Myostatin expression during development and chronic stress in zebrafish (*Danio rerio*)

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Abstract

Myostatin, a member of the transforming growth factor- β superfamily, is a negative regulator of skeletal muscle mass in mammals. We have studied myostatin expression during embryonic and post-hatching development in zebrafish by semiquantitative RT-PCR. The transcript is present in just-fertilized eggs and declines at 8 h post-fertilization (hpf), suggesting a maternal origin. A secondary rise occurs at 16 hpf, indicating the onset of embryonic transcription at the time of muscle cell differentiation. The level of myostatin mRNA decreases slightly at 24 hpf, when somitogenesis is almost concluded, and rises again at and after hatching, during the period of limited muscle hyperplastic growth that is typical of slow-growing, small fish. In the adult muscle, we found the highest expression of myostatin mRNA and protein, which were detectable by Northern and Western blot analyses respectively.

Although only the precursor protein form was revealed in the adult lateral muscle, we demonstrated that zebrafish myostatin is proteolytically processed and secreted in cultured cells, as is its mammalian counterpart. These results suggest that myostatin may play an important regulatory role during myogenesis and muscle growth in fish, as it does in mammals.

In chronically stressed fish, grown from 16 days post-fertilization to adulthood in an overcrowded environment, we observed both depression of body growth and a diminished level of myostatin mRNA in the adult muscle, as compared with controls. We propose that chronic stunting in fish brings about a general depression of muscle protein synthesis which does not spare myostatin.

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Introduction

Myostatin (GDF-8), a member of the transforming growth factor (TGF)- β superfamily, is a negative regulator of skeletal muscle mass. Its function was first suggested by McPherron *et al.* (1997), who generated myostatin knock-out mice that were characterized by a widespread increase in skeletal muscle mass, with a two to three times increase in individual muscle weight, due to a combination of muscle cell hyperplasia and hypertrophy. Similarly, mutations of the myostatin gene in some breeds of 'double muscled' cattle, that presumably lead to inactive proteins, were also associated with muscle growth (Grobet *et al.* 1997, 1998, Kambadur *et al.* 1997, McPherron & Lee 1997, Cappucio *et al.* 1998). Moreover, a hypermuscular mouse mutant (*Cmpt*) (Szabó *et al.* 1998) shows a mutation in the putative propeptide region of the myostatin gene, which possibly interferes with the proper protein folding, dimerization and secretion (Gray & Mason 1990).

These data suggest that myostatin performs a key role in controlling muscle mass during development. In fact, in mouse embryos, myostatin is expressed very early during

development and its expression appears to be restricted to the myotome compartment of developing somites (McPherron & Lee 1997). Studies in cattle (Kambadur *et al.* 1997, Bass *et al.* 1999), pigs (Ji *et al.* 1998) and chickens (Kocamis *et al.* 1999) have reported a similar myostatin expression pattern that begins early during development and continues throughout embryonic life. Myostatin mRNA abundance coincides roughly with the period of primary and secondary fibre formation, showing a reduction at hatching/birth and postnatally that may reflect the reduction in myogenic and myogenic events.

In adult animals, skeletal muscle myostatin expression has been reported in several vertebrate species (Grobet *et al.* 1997, Kambadur *et al.* 1997, McPherron & Lee 1997, Gonzalez-Cadavid *et al.* 1998, Ji *et al.* 1998, Sharma *et al.* 1999, Østbye *et al.* 2001, Rescan *et al.* 2001, Roberts & Goetz 2001, Rodgers *et al.* 2001). Although myostatin is predominantly expressed in the skeletal muscle, some reports have shown its occurrence in the Purkinje fibres and cardiomyocytes of the sheep heart (Sharma *et al.* 1999), in porcine lactating mammary gland (Ji *et al.* 1998) and, more recently, in several tissues of tilapia (Rodgers

et al. 2001), Atlantic salmon (Østbye *et al.* 2001), rainbow trout (Rescan *et al.* 2001) and in the brain and ovary of brook trout (Roberts & Goetz 2001).

The role of myostatin in the adult, differentiated muscle has not yet been fully clarified. Several reports have shown that muscle atrophy, induced by muscle unloading (Carlson *et al.* 1999, Wehling *et al.* 2000), exposure to a microgravity environment (Lalani *et al.* 2000) or associated with a pathological condition (Gonzalez-Cadavid *et al.* 1998), is accompanied by increments in muscle myostatin mRNA expression, muscle myostatin content and serum myostatin-related protein concentration (Gonzalez-Cadavid *et al.* 1998). The myostatin level returns to normal values after reloading (Wehling *et al.* 2000).

On the other hand, myostatin expression declines during the first phase of muscle regeneration, characterized by a proliferation of the satellite cells, and goes up again during the differentiation of newly formed fibres (Kirk *et al.* 2000, Mendler *et al.* 2000). Moreover, myostatin expression was significantly reduced in two strains of mice that undergo muscle degeneration coupled with extensive regeneration (models of muscle dystrophy) (Zhu *et al.* 2000). *In vitro* proliferating myoblasts produced low amounts of myostatin mRNA, which increased only upon the induction of differentiation (Mendler *et al.* 2000, Ma *et al.* 2001, Artaza *et al.* 2002). Taken together, these results suggest a role, or at least an involvement, of myostatin in adult muscle mass changes.

Although the role of myostatin has been better studied in mammalian species, the isolation of myostatin cDNAs in different fish species (McPherron & Lee 1997, Østbye *et al.* 2001, Rescan *et al.* 2001, Roberts & Goetz 2001, Rodgers & Weber 2001, Rodgers *et al.* 2001) has shown a high level of conservation, even related to the mammalian and avian orthologues, especially in the bio-active domain, suggesting an equally well conserved biological function. In order to understand the role that myostatin may play in fish muscle development and growth, we have investigated the myostatin mRNA expression in zebrafish during embryonic and larval development and in the muscles of fish grown in an overcrowded environment.

Materials and Methods

Animals and sample collection

All procedures were performed in accordance with the Guidelines on the Handling and Training of Laboratory Animals by the Universities Federation for Animal Welfare (UFAW) and with the Italian animal welfare legislation (D.L. 116/92).

Zebrafish were maintained and bred in aquaria according to the Zebrafish Book (Westerfield 1995). In the case of fish grown under overcrowded conditions, three lots of 40 eggs each were initially maintained in 500 ml water. At

16 days post-fertilization (dpf), each lot was subdivided into two groups of 20 fish. The OC (overcrowded culture conditions) subgroups were grown in 500 ml water until 90 dpf, while the N (normal culture conditions) subgroups were raised in 3 l water until 50 dpf, then in 20 l water until 90 dpf. All subgroups had food available *ad libitum*.

Just-fertilized eggs, embryos at different stage of development (following the description of Kimmel *et al.* 1995), larvae (from hatching till 20 dpf) and adult muscle samples were immediately frozen in liquid nitrogen after collection, and stored at -80°C until use. Skeletal muscle tissues were collected from fish killed by decapitation after anaesthesia in 20% tricaine. For total mRNA extraction, samples (100 eggs or 100 mg skeletal muscle pooled from two or more animals) were powdered in liquid nitrogen using pestle and mortar and the RNA was isolated with the RNazol B method (Genenco, Florence, Italy). For whole mount *in situ* hybridization, embryos were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C , and stored in methanol at -20°C until use. For Western blot analysis, tissue samples were immediately used for protein isolation.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis and Southern blotting

To study the myostatin expression during zebrafish development, a total of between 0.25 and 2 μg total RNA isolated from embryos (0, 8, 16, 24, 48 and 72 h post fertilization (hpf)), 16-dpf larvae (fry) and adult lateral skeletal muscle were subjected to cDNA synthesis and amplification using the SuperScript One-Step RT-PCR system (Life Technologies Italia s.r.l., Milan, Italy) and two primers (forward primer: 161-TGC ATG CCA TCA AGT CCC AAA TTC-184; reverse primer: 681-GTT AAT CTC GAT GCC TCG GTT GGT-658) based on the zebrafish myostatin cDNA sequence (accession number: AF019626, McPherron & Lee 1997). The primers were designed to span the first putative intron in order to reveal any contaminating genomic DNA as a larger sized PCR fragment. The intron position was extrapolated from that of human (Gonzalez-Cadavid *et al.* 1998) and porcine (accession number: AJ237662) myostatins.

