The Journal of Experimental Biology 206, 4067-4079

doi:10.1242/jeb.00635

Analysis of *myostatin* gene structure, expression and function in zebrafish

Cheng Xu*, Gang Wu*, Yonathan Zohar and Shao-Jun Du†

Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore, MD 21202, USA

*These authors contributed equally to the project

†Author for correspondence (e-mail: dus@umbi.umd.edu)

Accepted 30 July 2003

Summary

Myostatin is a member of the TGF-β family that functions as a negative regulator of skeletal muscle development and growth in mammals. Recently, Myostatin has also been identified in fish; however, its role in fish muscle development and growth remains unknown. We have reported here the isolation and characterization of *myostatin* genomic gene from zebrafish and analysis of its expression in zebrafish embryos, larvae and adult skeletal muscles. Our data showed that *myostatin* was weakly expressed in early stage zebrafish embryos, and strongly expressed in swimming larvae, juvenile and skeletal muscles of adult zebrafish. Transient expression analysis revealed that the 1.2 kb zebrafish *myostatin* 5′ flanking sequence could direct green fluorescent protein (GFP) expression predominantly in muscle cells, suggesting that the *myostatin* 5′ flanking sequence contained regulatory elements required for muscle expression. To determine the biological function of Myostatin in fish, we generated a transgenic line that overexpresses the Myostatin prodomain in zebrafish skeletal muscles using a muscle-specific promoter. The Myostatin prodomain could act as a dominant negative and inhibit Myostatin function in skeletal muscles. Transgenic zebrafish expressing the Myostatin prodomain exhibited no significant change in myogenic gene expression and differentiation of slow and fast muscle cells at their embryonic stage. The transgenic fish, however, exhibited an increased number of myofibers in skeletal muscles, but no significant difference in fiber size. Together, these data demonstrate that Myostatin plays an inhibitory role in hyperplastic muscle growth in zebrafish.

Key words: zebrafish, *Danio rerio*, muscle, Myostatin, hyperplasia, transgenic fish.

Introduction

Myostatin (or GDF-8), a member of the Transforming Growth Factor-β (TGF-β) superfamily, was first identified in mice by McPherron et al. (1997) and has been demonstrated to negatively regulate skeletal muscle growth in several mammalian species. *Myostatin* knockout mice show a dramatic increase of skeletal muscle mass, and the increase results from a combination of hyperplasia and hypertrophy (McPherron et al., 1997). The 'Double muscle' breeds of cattle that have significantly more muscle mass than standard breeds were found to carry natural mutations in the *myostatin* gene (McPherron and Lee, 1997; Kambadur et al., 1997; Grobet et al., 1997, 1998). *In vitro* studies have demonstrated that Myostatin functions by inhibiting myoblast proliferation and differentiation (Thomas et al., 2000; Rios et al., 2001; Taylor et al., 2001; Langley et al., 2002). This is, in part, accomplished by downregulating myogenic gene expression (Langley et al., 2002; Amthor et al., 2002). The *myostatin* gene has been cloned from over 20 different vertebrate species including several fish species **(**McPherron and Lee, 1997; Rodgers and Weber, 2001; Rodgers et al., 2001; Maccatrozzo et al., 2001a,b, 2002; Kocabas et al., 2002; Roberts and Goetz, 2001; Ostbye et al., 2001; Rescan et al., 2001; Radaelli et al., 2003**)**. In some fish species, two distinct *myostatin* genes were found (Roberts and Goetz, 2001; Ostbye et al., 2001; Rescan et al., 2001). Comparison of Myostatin sequences revealed that Myostatin was extremely well conserved throughout evolution. Remarkably, the murine, rat, human, porcine, chicken and turkey Myostatin sequences are all identical in the active C-terminal region of the protein, suggesting that the function of this gene might be conserved in all vertebrates. However, this has not been tested.

Like other members of the TGF-β family, Myostatin is synthesized as a prepro-peptide that undergoes two steps of proteolytic cleavage to generate the biologically active C-terminal domain (Thomas et al., 2000; Rios et al., 2001). The bioactive C-terminal domain dimerizes and binds to membrane receptors on target cells (Thomas et al., 2000). The mature TGF-β C-terminal dimer often forms an inactive complex with the N-terminal prodomain of TGF-β (McPherron and Lee, 1996). This observation suggested that the Myostatin C-terminal active domain might also exist as a secreted latent complex with the Myostatin prodomain (Lee and McPherron,

1999; Thies et al., 2001). Recently, Thies and colleagues (2001) showed that Myostatin prodomain was able to bind to the bioactive Myostatin C-terminal active domain and inhibit its biological activity, presumably by preventing Myostatin active domain from binding to its receptor on the cell surface. Transgenic mice expressing the Myostatin prodomain in muscle cells showed enhanced muscle growth similar to *myostatin* knockout mice (Lee and McPherron, 2001; Yang et al., 2001).

Studies in mice and cattle have demonstrated that *myostatin* mRNA was specifically expressed in developing somite and skeletal muscles (McPherron et al., 1997; Kambadur et al., 1997). Recent studies, however, revealed that in addition to muscle cells, *myostatin* mRNA was also expressed in several other tissues, such as cardiomyocytes, mammary glands and, at a lower level, in adipose tissue (Ji et al., 1998; Sharma et al., 1999). In fish, *myostatin* mRNA was found to be expressed in muscles, eyes, gill filaments, spleen, ovaries, gut, brain and, to a lesser extent, in testes (Rodgers et al., 2001; Maccatrozzo et al., 2001a). *Myostatin* expression in skeletal muscles appeared to be restricted to certain types of muscle fibers. Carlson et al. (1999) demonstrated that *myostatin* mRNA was mainly expressed in fast muscle fibers in mice. Roberts and Goetz (2001) reported that *myostatin* mRNA was primarily expressed in red muscles in brook trout, king mackerel, and yellow perch, but expression in the little tunny is in the white muscles. Consistent with the study by Roberts and Goetz (2001), Rescan et al. (2001) showed that trout *myostatin-2* mRNA was predominantly expressed in red muscles. It remains to be determined if Myostatin is involved in different muscle growth of fast and slow muscles in fish.

Although *myostatin* cDNA has been cloned from zebrafish (McPherron and Lee, 1997), the temporal and spatial pattern of its expression is unknown. It is not clear when and where it is expressed in zebrafish embryos. Is it expressed in developing somites and skeletal muscles as in mammals? Is the muscle expression restricted to specific types of muscle fibers? Moreover, does Myostatin inhibit muscle growth in fish? We have reported here the isolation and characterization of *myostatin* genomic gene from zebrafish and analysis of its expression in zebrafish embryos, larvae and adult skeletal muscles. Our data showed a weak *myostatin* expression in early stage embryos and a strong expression in swimming larvae and juveniles, and the skeletal muscles of adult zebrafish. Overexpression of the Myostatin prodomain in skeletal muscles of transgenic zebrafish had no effect on the expression of myogenic regulatory genes. However, the adult transgenic fish showed hyperplasia in their skeletal muscles compared with non-transgenic controls.

