Tmyostatin 1; Rescan et al., 2001) and brook trout (ov MSTN; Roberts and Goetz, 2001). Transcripts of salmonid *MSTN* homologous to *MSTN1* have been found in multiple tissues, suggesting constitutive expression (Ostbye et al., 2001; Rescan et al., 2001; Roberts and Goetz, 2001). On the other hand, RNA expression of the *MSTN2* orthologs in rainbow trout and brook trout are limited to brain and muscle tissue (Rescan et al., 2001; Roberts and Goetz, 2001). In addition, these transcripts are differentially expressed in muscle according to muscle type, sex, age and reproductive status (Rescan et al., 2001; Roberts and Goetz, 2003). In the present study, levels of MSTN2 were significantly different in transgenic coho salmon compared to controls in both red and white muscle. The combined data on coho salmon and trout suggest that, in salmonids, MSTN2 is the relevant transcript in muscle and probably functions to regulate growth.

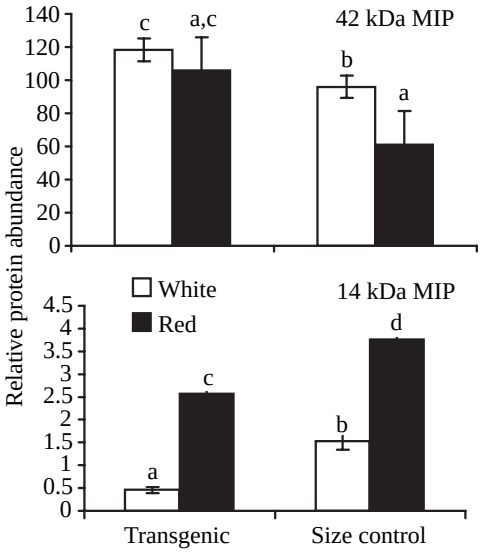

Fig. 4. Relative protein abundance of MSTN immunoreactive protein (MIP) in white and red muscle from transgenic and size-control coho salmon. Abundance of the 42 kDa MIP (top) and the 14 kDa MIP (bottom). Values are means ± S.E.M. (N=8) at each sampling time. For each graph, bars that do not share identical lowercase letters are significantly different when compared separately within fish group (transgenic, size control) or tissue type (white muscle, red muscle) at $P \leqslant 0.05$.

MSTN is synthesized as a precursor protein that is proteolytically cleaved to produce an N-terminal propeptide and a smaller C-terminal peptide, which is the mature biologically active form. In the present study, the two immunoreactive bands of 42 and 14 kDa recognized in coho muscle by western analysis presumably represent the precursor MSTN protein and the C-terminal, bioactive domain, respectively (Roberts and Goetz, 2003). This conclusion is based on the predicted molecular masses and results reported for other species using different antibodies (Rodgers et al., 2001; Vianello et al., 2003; Zimmers et al., 2002), and is consistent with our previous studies on brook trout (Roberts and Goetz, 2003). There was less mature MSTN protein (14 kDa MIP) in muscle taken from transgenic fish compared to controls. In addition, expression of *MSTN2* RNA was significantly less in white muscle from transgenic fish compared to size controls, and in northern blots, expression of *MSTN2* was lowest in age control fish. These data support the hypothesis that MSTN acts as an inhibitor of muscle growth in teleosts, since fish with the slowest growth rates (size controls) had the highest expression levels of *MSTN2* RNA and bioactive protein (14 kDa MIP). The faster growth rates in the transgenic fish used in this study have been attributed to hyperplasia (Hill et al., 2000). Recently, Xu et al. (2003) reported that zebrafish overexpressing the MSTN prodomain exhibited an increased number of muscle fibers. The MSTN prodomain is able to bind to the mature MSTN domain, presumably inhibiting MSTN from binding to a receptor and blocking its function (Hill et al., 2002). Therefore, the decreased expression of *MSTN* in coho salmon with increased muscle hyperplasia suggests that MSTN2 is an inhibitor of muscle hyperplasia in teleosts.

The results of the present study also suggest that MSTN could be a mechanism by which the growth-regulating effects of GH are realized, at least in part. That is, transgenic salmon that have elevated GH levels also had decreased levels of MSTN. This is consistent with the finding of Liu et al. (2003), who found that *in vitro* and *in vivo* treatment with GH decreased *MSTN* mRNA expression. Growth hormone response elements and GH cell-specific elements have been identified in the promoter region of the *MSTN* gene (Taylor et al., 2001; Roberts and Goetz, 2003). GH signal transduction pathways could therefore directly inhibit *MSTN* transcription, increasing growth. In addition to transcriptional regulation, GH could also regulate MSTN at the translational level. In the present study, western analysis of the unprocessed and processed MIPs did indicate that differential protein processing of MSTN occurred. At the time of sampling, there were higher levels of precursor MSTN (42 kDa) in transgenic white muscle tissue and lower levels of processed MSTN (14 kDa) compared to controls. One explanation for this is that in the faster growing fish, less MSTN is being proteolytically cleaved to the mature 14 kDa protein. Regulation of MSTN proteolysis is not well characterized. Researchers working with the C2C12 cell line of mouse myoblasts have shown that MSTN processing could be influenced by hydroxamate-based inhibitors of metalloproteinases (HIMPs; Huet et al., 2001). Wolfman et al. (2003) have shown that members of the bone morphogenetic protein-1/tolloid (BMP-1/TLD) family of metalloproteinases may be involved in activating myostatin *in vivo*. Ultimately, myostatin activity is likely to be controlled by several processes including transcription, translation, binding proteins and receptor binding.