After optimising the RT-PCR conditions and adjusting the number of cycles to be in the linear range of amplification for each sample, we performed semi-quantitative RT-PCR analysis in 25- μl reaction mixture containing 0.5 μg total mRNA, 5 pmol of each primer, 1 $\times$ reaction mix, and 0.5 μl RT-Platinum Taq mix, under the following conditions: 1 cycle at 54°C for 30 min and 95°C for 2 min (reverse transcription); 35 cycles at 95°C for 30 s, 61°C for 30 s, and 72°C for 45 s, and 1 cycle at 72°C for 10 min (amplification). Blanks were carried out in the absence of template RNA.

In order to check the quality and quantity of the RNA utilized for the semi-quantitative analysis, the total RNA

dilutions used for each myostatin amplification were run on agarose gel. The quantification of template 28S ribosomal RNA and of the amplified products was carried out by measuring the relative intensity of the bands stained by ethidium bromide using the Quantity One quantitation software (Bio-Rad Laboratories s.r.l., Milan, Italy).

The identity of the amplified 521-bp fragment obtained from 72-hpf embryos, 16-dpf larvae and adult muscle was confirmed by sequencing with the ABI PRISM Dye Terminator Cycle Sequencing Core Kit (Applied Biosystems, Monza, Italy). Electrophoresis of sequencing reactions was completed in an ABI PRISM automated sequencer, model 377, version 2.1.1. For all the developmental stages examined, the identity of amplified products was confirmed by Southern blot analysis. An aliquot (1/10) of all RT-PCR products was electrophoresed in 1.0% agarose gel, transferred onto a charged nylon membrane (Hybond N+; Amersham Pharmacia Biotech Italia, Milan Italy), baked at 80 °C for 2 h, pre-hybridized at 42 °C for 2 h in a buffer containing 5 × SSC (standard saline citrate), 50% formamide, 0.1% N-lauroylsarcosine, 0.02% sodium dodecyl sulphate (SDS), 2% polyvinylpyrrolidone, and then hybridized overnight at 42 °C in fresh hybridization buffer. This contained a digoxigenin-labelled cDNA myostatin probe synthesized by PCR (Roche Diagnostics, Milan, Italy) utilizing the same primers used for the RT-PCR reactions and a plasmid containing the complete zebrafish myostatin cDNA as a template (kindly provided by Dr S Lee, Johns Hopkins University School of Medicine, Baltimore, MD, USA; EMBL: AF019626). After hybridization, the membrane was washed and detection was performed as described in the DIG System User's Guide for Filter Hybridization provided by Roche, using the NBT/BCIP colorimetric detection system.

Rapid amplification of cDNA ends (RACE)

5'-RACE was carried out using the 5'-RACE System (GibcoBRL, Milan, Italy) following the manufacturer's instructions. Briefly, cDNA was synthesized by incubating, at 50 °C for 1 h, 2 µg total RNA from adult zebrafish muscle or 48-hpf embryos in 25 µl of the required reaction mix, containing 4 pmol of the first specific primer for zebrafish myostatin (212-GGA GCC TGC TTG AGT CGG AG-193; AF019626). Terminal transferase was then used to add a homopolymeric C-tail to the 3'-end of the first-strand cDNA purified with a GlassMAX DNA Isolation Spin Cartridge Purification kit (GibcoBRL). The tailed cDNA was amplified by PCR, using a second (nested) specific primer (179-TGG GAC TTG ATG GCA TGC AG-160) and the oligo dG-abridged anchor primer (5'-GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG-3'). The primary PCR product was then nested-amplified using a third specific primer (122-GAA CAC AGC TCG CTT TCC-105) and the

abridged universal amplification primer (5'-GGC CAC GCG TCG ACT AGT AC-3'). The resultant amplified product was purified from the sliced gel band and directly sequenced.

Northern blot analysis

To study the myostatin expression in the adult skeletal muscle and possible differences in the myostatin expression level between zebrafish grown under normal or overcrowded conditions, we performed Northern blot analysis on total RNA extracted from lateral skeletal muscle. Because of the small size of the muscle in OC fish, Northern analysis was performed on pooled tissues from animals belonging to each of the three different OC or N subgroups, and repeated at least three times for each subgroup.

Northern analysis was carried out according to Sambrook *et al.* (1989). Fifteen or twenty micrograms total mRNA were run on 1% formaldehyde-agarose gel and transferred to a Hybond N+ membrane (Amersham). The membrane was prehybridized in 5 × SSC, 50% formamide, 0.1% N-lauroyl-sarcosine, 0.02% SDS, 2% Blocking Reagent (Roche) and hybridized overnight at 68 °C in the same solution containing 60 ng/ml digoxigenin-labelled myostatin riboprobes, prepared with the DIG RNA labelling kit (Roche) using as a template Dr Lee's plasmid with the complete zebrafish myostatin cDNA. The complete plasmid sequencing showed the presence of the 5'- and 3'-untranslated terminal regions (UTRs) that were not included in the deposited sequence (AF019626).

A mixture of two antisense riboprobes was used, one covering a 846-base fragment of the zebrafish myostatin cDNA clone corresponding to 67 bases of the 5'-UTR before the ATG and the pro-region portion (bases 1–779) of the coding region and the second one covering a 353-base fragment of the zebrafish myostatin 3'-UTR corresponding to bases 1171–1523. Probes corresponding to the C-terminal region of myostatin were excluded in order to avoid any possible crossreactivity with other members of the TGF-β superfamily. Membranes were washed in 2 × SSC and 0.1% SDS twice for 15 min at room temperature, and in 0.2 × SSC and 0.1% SDS twice for 15 min at 65 °C. They were then incubated with the anti-DIG-AP (Roche) and the signal detected using the CPD-Star DIG Luminescent Detection Kit (Roche) according to the manufacturer's protocol. Bands were visualized by autoradiography. Uniformity of loading and RNA integrity were verified by methylene blue staining of total RNA transferred on the membrane before hybridization.

In situ hybridization on whole-mount embryos

Whole-mount *in situ* hybridization was performed in fixed zebrafish embryos at 11, 12, 14, 18, 24 and 48 hpf,

according to Thisse *et al.* (1993), using the following riboprobes labelled with digoxigenin (Roche): an 846-base zebrafish myostatin antisense probe, identical to the longer probe used for Northern blot analysis; a mixture of two 353- and 415-base zebrafish myostatin antisense probes, the first one identical to the other probe used for Northern blot analysis, the second one corresponding to a part of the pro-region (bases 365–779) in the coding region. The corresponding sense riboprobes were used as negative controls. In some cases, a double-colour hybridization was performed by adding a zebrafish MyoD cDNA (kindly provided by Dr Francesco Argenton, University of Padova, Italy) antisense riboprobe labelled with fluorescein (Roche), as a marker of muscle cell lineage.