Materials and methods

Isolation and characterization of zebrafish Myostatin genomic gene

The genomic gene of zebrafish *myostatin* was isolated as several DNA fragments by polymerase chain reaction (PCR) from the zebrafish GenomeWalker libraries (Du et al., 2003). A 1.2 kb DNA fragment containing the 5′ flanking sequence and part of the first exon were generated by PCR using the *myostatin* specific primers (*myostatin* E1.1/E1.2) close to the ATG start codon together with the adapter primers AP1 and AP2 at the 5′ end of the flanking region. A 2.5 kb fragment including the 3′ flanking region and part of the third exon were amplified from the libraries using a set of *myostatin* 3′ specific primers (*myostatin* E3.1/E3.2) together with the AP1 and AP2 primers. The middle part of the gene, including part of the first exon, intron 1, exon 2, intron 2 and part of exon 3, was generated by regular PCR from zebrafish genomic DNA using two sets of *myostatin*-specific primers, *myostatin* E1.3/E2.1R and *myostatin* E2.1F/E3.3. PCR for GenomeWalker was carried out in 50 μl reaction containing 1 μmol l^{-1} of each primer, four dNTPs at 0.2 mmol l^{-1} for each, and 5 units of Advantage Tth polymerase (Clontech, Palo Alto, CA, USA). PCR was carried out in two-step cycle program with 7 cycles at 94°C for 25 s and 72°C for 3 min, and followed by 32 cycles at 94°C for 25 s and 67°C for 3 min. The regular PCR was carried out in a 50 μl reaction solution containing 1 μmol l^{-1} of each primer, four deoxyribonucleotide triphosphates at 0.2 mmol l^{-1} for each, and 2.5 units of Taq DNA polymerase (Promega, Madison, WI, USA). PCR was carried out for 30 cycles. Each cycle included 30 s at 94°C, 30 s at 60°C and 3 min at 72°C. All DNA fragments were cloned into pGEM-T Easy Vector (Promega) and sequenced with an ABI automated DNA sequencer. Sequence alignment and searches were performed using the BLAST, TRANSFAC and TFSEARCH databases: *Ap1*, 5′-GTAATACGACTCACTATAGGGC-3′; *Ap2*, 5′-ACTATAGGGCACGCGTGGT-3′; *myostatin* E1.1, 5′-GCTGATGTTTGGAGCCTGCTTGAGTCG-3′; *myostatin* E1.2, 5′-CTTGAGTCGGAGTTTGCTAAGAATTTG-3′; *myostatin* E1.3, 5′-CAAATTCTTAGCAAACTCCGACTCAAG-3′; *myostatin* E2.1R, 5′-CTGCCAAGACGTGACTCCTGCGTTCA-3′; *myostatin* E2.1F, 5′-TGAACGCAGGAGTCACGTCTTGGCAG-3′; *myostatin* E3.1, 5′-ACTCCCACCAAGATGTCTCCCATCAAC-3′; *myostatin* E3.2, 5′-GGCAAAGAGCAGCTCATCTACGGCAAG-3′; *myostatin* E3.3, 5′-CTTGCCGTAGATGATCTGCTCTTTGCC-3′;

Mapping of zebrafish myostatin gene

The chromosomal position of zebrafish *myostatin* gene was mapped using the LN 54 radiation hybrid panel generated by Hukriede et al. (1999). The panel was produced by irradiation of zebrafish fin fibroblasts prior to fusion with mouse B78 melanoma cells. DNA from 93 hybrid cell lines was used for PCR analysis to detect the presence of the zebrafish *myostatin* gene using E1.3/E2.1R primers. The PCR was done as described above. The results of the PCR from the radiation hybrid panel were scored according to Hudson et al. (1995) using a web-based interface RHVECTOR at http://mgchd1.nichd.nih.gov:8000/zfrh/beta.cgi.

Construction of myostatin-GFP plasmid

The *myostatin-GFP* gene construct was obtained by amplifying the 5′ flanking sequence of zebrafish *myostatin* by

PCR using the *Ap2* primer at the 5′ and a gene-specific primer *myostatin*5R1 (5′-GCGTCGACGTTCCAAGGCGTGCTAA-AGGATG-3′) based on the 5′ UTR sequence of the zebrafish *myostatin* gene. To minimize mutation introduced by PCR, pfu DNA polymerase was used in the PCR reaction. PCR was carried out in a 50 μl reaction solution containing 1 μmol l^{-1} of each primer, four deoxyribonucleotide triphosphates at 0.2 mmol l^{-1} for each, and 2.5 units of pfu DNA polymerase (Stratagene, La Jolla, CA, USA). PCR was carried out for 35 cycles. Each cycle included 30 s at 94°C, 30 s at 60°C and 5 min at 72°C. A *Sal*I site was introduced at the end of the 5′ UTR primer. The 1.2 kb 5′ flanking sequence was first cloned into the *Sma*I site of Bluescript SK. The 5′ flanking sequence was then released from the Bluescript SK vector by *Sal*I digestion and cloned into the *Sal*I site of the GFP construct (Du and Dienhart, 2001). The resulting plasmid was confirmed by DNA sequencing and designated as *myostatin-GFP*.

Construction of the mylc-MSTN^{pro} transgene encoding the myostatin prodomain

To construct the transgene encoding the Myostatin prodomain, the complete coding region of zebrafish *myostatin* was first amplified by PCR using pfu DNA polymerase. A *Bam*HI and a *Xho*I site were introduced by the PCR primers at the 5′ and 3′, respectively. 5′-primer: 5′-GGATCCAACAT-GCATTTTACACAGGTTTT-3′, 3′-primer: 5′-CTCGAGGG-TTCATGAGCAGCCACAGCGG-3′. PCR was carried out in a 50 μl reaction solution containing 1 μmol l^{-1} of each primer, four deoxyribonucleotide triphosphates at 0.2 mmol l^{-1} each, and 2.5 units of pfu DNA polymerase (Stratagene). PCR was carried out for 30 cycles. Each cycle included 30 s at 94°C, 30 s at 60°C, and 3 min at 72°C. The PCR fragment was purified on a gel and then cloned into a plasmid Bluescript SK *Sma*I site. To generate the Myostatin prodomain construct, a stop codon (TAA) was introduced immediately after the RIRR proteolytic cleavage site at position 783 in the *myostatin* coding region using the QuickChange Site Directed Mutagenesis Kit (Stratagene). The gene construct encoding the Myostatin prodomain and its signal peptide were released from the plasmid by *Eco*RI digestion and cloned downstream of the rat *mylc* promoter/enhancer at the *Eco*RI site (Donoghue et al., 1991). In addition, SV 40 polyadenylation and transcription termination signals were included at the 3′ end of the Myostatin preprodomain to ensure proper transcription termination and polyadenylation of the mRNA encoding the Myostatin prodomain.