In transgenic and control coho embryos, *MSTN1* and *MSTN2* RNA expression was initially observed at about the time of eying, and the 42 kDa MIP was just detected prior to hatching. These results are similar to what has been observed previously in wild-type brook trout embryos (Roberts and Goetz, 2003). In teleost species with only one form of MSTN, RNA expression has been observed from stages just after fertilization throughout development (Kocabas et al., 2002; Rodgers et al., 2001; Vianello et al., 2003; Xu et al., 2003). Interestingly, in the present study *MSTN1* RNA expression increased above baseline values in transgenic coho salmon embryos earlier than in controls (Fig. 3). While the biological role of MSTN1 is unknown, this suggests that MSTN1 might be involved in distinct developmental processes, as certain events might be initiated earlier in fast-growing transgenic fish. The absence of the mature 14 kDa MIP in newly hatched coho embryos is identical to the result obtained in brook trout (Roberts and Goetz, 2003), and suggests either a rapid turnover of the active peptide or the build-up of the precursor without proteolytic processing. In general, the expression of MIP just after hatching could be related to changes that occur in the embryo as it starts to use somatic musculature for conventional locomotion

While the present study suggests roles for MSTN and GH in muscle development and growth in fish, there are still some aspects of *MSTN* expression that are not fully understood. One of the most complex aspects of *MSTN* expression is its differential expression across muscle tissue type. *MSTN2* ortholog RNA levels are higher in red muscle than white muscle in brook trout and rainbow trout (Rescan et al., 2001; Roberts and Goetz, 2001). In the present study, this was also the case in transgenic coho salmon. However, in size-control coho salmon, levels of *MSTN2* were not different between muscle types. *MSTN2* ortholog expression levels were also the same in white and red muscle for Atlantic salmon (Ostbye et al., 2001). One explanation for the variation in *MSTN2* ortholog expression in fish could be that expression is associated with changes that occur in developing muscle tissue. Salmonids undergo a distinct transformation in behavior and physical characteristics between the first juvenile stage (parr) and older juveniles (known as smolts in anadromous salmonids; Hoar, 1988). During this transformation, significant changes occur in red muscle kinetics, including activation time, relaxation time and maximum shortening velocity (Coughlin et al., 2001). In addition, there are biochemical changes occurring in red muscle that are apparent from shifts in myosin heavy chain expression (Weaver et al., 2001). In nontransgenic coho and Atlantic salmon, in which the levels of *MSTN2* ortholog were similar between muscle fiber types, the fish were generally smaller and younger than the brook and rainbow trout studied. Thus, it is possible that the differential expression is related to the age of the fish from which samples were obtained. Wild-type coho and Atlantic salmon might not have completed this transformation, whereas the other salmonids that were studied had. With coho salmon, Hill et al. (2000) observed that the effects of GH transgene expression on muscle anatomy were more pronounced in red muscle. Thus, it is possible that the changes associated with development (mentioned above) had already occurred in transgenic fish and that is why the *MSTN2* expression pattern in transgenic coho salmon muscle type is more like that observed in the older rainbow and brook trout. In the present study, western analysis indicated higher expression of the bioactive 14 kDa MIP in red muscle compared to white muscle, regardless of GH transgene expression. Higher levels of MSTN immunoreactivity in red muscle have also been observed in other fish (Radaelli et al., 2003). However, the difference in muscle expression may not be related to the same factors affecting RNA expression. Instead, decreased levels of the bioactive MSTN protein in white muscle compared to red muscle could be associated with the continual fiber recruitment and hypertrophy that occur in adult salmonids. Therefore, lower levels of the active protein would be expected to occur if MSTN is a negative regulator of muscle growth.

The present study used GH transgenic salmon as an experimental system to characterize the role of MSTN in fast growing animals and to examine the relationship of MSTN and GH expression. Decreased *MSTN* expression in faster growing fish suggests that MSTN does act as a negative regulator of muscle growth in fish, as it does in mammals. Specifically, *MSTN2* RNA expression appears to be involved in muscle hyperplasia, as this has been shown to be a primary mechanism of increased growth in GH transgenic coho salmon. The results of this study also provide evidence that the anabolic effects of GH could be mediated through MSTN. Further research is needed to characterize the significance of the GH–MSTN signaling pathway in controlling vertebrate muscle growth.

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