Myostatin expression in cultured cells

The cDNA coding for zebrafish myostatin was cloned into the CS2⁺ expression vector (Turner & Weintraub 1994), under the control of the simian CMVIE94 enhancer/promoter. 293T cells (kindly provided by Dr Stefano Piccolo, University of Padova, Italy), seeded at 30% confluence, were cultured for 24 h in DMEM (Sigma-Aldrich s.r.l., Milan, Italy) supplemented with 10% fetal calf serum (FCS), 0.6 g/l L-glutamine, 0.1 U/ml penicillin and 0.1 U/ml streptomycin, and transiently transfected by calcium phosphate precipitation, according to Gilman (1988), using 10, 20 or 40 µg of the plasmid CS2⁺-myostatin and 0.5 µg of the plasmid RSV-βGal. The medium was changed 21 h later with fresh medium without FCS.

Forty-eight hours after transfection, the conditioned medium with added 10 µM phenylmethylsulphonyl-fluoride (PMSF) was concentrated by centrifugation in a Centriplus YM 3 spin column (Millipore Corporation, Bedford, MA, USA). Cells were scraped from the dish in a lysis buffer (100 mM tricine, pH 7.8, 2 mM EDTA, 1% Triton X-100, 15% glycerol, and 1 mM dithiothreitol (DTT)) and centrifuged at 12 000 g for 5 min. β-D-galactosidase activity was determined in the supernatant, as described by Sambrook *et al.* (1989), to check for transfection efficiency. For successive analyses of cells transfected with different plasmid amounts, the same quantities of concentrated conditioned medium or lysed cell supernatant were used, after dilution to correct for transfection efficiency.

Protein isolation

Adult zebrafish muscle and liver (used as a negative control) were homogenized in a buffer containing 40 mM K₂HPO₄, 10 mM KH₂PO₄, 1 mM EDTA, 65 mM KCl, 1 mM DTT, 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin A, and 2 µg/ml aprotinin, and centrifuged at 1000 g for

10 min. Protein concentration in the supernatants was determined by the Bradford method (Protein Assay Kit, Bio-Rad Laboratories), using BSA as a standard.

Antibody production and Western blot analysis

To prepare an antiserum against zebrafish myostatin, a histidine-tagged myostatin fusion protein was expressed in bacteria using the pQE31 expression system (Qiagen, Milan, Italy). Briefly, the cDNA encoding the C-terminal region of zebrafish myostatin, spanning amino acids 267 to 374, was amplified by PCR, as a SacI fragment, and cloned into the pQE31 vector in frame with codons for 6 histidine residues at the N-terminus. The resulting construct was transfected into M15[pREP4] *Escherichia coli* strain and the myostatin fusion protein was induced for 5 h by adding 1 mM isopropyl-thio-β-galactoside (IPTG) to 1 l bacterial culture at an optical density (OD) of 0.7 (600 nm).

Bacteria were then collected by centrifugation, resuspended and incubated for 1 h in 5 ml lysis buffer/g bacterial pellet (6 M guanidine hydrochloride, 100 mM NaH₂PO₄, 10 mM Tris-HCl, pH 8, and 10 mM 2-mercaptoethanol). The lysate was centrifuged at 12 000 g for 15 min and myostatin was purified from the supernatant using a nickel-chelated column. Eluate fractions containing myostatin were pooled and used to inject rabbits. All immunizations were carried out by Cymbus Biotechnology Limited (Chandler Ford, Hants, UK). The IgG fraction from immune serum was purified using the Protein-A Sepharose CL-4B column (Sigma-Aldrich), according to the low salt procedure (Lane 1988).

For antibody validation, the purified antibody was used in a Western blot analysis of protein from induced and non-induced bacteria, and of different quantities of purified recombinant zebrafish myostatin protein. Rabbit pre-immune serum was used as a control on induced bacteria.

The concentrated medium and the supernatant of lysed myostatin-transfected 293T cells, and protein extracted from zebrafish muscle and liver were analysed by Western blotting. Cells transfected with the CS2⁺ expression vector alone were used as a negative control. Proteins were separated on 12% sodium dodecyl sulphate-polyacrylamide gel, according to Laemmli (1970), and transferred onto PVDF membrane (Roche). The filters were blocked in 5% BSA prepared in TBS-T (20 mM Tris-HCl, pH 7.6, 137 mM NaCl and 0.1% Tween-20) and incubated for 1 h at room temperature with 1/1500 dilution of the purified zebrafish myostatin antibody in TBS-T. Filters were then washed (3 × 5 min; 1 × 15 min) with TBS-T buffer and further incubated for 1 h at room temperature with 1/30 000 dilution of goat horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (Pierce, Milan, Italy) in TBS-T. Membranes were washed as

mentioned above and HRP activity was detected using the SuperSignal Chemiluminescent kit (Pierce) according to the manufacturer's protocol. In the case of liver and muscle, the primary antibody washing was performed either as reported above or in the presence of 0.3 M NaCl.

Statistical analysis

Data were expressed as means $\pm$ S.E.M. The body weight differences between fish grown under overcrowded or normal conditions were analysed by the Student's *t*-test.

RT-PCR analysis was performed on the total RNA extracted from three different lots for each stage examined, and was repeated at least three times for each lot. Densitometric values of cDNA bands were expressed as myostatin/28S rRNA ratios, the latter being a better visual indicator of RNA integrity because it is more readily degraded than 18S rRNA (Ivell 1998, Thellin *et al.* 1999). Data were then transformed as percentages of the myostatin expression in adult muscle (equal to 100) and analysed by one-way ANOVA. Comparison among densitometric data measured at each age was performed by Bonferroni's test. The differences among groups were considered significant at $P < 0.05$.

Results

Expression of myostatin during zebrafish development

In situ hybridization analysis on whole-mount zebrafish embryos during (11, 12, 14, and 18 hpf) and after (24 and 48 hpf) somitogenesis, using an antisense riboprobe specific for zebrafish myostatin, failed to reveal any specific signal (data not shown). This is in contrast with the results obtained in mice, where myostatin expression was easily detectable in the myotome compartment during somitogenesis using the same technique (McPherron & Lee 1997). *In situ* hybridization on whole-mount zebrafish embryos at 14, 18 and 24 hpf, using a riboprobe for *MyoD*, a marker of muscle cell lineage, gave the expected pattern of expression (Weinberg *et al.* 1996), showing that the negative result obtained with the myostatin riboprobe was not due to technical problems.

When a more amplificative technique was used, such as the semiquantitative RT-PCR on total mRNA extracted from embryos at different stages of development, we could detect the expression of myostatin messenger. Figure 1A shows a representative image of the temporal expression of the transcript. The developmental profile is reported in Fig. 1C, where the levels of myostatin mRNA expression at different stages are displayed as percentages relative to the expression in adult muscle. The myostatin transcript was detectable in just-fertilized eggs (0 hpf), and its signal intensity declined at 8 hpf (75% epiboly), a pattern consistent with the transfer and degradation of maternally

transcribed RNA. A secondary rise at 16 hpf (14-somite stage), to a level similar to that found in just-fertilized eggs, indicates the onset of myostatin gene embryonic transcription. Myostatin mRNA concentration showed a nonsignificant decrement at 24 hpf, when somite formation is concluded, followed by an increase until hatching and 16 dpf, when similar levels were found. The adult muscle always showed the highest level of myostatin mRNA expression, compared with all the developmental stages examined.

The sequencing of the more abundant transcripts (72 hpf, 16 dpf, and adult muscle) and Southern blot analysis at all stages examined, using a probe specific for zebrafish myostatin (Fig. 1B), confirmed the identity of the amplified products. Moreover, the 5'-RACE analysis of the myostatin mRNA expressed in 48-hpf embryos and adult skeletal muscle revealed a unique transcription start site for both developmental stages, located at -79 nt from the first in-frame ATG (sequence deposited with the accession number AJ318758). The sequence of the amplified products was identical in both cases.