Microinjection in zebrafish embryos

The plasmids were diluted in distilled water to a final concentration of 50 μg ml^{-1}. Phenol Red was added to the injection solution at a final concentration of 0.1% to facilitate visualization during microinjection. Approximately 2 nl of DNA solution were microinjected into the cytoplasm of zebrafish embryos at the one- or two-cell stage. Microinjection was carried out under a dissection microscope (MZ8, Leica, Deerfield, IL, USA) using a PLI-100 pico-injector (Medical System Corp., New York, NY, USA). 500 embryos were injected for each construct.

Whole-mount in situ hybridization and antibody staining

The whole-mount *in situ* hybridization and antibody staining was carried out as previously described (Du and Dienhart, 2001). The Dig-labeled probe was synthesized by T7 RNA polymerase *in vitro* using *Bam*HI linearized plasmid pBS-MSTN that contains the full-length zebrafish *myostatin* cDNA (McPherron and Lee, 1997). For embryos of 24 h and older, proteinase K treatment was performed to enhance the permeability of the embryos, as described by Du and Dienhart (2001).

Expression analysis of the endogenous myostatin gene and the prodomain transgene by RT-PCR

Total RNA was extracted from zebrafish *Danio rerio* embryos, larvae, juvenile and adult muscle with TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesized using the first strand cDNA synthesis kit (Life Sciences Inc., St Petersburg, FL, USA). The expression of *myostatin* mRNA was analyzed by reverse transcriptase (RT)-PCR using two pairs of *myostatin* primers. One pair is a set of transgene-specific primers (*myostatin*5pc/E2.1r). The *myostatin*5pc primer (5′-CTGCAGCCCGGATCCAACATGCAT-3′) was based on part of the 5′ UTR sequence of the rat *mylc* promoter and part of the *myostatin* coding region near the ATG site. The E2.1R primer (5′-CTGCCAAGACGTGACTCCTGCGTTCA-3′) was based on part of the sequence in *myostatin* exon 2. PCR using this pair of primers generated a 627 bp fragment from the *mylc-myostatin*^{pro} transgene. Another pair of primers was *myostatin* E1.3/E2.1R. E1.3 (5′-CAAATTCTTAGCAAAC-TCCGACTCAAG-3′) was based on *myostatin* exon 1, while primer E2.1R was from the exon 2 sequence shown above. PCR using this pair of primers generated a 432 bp fragment from the endogenous *myostatin* gene and the prodomain transgene. Elongation factor 1-α (Ef-1α) was used as RT-PCR control. The primers for the Ef-1α 5′ and 3′ primers were EF-1α-5′ (GCATACATCAAGAAGATCGGC) and Ef-1α-3′ (GCAGCCTTCTGTGCAGACTTTG), respectively. PCR was carried out for 35 cycles (30 s at 94°C, 30 s at 60°C and 1 min at 72°C) in a 25 μl reaction solution containing 1 μmol l^{-1} of each primer, four deoxyribonucleotide triphosphates at 0.2 mmol l^{-1} for each, and 0.5 units of Taq DNA polymerase (Promega). 20 μl of the amplified product was analyzed by electrophoresis on a 1% agarose gel.

PCR screening of founder transgenic fish and transgenic offspring

To screen germline transgenic fish, DNA was extracted from a batch of 100 F_1 embryos from each cross. Briefly, 500 μl of lysis buffer (50 mmol l^{-1} KCl, 10 mmol l^{-1} Tris, pH 8.8, 1.5 mmol l^{-1} $MgCl_2$, 0.1% Triton X-100) was added to a group of 100 embryos, 48 h.p.f. (hours post fertilization). The embryos were gently homogenized in the lysis buffer. The

homogenate was boiled for 5 min, and was then digested with proteinase K (100 μg ml^{-1}) for 1 h at 55°C. Proteinase K was inactivated by boiling for 10 min after the digestion. The samples were centrifuged at 12 000 ***g*** for 5 min and 1.5 μl of the supernatant was used for the PCR reaction. PCR was carried out in a 25 μl reaction solution containing 1 μmol l^{-1} of each primer, four deoxyribonucleotide triphosphates at 0.2 mmol l^{-1} for each, and 0.5 units of Taq DNA polymerase (Promega). PCR was carried out for 35 cycles. Each cycle included 30 s at 94°C, 30 s at 60°C and 1 min at 72°C. 10 μl of the amplified product was analyzed by electrophoresis on a 1% agarose gel.

To screen adult transgenic offspring, DNA was extracted from a small portion of the caudal fin as described (Du and Dienhart, 2001) and used for PCR analysis. PCR was carried out using transgene-specific primers (mylc-p2/E2.1R) based on the *mylc* 5′ flanking sequence (mylc-p2, 5′-CACCACT-GCTCTTCCAAGTGTCA-3′**)** and part of exon 2 (E2.1R) of zebrafish *myostatin*. This PCR yields a 704 bp product. Control PCR primers HH-F (5′-GGACGGTGACACTTGGTGATG-3′) and HH-R (5′-CGAGTGGATGGAAAGAGTCTC-3′) were derived from the sense and antisense strand of the *tiggy winkle hedgehog* exon 3 sequence, respectively. PCR using this set of control primers produced a 615 bp DNA fragment. PCR reaction was carried out as described above for the embryos.

Real-time PCR analysis of myogenic gene expression

Expression of *MyoD*, *Myf5* and *myogenin* mRNAs was analyzed by real-time PCR in transgenic and non-transgenic fish at 2, 7, 45 and 240 days of age. For 2- and 7-day-old fish, total RNA was extacted from 100 embryos or larvae using TRIzol. For 45-day-old fish, individual fish were used for RNA extraction. For 240-day-old fish, ~60 mg of muscle tissue was used for RNA extraction. The total RNA was digested with DNAses to remove endogenous DNA contamination. First-strand cDNA was synthesized by reverse transcriptase using oligo-dT primer (RETROscript Kit, Ambion, Austin, TX, USA). Real-time RT-PCR was carried out using the following primers. *MyoD* was amplified using *MyoD* 5′- and 3′- primers. The PCR product was a 288 bp fragment. Similarly, *Myf5* and *myogenin* were amplified with their specific primers that generated a 482 bp and 319 bp fragment, respectively: MyoD 5′: 5′-AGACGGAACAGCTATGACAGCTCT-3′; MyoD 3′, 5′-ATTTTAAAGCACTTGATAAATG-3′; Myf-5 5′, 5′-CAC-TCAGAAACCTTCAACACCAA-3′; Myf5 3′, 5′-ATGCTCT-CTGAGCAGCTGGAGGA-3′; Myogenin 5′, 5′-TCTAGT-GATCAGGGCTCTGGCAGCA-3′; Myogenin 3′, 5′-TAAGC-CCTCCAAGGCTTGTCTAACTTGC-3′.