Myostatin mRNA expression in the adult zebrafish skeletal muscle and in cultured cells

As shown by RT-PCR analysis, myostatin mRNA is well expressed in the adult zebrafish skeletal muscle, at a level that is detectable even using a less amplificative technique, such as Northern blotting: a unique 2.8-kb transcript was observed using 15 μ g adult zebrafish lateral muscle probed with an anti-sense RNA specific for zebrafish myostatin (Fig. 2). This is in accordance with previous reports on several mammalian species (McPherron & Lee 1997, Gonzalez-Cadavid *et al.* 1998, Ji *et al.* 1998, Mendler *et al.* 2000) and on trout (Roberts & Goetz 2001), while it is in contrast with results on another fish species, tilapia, where myostatin mRNA expression was not detectable in the adult skeletal muscle, even using polyadenylated RNA (Rodgers *et al.* 2001).

To characterize the myostatin protein and its expression in the zebrafish skeletal muscle, we generated a polyclonal, affinity-purified antibody against a recombinant, histidine-tagged, zebrafish myostatin C-terminal region, expressed in *E. coli* and purified using a nickel-chelated column. As shown in Fig. 3A, the IgG fraction from rabbit immune serum detected a recombinant, histidine-tagged, 17 kDa myostatin protein from a bacterial extract induced to express myostatin, but no myostatin protein in a control non-induced extract, proving that the antibody specifically recognizes the myostatin recombinant protein. Furthermore, pre-immune rabbit serum did not detect any specific signal in the induced extract. In the Western blot analysis of affinity-purified, recombinant myostatin protein, the antibody recognized the protein in a manner that was proportional to the loaded amount (Fig. 3A). We used

Figure 1 Stage-related expression of myostatin mRNA in just-fertilized eggs (0 h post-fertilization, hpf), during embryo development (8, 16, 24 and 48 hpf), at or just after hatching (72 hpf), at the larval stage at 16 days of life (dpf), and in the adult muscle (adult muscle), as determined by semi-quantitative RT-PCR. (A) Representative images of ethidium bromide-stained gels. Total RNAs (0.5 µg) from whole embryos and 16-dpf and adult zebrafish muscles, were reverse transcribed and amplified using primers specific for zebrafish myostatin. Negative control: C-, no template. An aliquot (10/25) of each amplified sample was size-separated by agarose gel electrophoresis and stained with ethidium bromide. The expected 512-bp amplified product is indicated by an arrow (top panel). The 28S rRNA (lower panel) is presented as an indicator of the quality and quantity of the total RNA used in the reaction. (B) Southern blot analysis of the amplified product, using a digoxigenin-labelled cDNA probe specific for zebrafish myostatin. The signal was detected using a colorimetric system, after 20-min reaction. (C) RT-PCR analysis performed in triplicate on three different lots of total RNA from each stage examined. The quantification of the amplified product and 28S rRNA was carried out by densitometry of the respective ethidium bromide-stained bands. Values were expressed as myostatin/28S rRNA optical density (O.D.) ratios, calculated as percentages of the myostatin expressed in the adult muscle (equal to 100), and subjected to statistical analysis. Different letters indicate a statistically significant difference ($P < 0.05$; Bonferroni's test).

this antibody to characterize myostatin expression in the adult zebrafish lateral muscle.

By Western blot analysis on the muscle homogenate, washing the primary antibody in the presence of 0.3 M NaCl, we detected a unique 40 kDa immunoreactive band, that was not present in the liver homogenate used as a negative control (Fig. 3B). The size of this band is in accordance with the predicted mass of the unprocessed monomeric zebrafish myostatin precursor. Washing the

primary antibody in the absence of NaCl (less stringent washing), we observed also a 15 kDa band (Fig. 3C) that could correspond to the processed myostatin form, besides other faint bands. The same result was obtained (Fig. 3C) using the anti-human myostatin antibody B that is specific for a 16-amino acid peptide of the C-terminal region (kindly provided by Dr NF Gonzalez-Cadavid, Charles R Drew University, Los Angeles, CA, USA; Gonzalez-Cadavid *et al.* 1998).

Figure 2 Expression of myostatin mRNA in adult zebrafish skeletal muscle revealed by Northern blot analysis of total muscle RNA (20 and 15 µg) (upper panel). The blot was hybridized with two digoxigenin-labelled antisense riboprobes, specific for zebrafish myostatin, and the signal was visualized with a luminescent detection system after 40 min of film exposure. Transcript size and position relative to 28S and 18S rRNAs are shown on the right of the upper panel. In the lower panel, methylene blue-stained 28S and 18S rRNA bands are shown, to confirm the integrity of the total RNA used.

To further study *in vitro* zebrafish myostatin processing and secretion, the CS2⁺-myostatin plasmid (containing the zebrafish myostatin cDNA under the control of the cytomegalovirus (CMV) promoter) was transiently transfected into 293T cells, and the lysed-cell supernatant and conditioned medium analysed by Western blotting 48 h after transfection. As shown in Fig. 4 (right panel), two specific bands of approximately 52 and 15 kDa were detected in the conditioned medium of cells transfected with 10, 20 or 40 µg plasmid, whereas a unique 52 kDa specific band was present in the lysed-cell supernatant (Fig. 4, left panel). The same bands were also recognized by the anti-human myostatin antibody B (data not shown). Other bands present in both the myostatin-transfected cells and control cells transfected with the CS2⁺ vector alone were considered not specific.

The 15 and 52 kDa bands are in accordance with the values found in the literature for the myostatin protein expressed in mammalian cultured cells, corresponding

to the processed mature monomeric form and the unprocessed monomeric form respectively (McPherron *et al.* 1997). Hence, like mouse myostatin (McPherron & Lee 1997), zebrafish myostatin appears to be successfully proteolytically processed and secreted.

Myostatin expression in zebrafish grown under overcrowded conditions

Fish growth is markedly influenced by environmental and social factors (Wendelaar Bonga 1997). Zebrafish grown in an overcrowded (OC) environment from 16 dpf (free-swimming larvae) showed, at 90 dpf (adults), a delayed growth compared with fish raised under normal (N) conditions (Fig. 5A). To examine whether myostatin expression was altered in overcrowded fish, we subjected the total RNA extracted from the lateral skeletal muscle to Northern blot analysis. Myostatin mRNA was markedly higher in normally grown fish than in overcrowded fish (Fig. 5B). The experiment was repeated on three separate lots of zebrafish, obtaining similar results.

Discussion

Embryonic muscle development has been extensively studied in zebrafish (van Raamsdonk *et al.* 1978, Devoto *et al.* 1996), where slow and fast muscle fibres arise in this sequence from distinct populations of myoblasts.

In homeotherms, postnatal muscle development is mainly due to the hypertrophy of existing fibres (Goldspink 1972, Campion 1984). By contrast, in several fish species, hyperplastic recruitment of muscle fibres continues throughout much of the life cycle (Koumans & Akster 1995, Johnston 1999). After hatching, two main phases of muscle hyperplastic growth can be distinguished. The first is observed in yolk-sac larvae, where myoblasts proliferate in the dorsal and ventral regions of the myotomes (Veggetti *et al.* 1990, Brooks & Johnston 1993, Veggetti *et al.* 1993, Koumans *et al.* 1994, Rowleson *et al.* 1995). The second phase occurs post-metamorphically in white muscle only and is typical of large, fast-growing species, being absent or greatly reduced in slow-growing, small fish (van Raamsdonk *et al.* 1983, Weatherley & Gill 1984, Weatherley *et al.* 1988, Veggetti *et al.* 1993), such as zebrafish, in which post-metamorphic muscle growth results solely from hypertrophy of existing fibres.