Real-time PCR was carried on Sequence Detector (PRISM 7700; ABI, Foster City, CA, USA). Each reaction (50 μl) contained 3 μl cDNA and primers at a final concentration of 2 ng μl^{-1}. SYBR Green was included in the PCR reaction as described in the protocol of SYBR Green PCR (ABI). The samples were first heated to 50°C for 2 min followed by 95°C for 10 min. The PCR reactions were carried out for 45 cycles at 95°C for 15 s and 60°C for 1 min. SDS v1.7a software was used to define the cycle in which each sample attained the threshold value.

Growth evaluations and determination of muscle fiber size and number

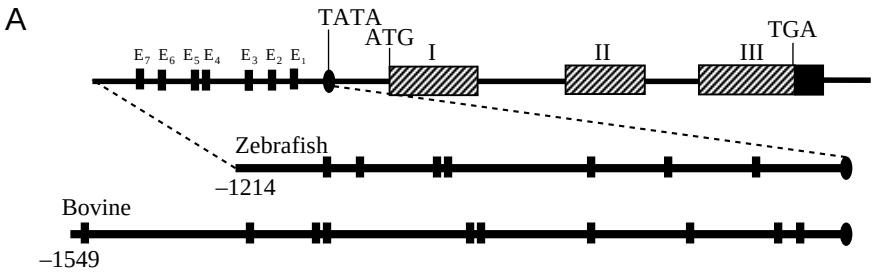
To determine the muscle structure in transgenic zebrafish at juvenile and adult stages, F_2 transgenic fish were generated by crossing transgenic males with wild-type females. Approximately 100 F_2 fish, including both transgenic and non-transgenic fish, were raised in the same tank. These fish were weighed at 2.5, 3 and 5.5 months of age, after anesthetization with 0.016% tricaine at two time points. The transgenic fish were identified by PCR using DNA extracted from the tail fins. Mean body mass was calculated for transgenic and non-transgenic males and females of different ages. For fiber size and number analysis, five individual fish from each group (female or male and transgene or wild type) were fixed with Bouin's solution for 24 h followed by routine paraffin sectioning and Haematoxylin/Eosin staining. A horizontal section at the base of the first pin of the anal fin was selected for quantification of the number of muscle fibers and measurement of the diameter of muscle fibers. A Student *t*-test was used to determine whether significant differences existed between wild-type and transgenic fish.

Results

Isolation and characterization of myostatin gene from zebrafish

To better understand the *myostatin* gene structure, function and regulation of expression in fish, the complete genomic sequence of zebrafish *myostatin* was isolated by polymerase chain reaction (PCR) using the GenomeWalker method as described in Materials and methods. Sequence analysis revealed that the zebrafish *myostatin* gene spanned 6.4 kb, including a 1.2 kb 5′ flanking sequence and a 5.2 kb transcriptional unit that includes the 3′ flanking sequence (GenBank accession number AY323521). Comparison with the zebrafish *myostatin* cDNA sequence revealed that the zebrafish *myostatin* gene contained three exons and two introns (Fig. 1A). The intron and exon junctions are highly conserved with *myostatin* genes of other vertebrate species. Sequence analysis of the zebrafish *myostatin* 5′ flanking sequence identified a putative 'TATA' box at position 110 bp upstream from the ATG start site (Fig. 1B). In addition, several putative MyoD binding sites (CAxxTG) that confer muscle-specificity, known as E boxes, were identified in the 5′-flanking region (Fig. 1B). Two of the potential E boxes (E4, E5) are closely located in the 5′ flanking sequence of the zebrafish *myostatin* gene. Of particular interest, two closely linked E boxes (E5, E6) were present in a similar position in the bovine *myostatin* promoter (Fig. 1A), and critical for its muscle-specific expression (Spiller et al., 2002). Considering that MyoD often functions as a dimer, the presence of closely linked E boxes in the conserved region of *myostatin* gene promoters suggests that MyoD may be involved in regulation of *myostatin* gene expression.

A



B

Zebrafish *myostatin* promoter sequence

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atcatgccctgttatcggtttattagcacataaatgttcacaggcatggtgtttagacgccaagcaacgtt -1144
ttgtgaacggcactgaaatgaaataatgaccgaaatattgcacattatccagcttattatttgtatacatg -1073
ccaaataaataaatggatttgaaatattaattcgcgttacaaatatgcttattacaagagaaacaaactga -1002
aactgatgggaaatacttgacaagtcttgtttacagtttagtgactgactgactgacatcacaattg  -931
                                                          E-box
cccaggtattcagtgaaccataatgtcatttttatttttttattttattagcctacaacatatgcagactag  -860
                                                    E-box
tgtatccaaaatattttctgatgtaaataggaatgtatgtattgtctagatctgtgttagttttgtttatt  -789
ttgcatgtcaccatcatacgtagaatttactttacaggtattcgactagaaagatctacatacctcacctg  -718
                                                          E-box
gttaacgcgttacacatgtagttccacaagatggcgcaatttatcctatccaccgtactaaatgaactgaa  -647
          E-box
aatgccgcaactacatgagtgtgttgaataaataagatgaactgtgattagttaagatcttactgctaaga  -576
cagaggaattgggctgacagataaatgcactgacagaagatacgttcatttttatccaattacttcttttc  -505
aaacaccacatgaaaaaagataaaggcaggaattgagaatattgggtgattttagcatgttattcactata  -434
      E-box
taaactagcaaattaaatgatataactacgaaaatgagtacggttattgtgttatatgcgtaaatataaca  -363
tgtagcctttgtcagctgcgtttgacaaaagacgcaaagtgtgcacgcacgaaaatatgtattttaaatgt  -292
          E-box
ttgtatttttcataaatctgcataatttctcaggttcaaatgatttccccacgagtcctcaaagatgtgtt  -221
                                    E-box
atacctctttaagagctgcgaaagaagtccagctctgtccattaggtttattgttgccagctcagccaatc  -150
attgaatcttacgacacaatagagtggccaaagttgcagtataaaaagccttgccgaatttaagcatgaca  -79
                                      TATA box
tctacttgtccggtgcgtggtgaggttcatttccatagcaaatcagaacatcaaacatcctttagcacgcc   -8
      +1
ttggaacATGCATTTTACACAGGTTTTAATTTCT...
       M  H  F  T  Q  V  L  I  S
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Fig. 1. The genomic structure and promoter sequence of zebrafish *myostatin* gene. (A) The zebrafish *myostatin* gene contains three exons and two introns with conserved exon and intron boundaries. Sequence analysis identified seven putative E box sites in the 5′ flanking sequence. Some of the E boxes are conserved compared with that in the bovine *myostatin* promoter. (B) The DNA sequence of zebrafish *myostatin* gene 5′ flanking region. The putative TATA box and the seven putative E box sites are underlined in the DNA sequence. The GenBank accession number is AY323521.

Zebrafish is widely viewed as an excellent system for genetic study of gene function. To map the position of the *myostatin* gene in zebrafish for future identification of potential *myostatin* mutants, linkage group analysis was carried out using the LN 54 radiation hybrid panel generated by Hukriede et al. (1999). The results placed the zebrafish *myostatin* gene in chromosome 9, at approximately 0.5 centiRay (1 cR is approximately 148 kb) from the genetic marker Z8363.

Characterization of myostatin mRNA expression in zebrafish

There is much evidence that *myostatin* mRNA is primarily expressed in developing somite and skeletal muscles in mammals. Recent studies in fish have also demonstrated that *myostatin* mRNA is predominantly expressed in skeletal muscles. However, its expression pattern in early stage fish embryos is not clear. A clear understanding of *myostatin* expression in fish embryos would provide important insights into the role(s) of Myostatin in regulating muscle development and growth in fish. To determine the temporal and spatial pattern of *myostatin* mRNA expression in zebrafish embryos, whole-mount *in situ* hybridization was carried out with zebrafish embryos at several developmental stages (14 h post fertilization to 4 days post fertilization). The results showed that *myostatin* mRNA could not be detected by *in situ* hybridization in zebrafish embryos (Fig. 2A,B), suggesting that *myostatin* mRNA was not expressed or only at a very low level in early stage zebrafish embryos. To confirm the *in situ* data and to further investigate its temporal pattern of expression in zebrafish, RT-PCR was used to analyze *myostatin* expression using total RNA from whole zebrafish embryos, larvae, juvenile and skeletal muscles of adult zebrafish. The results confirmed that *myostatin* mRNA was weakly expressed in the early stage zebrafish embryos (Fig. 2C). However, compared with day-4 embryo, *myostatin* mRNA expression appeared to be increased in 2-week-old swimming larvae, 1-month-old juveniles and skeletal muscle of 3-month-old adult zebrafish based on results from regular RT-PCR (Fig. 2C).

Analysis of the zebrafish *myostatin* 5′ flanking sequence revealed several putative E box sites that may be involved in its muscle-specific expression. To determine if the 5′ flanking sequence could drive gene expression in muscle cells, the 1.2 kb DNA sequence of zebrafish *myostatin* 5′ flanking region was ligated with the GFP reporter gene, and the DNA construct *myostatin-GFP* (Fig. 3) was microinjected into zebrafish

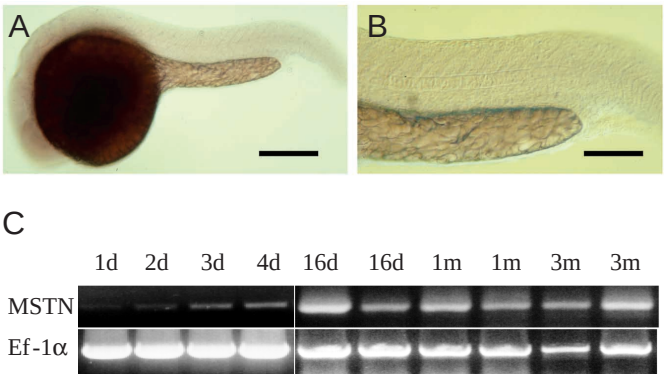

Fig. 2. Expression of *myostatin* in zebrafish embryos, larvae, juvenile and adult. (A,B) *In situ* hybridization showing little or no *myostatin* expression in developing somites of zebrafish embryos at 24 h.p.f. (hours post-fertilization). (C) RT-PCR results showing *myostatin* (MSTN) expression in early stage zebrafish embryos, 1–4 d (days post-fertilization), swimming larvae (16 d), juvenile (1 month) and adult skeletal muscles (3 months). Elongation factor 1α (Ef-1α) was used as control for RT-PCR. 16d, 1m and 3m are duplicated.

embryos for transient expression analysis. As indicated by GFP expression and anti-GFP antibody staining in the injected embryos (Fig. 3A,B,D,E), the zebrafish *myostatin* 5′ flanking sequence strongly targeted GFP expression in muscle cells of zebrafish embryos. Approximately 69% (*N*=148) of the injected embryos showed muscle expression, although some of the embryos also showed weak non-muscle expression in floor plate and head regions (Fig. 3C,F). These results indicate that the zebrafish *myostatin* 5′ flanking sequence contained regulatory elements required for muscle expression and possibly other regulatory sequences for *myostatin* expression in other tissues.

Production of transgenic zebrafish expressing myostatin prodomain in skeletal muscles

To investigate Myostatin function in fish muscle development and growth, a transgenic approach was used to express Myostatin prodomain in zebrafish muscle cells that could suppress Myostatin function. To identify a strong muscle-specific promoter with which to target the expression of the Myostatin prodomain in zebrafish skeletal muscles, we analyzed whether or not the rat *myosin light chain* (*mylc*) gene promoter and its 1/3 enhancer were muscle-specific in zebrafish. The *mylc* promoter/enhancer has been shown to be muscle-specific in other vertebrates (Donoghue et al., 1991; Lee and McPherron, 2001). The rat *mylc* gene promoter/enhancer was linked with the GFP reporter gene (Fig. 4A). The *mylc-GFP* construct was microinjected into zebrafish embryos. GFP expression was analyzed by direct observation using a fluorescence microscope or immunostaining using anti-GFP antibody. Over 80% (*N*=157) of the injected embryos exhibited muscle-specific GFP expression (Fig. 4C,D). The remaining