During mammalian and avian myogenesis, myostatin mRNA abundance peaks at the onset of secondary muscle fibre formation, reaches a minimum at birth and post-natally, and slightly increases again at adulthood (Ji *et al.* 1998, Kocamis *et al.* 1999, Oldham *et al.* 2001). In zebrafish, we have found that myostatin mRNA is already present in just-fertilized eggs (0 hpf) and declines thereafter (8 hpf), a pattern that is consistent with a maternal transfer of the transcript and its subsequent degradation. It

Figure 3 Expression of myostatin protein in adult zebrafish skeletal muscle revealed by Western blot analysis using a polyclonal antibody against a recombinant, polyhistidine-tagged carboxy-terminal domain of zebrafish myostatin protein. (A) Antibody validation. Protein extracts from non-induced (NI) and induced (I) bacteria (carrying the expression plasmid containing the recombinant, histidine-tagged myostatin), and decreasing amounts of affinity-purified myostatin recombinant protein (P) were electrophoresed on 12% polyacrylamide gel, blotted and probed with rabbit pre-immune (PI) serum or rabbit antiserum IgG fraction against zebrafish myostatin. Membrane was washed in the presence of 0.3 M NaCl. (B and C) Tissue detection of myostatin protein. (B) Protein extracts from lateral muscle (5 and 15 µg) or liver (15 µg), the latter used as a negative control, were electrophoresed on 12% polyacrylamide gel, blotted and probed with anti-zebrafish myostatin antibody. The signal was visualized with a luminescent detection system after 5-s exposure. The putative unprocessed myostatin protein (arrow) and molecular mass markers (left) are shown. (C) Protein extracts from lateral muscle (15 µg) were treated as before and probed with either anti-human myostatin antibody B (1) or anti-zebrafish myostatin antibody (2). Membranes were washed in the absence of NaCl. The signal was visualized after 15-s exposure. The arrows indicate the putative unprocessed (upper arrow) and processed (lower arrow) forms of zebrafish myostatin.

has been shown, in several animal species, that maternally inherited mRNAs and proteins are used to program the earliest stages of development, but are degraded by the mid-blastula transition, allowing genetic control of development to pass to zygotically synthesized transcripts (Pendersen 1998). Maternal myostatin mRNA may be either directly transcribed by maturing or pre-ovulatory oocytes or transferred from granulosa cells into the oocytes (Patino & Kagawa 1999).

Two forms of myostatin mRNA, with 92% identity at the nucleotide level, were isolated in the ovary of brook trout, where one form is predominantly expressed during ovulation (Roberts & Goetz 2001). Two isoforms were also identified in both the rainbow trout, in which myostatin II is expressed only in muscle and brain, while myostatin I is present ubiquitously (Rescan *et al.* 2001), and the Atlantic salmon, whose two myostatins occur in multiple tissues (Østbye *et al.* 2001).

Figure 4 Secretion and processing of zebrafish myostatin in 293T transiently transfected cells, revealed by Western blotting using an antibody against zebrafish C-terminal myostatin domain. Cells were transfected with 10, 20 or 40 µg of the CS2⁺-myostatin plasmid or with 40 µg of the CS2⁺ vector alone (C-). Forty-eight hours after transfection, the lysed cells supernatant (left panel) and the concentrated conditioned medium (right panel) were electrophoresed on 12% polyacrylamide gel, blotted and probed with rabbit anti-myostatin IgG fraction. The signal was visualized with a luminescent detection system after 5-s exposure. Expressed myostatin precursor (52 kDa) and mature protein (15 kDa), and molecular mass markers are indicated.

As the identity of myostatin mRNA in zebrafish eggs was verified by Southern blot analysis at low-stringency hybridization and washing temperature (42 °C), we might have failed to discriminate between very similar isoforms. For this reason, we cannot exclude the possibility that myostatin mRNA in zebrafish eggs is a different isoform of the transcript found in the embryo, larva and adult fish. On the other hand, we have demonstrated that myostatin mRNA expressed in yolk-sac larvae (72 hpf) and free-swimming larvae (16 dpf) is identical to the adult muscle form. Moreover, the 5'-UTR of the transcript in 48-hpf embryos is also identical to the adult one.

In general, myogenic transcription factor genes, such as MyoD, are expressed earlier than muscle-specific protein genes (MSP), consistent with their upstream, regulatory role (Xu *et al.* 2000). In zebrafish, the expression of MyoD during embryonic development starts at 7 to 7.5 hpf, prior to somite formation, and drops markedly at 24 hpf, when somitogenesis is completed (Weinberg *et al.* 1996). Our pattern of myostatin expression in zebrafish embryos was timed as expected with respect to MyoD expression. In fact, the level of embryonic myostatin mRNA rose between 8 and 16 hpf, after the onset of MyoD expression and muscle cell differentiation (Devoto *et al.* 1996, Bladgen *et al.* 2000), and declined only slightly at 24 hpf, when MyoD expression and somite formation were almost concluded (Stickney *et al.* 2000). Thereafter, myostatin mRNA concentration rose again until hatching and 16 dpf and reached the highest value in the adult muscle.

Since in zebrafish, as in other small teleost fish, red and white muscle fibres are well differentiated at hatching and the single wave of hyperplastic muscle growth after hatching is limited to some distinct germinal zones (Veggetti *et al.* 1993, Koumans & Akster 1995, Johnston 1999), the observed increase in myostatin mRNA content at these stages and, particularly, in adult fish is in keeping with the fact that, in mammals, myostatin negatively regulates skeletal muscle growth by inhibiting myoblast proliferation (Thomas *et al.* 2000, Taylor *et al.* 2001).

These data suggest a key regulatory role of myostatin during early muscle development in both fish and mammals. However, the level of myostatin expression during mammalian somitogenesis appears to be higher, because, contrary to mammals (McPherron *et al.* 1997), the transcript was not detectable in zebrafish by whole mount *in situ* hybridization up to 48 hpf. In developing tilapia, myostatin mRNA was first revealed by RT-PCR only after hatching (Rodgers *et al.* 2001), thus even later than in zebrafish.

As in mammals, myostatin mRNA was readily detectable in adult zebrafish skeletal muscle by Northern blot analysis. By contrast, myostatin mRNA levels in tilapia white skeletal muscle were too low to be shown by Northern blotting (Rodgers *et al.* 2001). The authors proposed that this result could reflect the type of fibres sampled, as a differential expression of myostatin among white and red fibres was noted in various fish species (Østbye *et al.* 2001, Rescan *et al.* 2001, Roberts & Goetz

Figure 5 Myostatin expression in skeletal muscle of zebrafish grown under overcrowded (OC) or normal (N) conditions. (A) Body weight differences (means $\pm$ S.E.M.) between zebrafish grown under overcrowded conditions since 16 dpf or under normal conditions ($***P < 0.001$; *t*-test) are shown. (B) Representative Northern blot of total mRNA (20 μ g) from 90-dpf zebrafish lateral muscle. The blot was hybridized with two digoxigenin-labelled antisense riboprobes, specific for zebrafish myostatin, and the signal was visualized with a chemiluminescent detection system after 1-h exposure. Transcript size and position relative to 28S and 18S rRNAs are shown on the right. In the lower panel, methylene blue-stained 28S and 18S rRNA bands are shown as indicators of equal RNA loading and integrity. The image is representative of three separate experiments conducted on three different subgroups of zebrafish grown under overcrowded or normal conditions.

2001). In our case, due to the small size of zebrafish, pools of whole (white and red) adult lateral muscle, from two or three different animals, were used for Northern blot analyses, thus averaging possible differences between fibre types.

Western blotting of adult zebrafish muscle extracts, using an antibody against the zebrafish myostatin

C-terminus, detected a 40 kDa band, in accordance with the expected apparent molecular mass of the unprocessed myostatin precursor. A 15 kDa fainter band, probably corresponding to the processed myostatin monomer, was revealed only after longer exposure and using a less stringent primary antibody washing. A similar result, confirming the specificity of our antibody, was obtained using the anti-human myostatin antibody B (Gonzalez-Cadavid *et al.* 1998), that has been extensively validated by its ability to detect both the precursor and the processed mammalian protein (Lalani *et al.* 2000, Taylor *et al.* 2001). This antibody was raised against a 16-amino acid sequence within the C-terminal portion of the myostatin that is identical in human and zebrafish proteins. Using less stringent washing conditions a 37 kDa unknown protein was detectable by our antibody, but at a lower level than the 15 kDa band. This protein was only barely detectable by the Gonzalez-Cadavid's antibody, inferring the possible signal aspecificity.

Although several authors found a different molecular mass for the processed myostatin in mammalian muscle extract, ranging between 26 and 30 kDa (Gonzalez-Cadavid *et al.* 1998, Sharma *et al.* 1999, Lalani *et al.* 2000, Sakuma *et al.* 2000, Thomas *et al.* 2000, Taylor *et al.* 2001) (with several kDa differences reported even for the same species by different authors, despite using the same antibody), a considerable debate remains open, since in some reports lower molecular masses have been found. This is the case for the rat, where an 18–19 kDa molecular mass has been reported for the mature protein (Mendler *et al.* 2000). It is also the case for the mouse (Zimmers *et al.* 2002), where the authors found a 12.5 kDa protein in Western blot analysis of the serum of the wild-type mice, but not in that of the GDF-8 null animals (*Mstn*^{-/-}). In C2C12 myoblast cells transfected or not with the myostatin cDNA (Ríos *et al.* 2001), a band of 12 kDa was found using the Gonzalez-Cadavid's antibody B. Expressing the complete myostatin propeptide in mammalian, non-muscular cells lines, different authors found a 12.5 to 15 kDa band (McPherron *et al.* 1997, Lee & McPherron 2001, Rodgers *et al.* 2001), corresponding to the mature monomer, that under non-reducing conditions shows an electrophoretic mobility consistent with that of the corresponding dimer. These results, together with other similar findings obtained expressing the C-terminal mature myostatin region in *E. coli* or *Drosophila* cells (Lalani *et al.* 2000, Taylor *et al.* 2001), suggest that the 26–30 kDa band found in several muscle extracts could be a mature myostatin dimer that does not fully dissociate despite the denaturing and reducing conditions employed in the Western blot analyses.

In our experiments the 15 kDa immunoreactive band we found in zebrafish muscle extracts, essentially consistent with the predicted molecular mass of the mature zebrafish myostatin monomer, was also detected in mammalian 293T cells, transiently transfected with the

full-length zebrafish myostatin cDNA, but not in the negative control transfected cells.

In particular, Western blot analysis, using the antibody against the zebrafish myostatin C-terminus, demonstrated the occurrence of a 52 kDa band, corresponding to the unprocessed precursor, in both the cell extract and the conditioned medium, and of a 15 kDa signal in the medium, but not inside the cells. The presence of both precursor and processed forms was also observed in the medium of Chinese hamster ovary (CHO) cells stably transfected with mouse myostatin cDNA (McPherron *et al.* 1997). This confirms that zebrafish myostatin is processed and secreted in a similar manner as its mammalian orthologues, and that the precursor is cleaved in conjunction with or just after its secretion, as has been demonstrated for TGF- β 1 (Miyazono *et al.* 1991). It is also possible that some unprocessed myostatin in the medium is due to leakage from dead cells.

The apparent molecular masses of the unprocessed zebrafish myostatin found in transfected 293T cell cultures, is in accordance with the values reported in the literature for mammalian myostatin proteins expressed in cultured CHO mammalian cells (McPherron *et al.* 1997). Nevertheless, the mass of 52 kDa for the unprocessed protein in cell culture is different from that of 40 kDa estimated in zebrafish skeletal muscle. This difference in molecular weight might be due to some sort of post-translational modification of the myostatin precursor, such as glycosylation and/or denaturation-resistant association with other proteins that might be prominent in mammalian but not in fish or other cells. In fact, expressing human myostatin in *E. coli* or *Drosophila* cells, Taylor and collaborators (2001) found a 45 kDa immunoreactive band for the myostatin monomer precursor, whereas a higher, approximately 49–52 kDa band, has previously been reported for the mammalian unprocessed myostatin expressed in mammalian muscle cells by several authors (Sharma *et al.* 1999, Lalani *et al.* 2000, Thomas *et al.* 2000). In our Western analysis the Gonzalez-Cadavid's antibody revealed a weak 50 kDa band in zebrafish muscle that was barely detectable by our antibody. This probably means that the above post-translational processing or binding is less important in fish (strong 40 kDa band and weak 50 kDa band) as compared with the results obtained by other authors in mammalian cells (strong 49–52 kDa and no 40 kDa band).

An inverse correlation between myostatin levels and muscle mass has been reported during muscle atrophy in mammals (Gonzalez-Cadavid *et al.* 1998, Lalani *et al.* 2000). Moreover, when rats were subjected to muscle thermal injury, a damage that provokes a sustained elevation of glucocorticoids, Lang and collaborators (2001) noticed an increase in the myostatin mRNA level in muscle, concomitantly with a reduction in the muscle protein content. Similarly, dexamethasone enhanced the transcriptional activity of myostatin gene promoter con-

structs transfected into skeletal muscle cells in culture (Ma *et al.* 2001).

In the experiment in which zebrafish were raised from 16 up to 90 dpf under stressful overcrowding conditions, we observed a marked depression of body growth, resulting in stunting of fish, as well as a reduction of muscle myostatin mRNA expression.

Although this result seems to be in contrast with some of the above findings in mammals, it is in agreement with the report by Rescan *et al.* (2001) showing that, in rainbow trout, myostatin II mRNA, predominantly expressed in slow-twitch fibres, dropped dramatically in wasting muscles during sexual maturation, while myostatin I mRNA, expressed equally in red and white fibres, remained unchanged. This indicates that the muscle wastage occurring in fish both during sexual maturation and the stunting of muscle mass due to overcrowding are not dependent upon the up-regulation of myostatin expression. Rather, in these cases, the quenching of myostatin mRNA levels seems to be correlated to persistent catabolic or scarcely anabolic states in fish muscles.

The lowering of myostatin mRNA in the muscles of crowded fish does not necessarily conflict with the facts that crowding is stressful to fish and that the stress-induced elevation of glucocorticoids may enhance myostatin transcription. Fish can adapt to overcrowding, and it was found that, in chronically overcrowded brown trout, plasma cortisol levels returned to normal after a few weeks while body growth was depressed (Pickering & Stewart 1984).

We did not measure plasma cortisol in the crowded zebrafish because the operation is technically complicated but, if this species can also hormonally adapt to overcrowding, it is conceivable that both cortisol and its up-regulation of myostatin expression were transitory and that the subsequent adjustment towards retarded body growth brought about a general depression of muscle protein synthesis which did not spare myostatin.

Further investigation is needed to determine whether the lower content of myostatin mRNA observed during wasting of normally developed muscles caused by protein mobilization for gonadal development in rainbow trout and the delay of muscle growth under overcrowding conditions in zebrafish rely on common or different regulatory mechanisms.