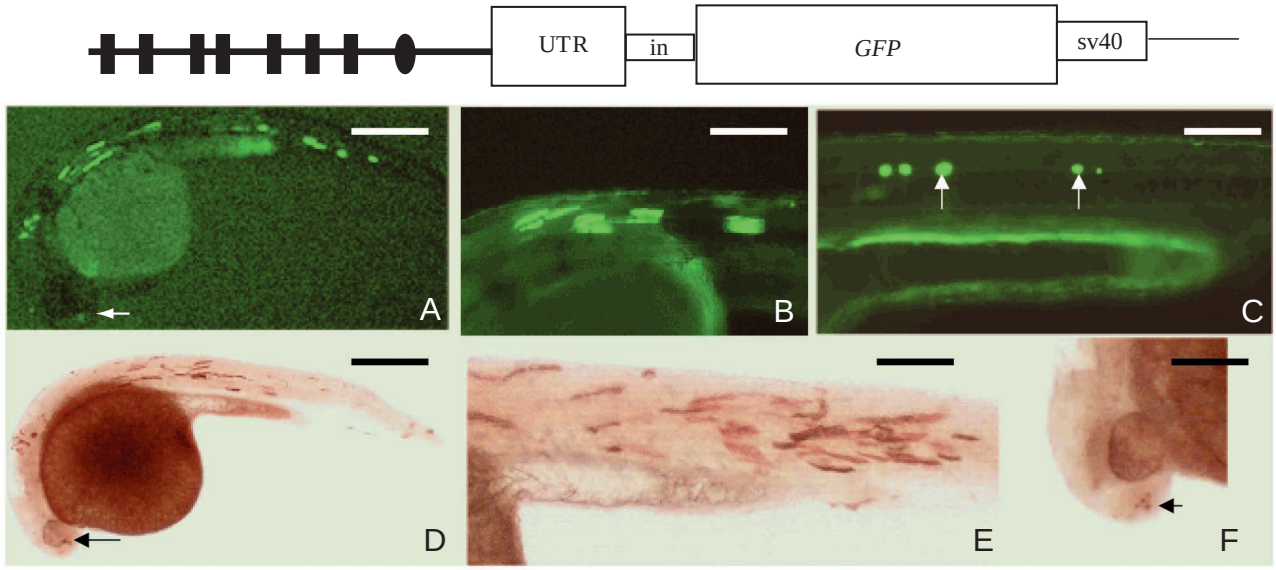

Fig. 3. Structure of *myostatin*-GFP (green fluorescent protein) construct and activity of *myostatin* promoter in zebrafish embryos. (Top) The *myostatin*-GFP construct contains a 1.2 kb zebrafish *myostatin* 5′ flanking sequence including the 5′UTR (5′-untranslated region). To ensure proper processing of the RNA transcripts, the rabbit β-globin intron-2 sequence (in) and the SV40 polyadenylation and transcription termination signals are included in the *myogenin-GFP* construct. (A) GFP expression in myofibers and forebrain region of zebrafish injected with the *myostatin-GFP* construct at 24 h.p.f. (hours post-fertilization). Forebrain expression is indicated by the white arrow. (B) Higher magnification view showing GFP expression in muscle fibers. (C) GFP expression in the floor plate (white arrows) of zebrafish embryos injected with the *myostatin-GFP* construct at 24 h.p.f. (D) Whole-mount anti-GFP staining confirming GFP expression in myofibers and brain (arrow) of injected zebrafish embryos (24 h.p.f.). (E) Higher magnification view of anti-GFP staining in muscle fibers. (F) Expression of GFP in the forebrain region (arrow) of a *myostatin-GFP* injected embryo, revealed by whole-mount antibody staining. Scale bars, 500 μm (A,D); 200 μm (B,C,E,F).

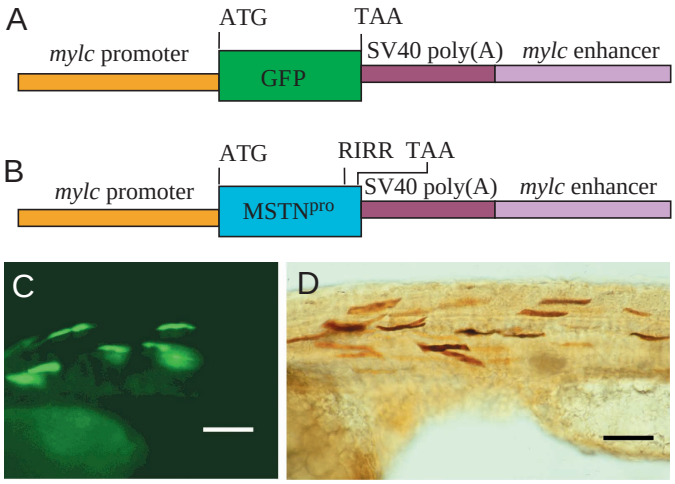

Fig. 4. Transient expression analysis of the rat *myosin* light chain (*mylc*) promoter in zebrafish embryos and structure of the *mylc-GFP* (A) and *mylc-MSTN^{pro}* (B) transgenes. The *mylc-GFP* gene construct (A) contains a 1.3 kb rat myosin light chain promoter (*mylc*) at the 5′, the 0.8 kb GFP coding region, a 0.8 kb SV40 poly(A) signal, and a *mylc* 1/3 enhancer from the rat *mylc* gene (Donoghue et al., 1991). The *mylc–MSTN^{pro}* gene construct (B) contains the 1.3 kb rat myosin light chain promoter (*mylc*) at the 5′, the 0.8 kb prodomain coding region of zebrafish *myostatin* (*MSTN^{pro}*), the 0.8 kb SV40 polyA signal, and the *mylc* 1/3 enhancer from the rat *mylc* gene. The ATG start codon, RIRR proteolytic site and the TAA stop codon are indicated. (C,D) Muscle-specific expression of GFP reporter gene in 24 h.p.f. zebrafish embryos injected with the *mylc-GFP*: (C) fluorescence, (D) immunostaining. Scale bar, 100 μm. The mosaic pattern of expression is typical for transient expression studies in zebrafish embryos (Westerfield et al., 1992; Du and Dienhart, 2001).

embryos that failed to express GFP in muscle cells did not exhibit GFP expression in other tissues. These data demonstrated that the rat *mylc* promoter/enhancer was muscle-specific in zebrafish and could be used to drive the expression of Myostatin prodomain in zebrafish muscle cells.