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References

- Artaza JN, Bhasin S, Mallidis C, Taylor W, Ma K & Gonzalez-Cadavid NF 2002 Endogenous expression and localization of myostatin and its relation to myosin heavy chain distribution in C2C12 skeletal muscle cells. *Journal of Cellular Physiology* **184** 356–363.
- Bass J, Oldham J, Sharma M & Kambadur R 1999 Growth factors controlling muscle development. *Domestic Animal Endocrinology* **17** 191–197.
- Bladgen CS, Currie PD, Ingham PW & Hughes SM 2000 Notochord induction of zebrafish slow muscle mediated by Sonic hedgehog. *Genes and Development* **11** 2163–2175.
- Brooks S & Johnston IA 1993 Influence of development and rearing temperature on the distribution, ultrastructure and myosin sub-unit composition of myotomal muscle fibre types in the plaice *Pleuronectes platessa*. *Marine Biology* **117** 501–513.
- Campion DR 1984 The muscle satellite cells; a review. *International Review of Cytology* **87** 225–251.
- Cappuccio I, Marchitelli C, Seracchioli A, Nardone A, Filippini F, Aimone-Marsan P & Valentini A 1998 A G–T transversion introduces a stop codon at the mh locus in hypertrophic Marchigiana beef subjects. *Animal Genetics* **29** (Suppl 1) 51.
- Carlson CJ, Booth FW & Gordon SE 1999 Skeletal muscle myostatin mRNA expression is fiber-type specific and increases during hindlimb unloading. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* **277** R601–R606.
- Devoto SH, Melançon E, Eisen JS & Westerfield M 1996 Identification of separate slow and fast muscle precursor cells *in vivo*, prior to somite formation. *Development* **122** 3371–3380.
- Gilman M 1988 Transfection of DNA into eukaryotic cells. In *Current Protocols in Molecular Biology*, pp 9.1.1–9.1.3. Eds FM Ausubel, R Brent, RE Kingston, DD More, TA Smith & K Struhl. New York: John Wiley & Sons.
- Goldspink G 1972 Postembryonic growth and differentiation of striated muscle. In *The Structure and Function of Muscle*, edn 2, vol. 1, p 179. Ed. GH Bourne. New York: Academic Press.
- Gonzalez-Cadavid NF, Taylor WE, Yarasheski K, Sinha-Hikim I, Ma K, Ezzat S, Shen R, Lalani R, Asa S, Mamita M, Nair G, Arver S & Bhasin S 1998 Organization of the human myostatin gene and expression in healthy men and HIV-infected men with muscle wasting. *PNAS* **95** 14938–14943.
- Gray AM & Mason AJ 1990 Requirement for activin A and transforming growth factor- β 1 pro-regions in homodimer assembly. *Science* **247** 1328–1330.
- Grobet L, Martin LJ, Poncelet D, Pirottin D, Browsers B, Riquet J, Schoeberlein A, Dunner S, Ménessier F, Massabanda J, Fries R, Hanset R & Georges M 1997 A deletion in the bovine myostatin gene causes the double-muscling phenotype in cattle. *Nature Genetics* **17** 71–74.
- Grobet L, Poncelet D, Royo LJ, Browsers B, Pirottin D, Michaux C, Ménessier F, Zanotti M, Dunner S & Georges M 1998 Molecular definition of an allelic series of mutations disrupting the myostatin function and causing double-muscling in cattle. *Mammalian Genome* **9** 210–213.
- Ivell R 1998 A question of faith – or the philosophy of RNA controls. *Journal of Endocrinology* **159** 197–200.
- Ji S, Losinski RL, Cornelius SG, Frank GR, Willis GM, Gerrard DE, Depreux FFS & Spurlock ME 1998 Myostatin expression in porcine tissues: tissue specificity and developmental and postnatal regulation. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* **275** R1265–R1273.
- Johnston IA 1999 Muscle development and growth: potential implications for flesh quality in fish. *Aquaculture* **177** 99–115.
- Kambadur R, Sharma M, Smith TPL & Bass JJ 1997 Mutations in myostatin (GDF-8) in double-muscling Belgian Blue and Piedmontese cattle. *Genome Research* **7** 910–915.
- Kimmel CB, Ballard WW, Kimmel SR, Ullmann B & Schilling TF 1995 Stages of embryonic development of the zebrafish. *Developmental Dynamics* **203** 253–310.
- Kirk S, Oldham J, Kambadur R, Sharma M, Dobbie P & Bass J 2000 Myostatin regulation during skeletal muscle regeneration. *Journal of Cellular Physiology* **184** 356–363.
- Kocamis H, Kirkpatrick-Keller DC, Richter J & Killefer J 1999 The ontogeny of myostatin, follistatin and activin-B mRNA expression during chicken embryonic development. *Growth, Development and Aging* **63** 143–150.
- Koumans JTM & Akster HA 1995 Myogenic cells in development and growth of fish. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology* **110** 3–20.
- Koumans JTM, Akster HA, Witkam A & Osse JWM 1994 Numbers of muscle nuclei and myosatellite cell nuclei in red and white axial muscle during growth of the carp (*Cyprinus carpio* L.). *Journal of Fish Biology* **44** 391–408.
- Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** 680–685.
- Lalani R, Bhasin S, Byhower F, Tarnuzzer R, Grant M, Shen R, Asa S, Ezzat S & Gonzalez-Cadavid NF 2000 Myostatin and insulin-like growth factor-I and -II expression in the muscle of rats exposed to the microgravity environment of the NeroLab space shuttle flight. *Journal of Endocrinology* **167** 417–428.
- Lane D 1988 Storing and purifying antibodies. In *Antibodies. A Laboratory Manual*, p 310. Ed. I Harlow. Cold Spring Harbor, New York, USA: Cold Spring Harbor Laboratories Press.
- Lang CH, Silvis C, Nystrom G & Frost RA 2001 Regulation of myostatin by glucocorticoids after thermal injury. *FASEB Journal* **15** 1807–1809.
- Lee S-J & McPherron AC 2001 Regulation of myostatin activity and muscle growth. *PNAS* **98** 9306–9311.
- Ma K, Mallidis C, Artaza J, Taylor W, Gonzalez-Cadavid N & Bhasin S 2001 Characterization of 5'-regulatory region of human myostatin gene: regulation by dexamethasone *in vitro*. *American Journal of Physiology - Endocrinology and Metabolism* **281** E1128–E1136.
- McPherron AC & Lee S-J 1997 Double muscling in cattle due to mutations in the myostatin gene. *PNAS* **94** 12457–12461.
- McPherron AC, Lawler AM & Lee S-J 1997 Regulation of skeletal muscle mass in mice by a new TGF- β superfamily member. *Nature* **387** 83–90.
- Mendler L, Zádor E, Ver Heyen M, Dux L & Wuytack F 2000 Myostatin levels in regenerating rat skeletal muscles and in myogenic cell cultures. *Journal of Muscle Research and Cell Motility* **21** 551–563.
- Miyazono K, Olofsson A, Colosetti P & Heldin C-H 1991 A role of the latent TGF- β 1-binding protein in the assembly and secretion of TGF- β 1. *EMBO Journal* **10** 1091–1101.
- Oldham JM, Martyn JAK, Sharma M, Jeanplong F, Kambadur R & Bass JJ 2001 Molecular expression of myostatin and MyoD is greater in double-muscling than normal-muscling cattle fetuses. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **280** R1488–R1493.