A gene encoding the zebrafish Myostatin prodomain was linked with the *mylc* promoter/enhancer (Fig. 4B). The resultant construct *mylc-myostatin^{pro}* was microinjected into zebrafish embryos for transgenic fish production. Two independent germline transgenic founders (1 and 33) were identified (out of 184) by PCR screening of their F_1 embryos. To determine the temporal and spatial expression of the transgene encoding the Myostatin prodomain, whole-mount *in situ* hybridization was used to analyze the expression of *myostatin* prodomain transgene in F_1 embryos at 14, 16 and 24 h.p.f. Because the expression of the endogenous *myostatin* gene was undetectable by whole-mount *in situ* hybridization at these stages (Fig. 2A,B), the *in situ* expression pattern, thus represented the expression of the transgene. The results showed that the *myostatin* transgene was strongly expressed in developing somite and embryonic muscles of the transgenic fish embryos (Fig. 5A,C,E). The two transgenic lines exhibited different patterns of transgene expression. In line 33, the transgene was exclusively expressed in skeletal muscles, and it appeared that the expression was fast-fiber specific (Fig. 5H,I). In contrast, line 1 showed expression in skeletal muscle and weak expression in the brain and spinal cord (data not shown). Because line 33 exhibited a strong muscle-specific expression, this transgenic line was primarily used in this study.

The inheritance of the transgene in line 33 was analyzed in the F_1, F_2 and F_3 generations. The transgene was inherited to the F_2 and F_3 generations in a Mendelian fashion, suggesting that it was integrated in a single site (data not shown). To determine if the transgene was expressed in skeletal muscles of adult transgenic fish, two sets of RT-PCR were employed to analyze the expression of the transgene and the endogenous *myostatin* gene (Fig. 6). The results showed that the transgene was strongly expressed in the skeletal muscles of adult transgenic fish. RT-PCR using the transgene-specific primers produced a predicted PCR product from the skeletal muscles of the transgenic fish (Fig. 6). In contrast, no PCR product of the transgene was found in non-transgenic fish. RT-PCR using another set of primers that amplify RNA transcripts from both the transgene and the endogenous *myostatin* gene generated a PCR product from both the transgenic and the non-transgenic fish (Fig. 6). Moreover, there were clearly more PCR products generated from the transgenic fish than the non-transgenic control (Fig. 6). This was probably because the transgenic fish contained RNA transcripts from both the endogenous *myostatin* gene and the transgene, whereas in the non-transgenic fish, the PCR product was solely derived from the endogenous gene transcripts. The PCR fragments were cloned, sequenced and confirmed to be derived from the *myostatin* gene or the transgene. Together with the *in situ* data (Fig. 5), these studies demonstrated that the *myostatin* prodomain transgene was inherited and expressed in a muscle-specific manner in the developing somite of transgenic zebrafish embryos. Moreover, this transgene is strongly expressed in adult skeletal muscles of the transgenic fish.

Inhibiting myostatin function induced hyperplasia in skeletal muscles of transgenic fish

The development and growth of skeletal muscles were analyzed in the transgenic fish at the morphological, histological and molecular levels. Overall, there were no obvious morphological changes with the transgenic fish. Transgenic fish showed normal development and growth. To determine if inhibiting Myostatin function affected the expression of myogenic regulatory genes, we analyzed the expression of *MyoD*, *myogenin* and *Myf-5* in transgenic fish embryos by *in situ* hybridization. There was no significant change in *MyoD*, *myogenin* or *Myf5* expression in transgenic zebrafish embryos (Fig. 7). In addition, we analyzed the formation of embryonic myofibers by F59 and MF20 antibody staining, and found no difference between transgenic and non-transgenic fish (data not shown). These results demonstrate that Myostatin may not be involved in myogenesis in early stage embryos. To determine if inhibiting Myostatin function affected

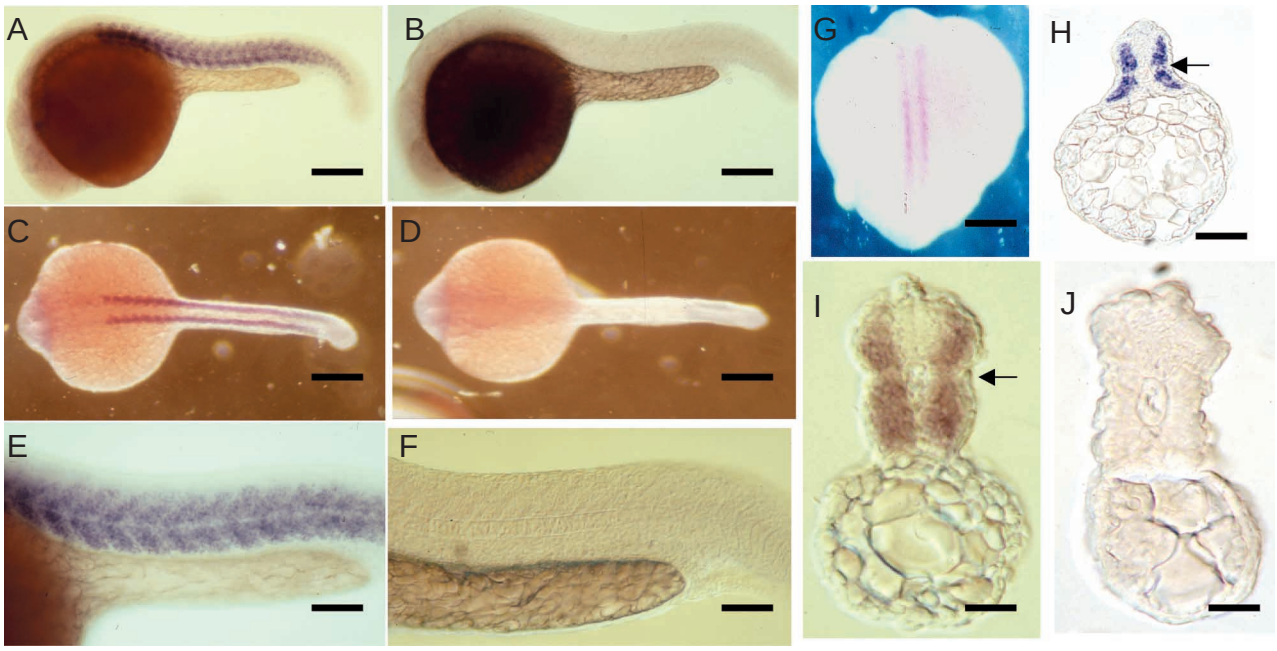

Fig. 5. Expression analysis of the *mylc-MSTN*^{pro} transgene in F_1 transgenic embryos by *in situ* hybridization. The expression of the transgene was visualized by *in situ* hybridization with a probe that could hybridize with transcripts of both the endogenous *myostatin* gene and the prodomain transgene. (A,C,E,G–I) Expression of Myostatin prodomain transcripts in transgenic fish embryos of line 33. Strong expression of Myostatin prodomain was found in transgenic fish embryos. (B,D,F,J) *In situ* results of non-transgenic zebrafish embryos using the same probe. All embryos were analyzed at 24 h.p.f. except G (16 h.p.f.). (H–J) Cross sections showing the fast muscle-specific expression of the transgene directed by the rat *mylc* promoter. Note the absence of staining in the slow muscle pioneer region at the myoseptum region of the somite (arrow). Scale bars, 500 μm (A–D); 200 μm (E,F); 150 μm (G–J).