- Østbye T-K, Galloway TF, Nielsen C, Gabestad I, Bardal T & Andersen Ø 2001 The two myostatin genes of Atlantic salmon (*Salmo salar*) are expressed in a variety of tissues. *European Journal of Biochemistry* **268** 1–10.
- Patino Ri & Kagawa H 1999 Regulation of gap junctions and oocyte maturational competence by gonadotropin and insulin-like growth factor-I in ovarian follicles of red seabream. *General and Comparative Endocrinology* **115** 454–462.
- Pendersen RA 1998 Early mammalian embryogenesis. In *The Physiology of Reproduction*, vol 1, pp 187–230. Eds E Knobil, JD Neill, LL Ewing, GS Greenwald, CL Markeret, DW Pfaff. New York: Raven Press.
- Pickering AD & Stewart A 1984 Acclimation of the interrenal tissue of the brown trout, *Salmo trutta* L., to chronic crowding stress. *Journal of Fish Biology* **24** 731–740.
- van Raamsdonk W, Pool CW & te Kronnie G 1978 Differentiation of muscle fiber types in the teleost *Brachydanio rerio*. *Anatomy and Embryology* **153** 137–155.
- van Raamsdonk W, Mos W, Smit-Onel MJ, van der Laarse WJ & Fehres R 1983 The development of the spinal motor column in relation to the myotomal muscle fibers in the zebrafish (*Brachydanio rerio*). *Anatomy and Embryology* **167** 125–139.
- Rescan P-Y, Jutel I & Rallière C 2001 Two myostatin genes are differentially expressed in myotomal muscles of the trout (*Oncorhynchus mykiss*). *Journal of Experimental Biology* **204** 3523–3529.
- Ríos R, Carneiro I, Arce VM & Devesa J 2001 Myostatin regulates cell survival during C2C12 myogenesis. *Biochemical and Biophysical Research Communications* **280** 561–566.
- Roberts SB & Goetz FW 2001 Differential skeletal muscle expression of myostatin across teleost species, and the isolation of multiple myostatin isoforms. *FEBS Letters* **491** 212–216.
- Rodgers BD & Weber GM 2001 Sequence conservation among fish myostatin orthologues and the characterization of two additional cDNA clones from *Morone saxatilis* and *Morone americana*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **129** 597–603.
- Rodgers BD, Weber GM, Sullivan CV & Levine MA 2001 Isolation and characterization of myostatin complementary deoxyribonucleic acid clones from two commercially important fish: *Oreochromis mossambicus* and *Morone chrysops*. *Endocrinology* **142** 1412–1418.
- Rowlerson A, Mascarello F, Radaelli G & Veggetti A 1995 Differentiation and growth of muscle in the fish *Sparus aurata* (L): II. Hyperplastic and hypertrophic growth of lateral muscle from hatching to adult. *Journal of Muscle Research and Cell Motility* **16** 223–236.
- Sakuma K, Watanabe K, Sano M, Uramoto I & Totsuka T 2000 Differential adaptation of growth and differentiation factor 8/myostatin, fibroblast growth factor 6 and leukaemia inhibitory factor in overloaded, regenerating and denervated rat muscles. *Biochimica et Biophysica Acta* **1497** 77–88.
- Sambrook J, Fritsch EF & Maniatis T 1989 *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor, New York, USA: Cold Spring Harbor Laboratories Press.
- Sharma M, Kambadur R, Matthews KG, Somers WG, Devlin GP, Conaglen JV, Fowke PJ & Bass JJ 1999 Myostatin, a transforming growth factor- β superfamily member, is expressed in heart muscle and is upregulated in cardiomyocytes after infarct. *Journal of Cellular Physiology* **180** 1–9.
- Stickney HL, Barresi MJF & Devoto SH 2000 Somite development in zebrafish. *Developmental Dynamics* **219** 287–303.
- Szabó G, Dallmann G, Müller G, Patthy L, Soller M & Varga L 1998 A deletion in the myostatin gene causes the compact (Cmpt) hypermuscular mutation in mice. *Mammalian Genome* **9** 671–672.
- Taylor WE, Bhasin S, Artaza J, Byhower F, Azam M, Willard DH, Kull FC & Gonzalez-Cadavid N 2001 Myostatin inhibits cell proliferation and protein synthesis in C(2)C(12) muscle cells. *American Journal of Physiology – Endocrinology and Metabolism* **280** E221–E228.
- Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, Hennen G, Grisar T, Igout A & Heinen E 1999 Housekeeping genes as internal standards: use and limits. *Journal of Biotechnology* **75** 291–295.
- Thisse TB, Schilling TF & Postlethwait JH 1993 Structure of the zebrafish *snail1* gene and its expression in wild-type, spadetail and no tail mutant embryos. *Development* **119** 1203–1215.
- Thomas M, Langley B, Berry C, Sharma M, Kirk S, Bass J & Kambadur R 2000 Myostatin, a negative regulator of muscle growth, functions by inhibiting myoblast proliferation. *Journal of Biological Chemistry* **275** 40235–40243.
- Turner DL & Weintraub H 1994 Expression of achaete-scute homolog 3 in *Xenopus* embryos converts ectodermal cells to neural fate. *Genes and Development* **8** 1434–1447.
- Veggetti A, Mascarello F, Scapolo PA & Rowlerson A 1990 Hyperplastic and hypertrophic growth of lateral muscle in *Dicentrarchus labrax* (L.). An ultrastructural and morphometric study. *Anatomy and Embryology* **182** 1–10.
- Veggetti A, Mascarello F, Scapolo PA, Rowlerson A & Candia Carnevali MD 1993 Muscle growth and myosin isoform transitions during development of a small teleost fish, *Poecilia reticulata* (Peters) (Atheriniformes, Poeciliidae): a histochemical, immunohistochemical, ultrastructural and morphometric study. *Anatomy and Embryology* **187** 353–361.
- Weatherley AH & Gill HS 1984 Growth dynamics of white myotomal muscle fibres in the bluntnose minnow, *Pimephales notatus* Rafinesque, and comparison with rainbow trout, *Salmo gairdneri* Richardson. *Journal of Fish Biology* **25** 13–24.
- Weatherley AH, Gill HS & Lobo AF 1988 Recruitment and maximal diameter of axial muscle fibres in teleosts and their relationship to somatic growth and ultimate size. *Journal of Fish Biology* **33** 851–859.
- Wehling M, Cai B & Tidball JG 2000 Modulation of myostatin expression during modified muscle use. *FASEB Journal* **14** 103–110.
- Weinberg ES, Allende ML, Kelly CS, Abdelhamid A, Murakami T, Andermann P, Doerre OG, Grunwald DJ & Riggleman B 1996 Developmental regulation of zebrafish *MyoD* in wild-type, *no tail* and *spadetail* embryos. *Development* **122** 271–280.
- Wendelaar Bonga SE 1997 The stress response in fish. *Physiological Reviews* **77** 591–625.
- Westerfield M 1995 *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Danio rerio)*, edn 3. Eugene, OR, USA: University of Oregon Press.
- Xu Y, He J, Wang X, Lim TM & Gong Z 2000 Asynchronous activation of 10 muscle-specific protein (MSP) genes during zebrafish somitogenesis. *Developmental Dynamics* **219** 201–215.
- Zhu X, Hadhazy M, Wehling M, Tidball JG & McNally EM 2000 Dominant negative myostatin produces hypertrophy without hyperplasia in muscle. *FEBS Letters* **474** 71–75.
- Zimmers TA, Davies MV, Koniaris LG, Haynes P, Esqueda AF, Tomkinson KN, McPherron AC, Wolfman NM & Lee S-J 2002 Induction of cachexia in mice by systemically administered myostatin. *Science* **296** 1486–1488.

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