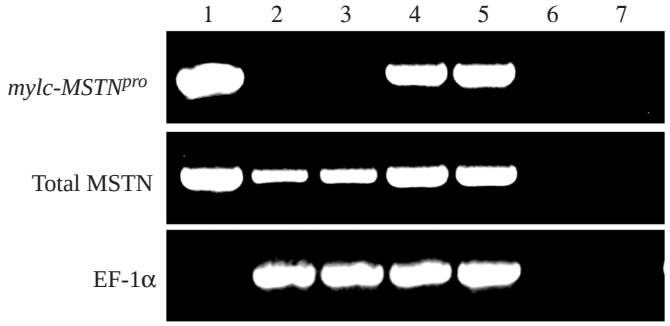

Fig. 6. Expression of the prodomain transgene and the endogenous *myostatin* gene in skeletal muscles of adult transgenic and non-transgenic zebrafish. For each sample, RT-PCR was performed using three different sets of primers that are specific for the transgene (*mylc-MSTN*^{pro}), the endogenous *myostatin* gene and the transgene (Total MSTN), and the elongation factor 1-α (Ef-1α) mRNA transcripts, respectively. Lane 1, RT-PCR results using *mylc-myostatin*^{pro} plasmid DNA as positive control. Lanes 2 and 3, RT-PCR results showing the expression of the endogenous gene (total MSTN) and EF-1α in the skeletal muscle of two individual non-transgenic fish (2 and 3). Lanes 4 and 5, RT-PCR results showing the expression of the Myostatin prodomain transgene (*mylc-MSTN*^{pro}), the endogenous *myostatin* gene and the transgene (total MSTN) and the EF-1α. Lane 6, RT minus control of non-transgenic fish. Lane 7, RT minus control of transgenic fish.

the expression of myogenic regulatory genes at later stage when *myostatin* mRNA expression was increased, we analyzed the expression of *MyoD*, *myogenin* and *Myf-5* in transgenic fish at days 7, 45 and 240 by quantitative RT-PCR. The results showed the expression levels of *MyoD*, *myogenin* and *Myf-5* were very similar between transgenic and non-transgenic fish (Fig. 8, Table 1). Statistical analysis did not reveal any significant difference in myogenic gene expression between transgenic and non-transgenic fish at 2, 7, 45 and 240 days of age.

To determine whether or not Myostatin plays a role in regulating muscle growth in adult fish, F_2 transgenic fish were generated by crossing F_1 (hemizygous) transgenic males with transgenic females, or transgenic males with non-transgenic females. The F_2 generations from these crosses contained both homozygous and hemizygous transgenic fish as well as non-transgenic offspring. The body mass and muscle structures of the transgenic and non-transgenic fish were compared at several developmental stages. The hemizygous transgenic fish did not grow significantly larger than the non-transgenic siblings when analyzed at 3 and 5.5 months of age (Table 2). However, there appeared to be a significant increase in mass (10–15%) when homozygous transgenic fish were included in the analysis at 2.5 months of age (Table 2).

To determine whether or not the muscle growth was affected in the transgenic fish, samples of representative fish from transgenic and non-transgenic sibling were sectioned to determine fiber size and for number analysis at 3 and 5.5

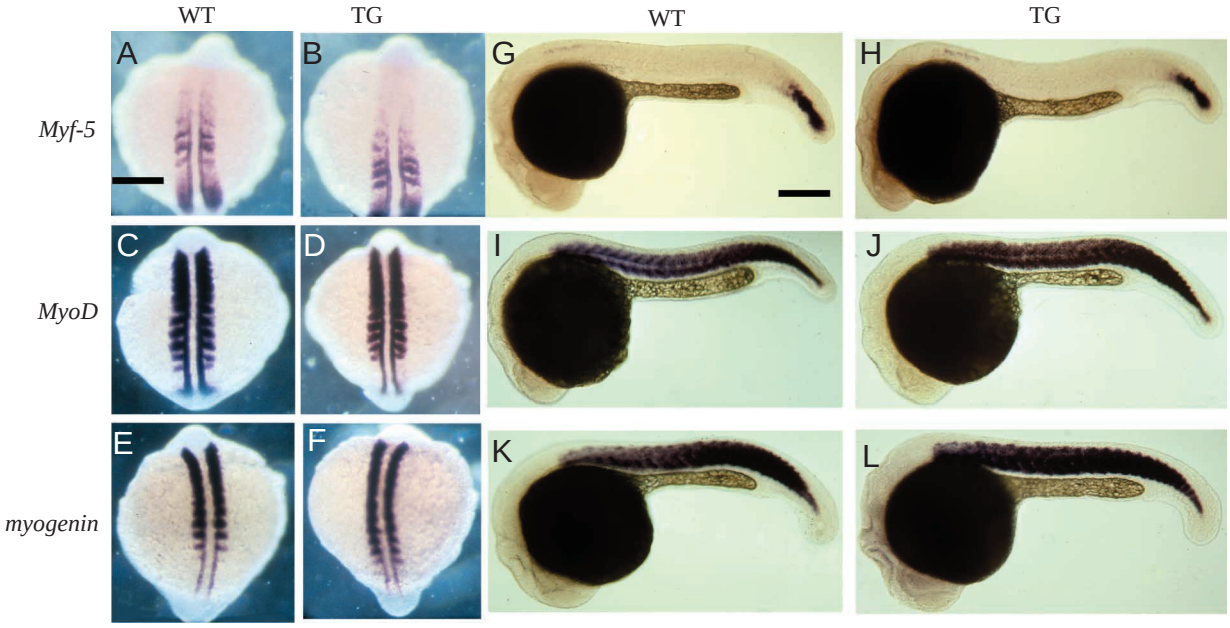

Fig. 7. *In situ* hybridizations comparing myogenic gene expression in transgenic and non-transgenic fish embryos. (A,B,G,H) *Myf-5* expression in transgenic (TG; B,H) and wild-type (WT; A,G) zebrafish embryos. (C,D,I,J) *MyoD* expression in transgenic (D,J) and wild-type (C,I) zebrafish embryos. (E,F,K,L) *myogenin* expression in transgenic (F,L) and wild-type (E,K) zebrafish embryos. (A–F) 16 h.p.f. embryos; scale bar, 200 μm. (G–L) 24 h.p.f. embryos; scale bar, 500 μm.

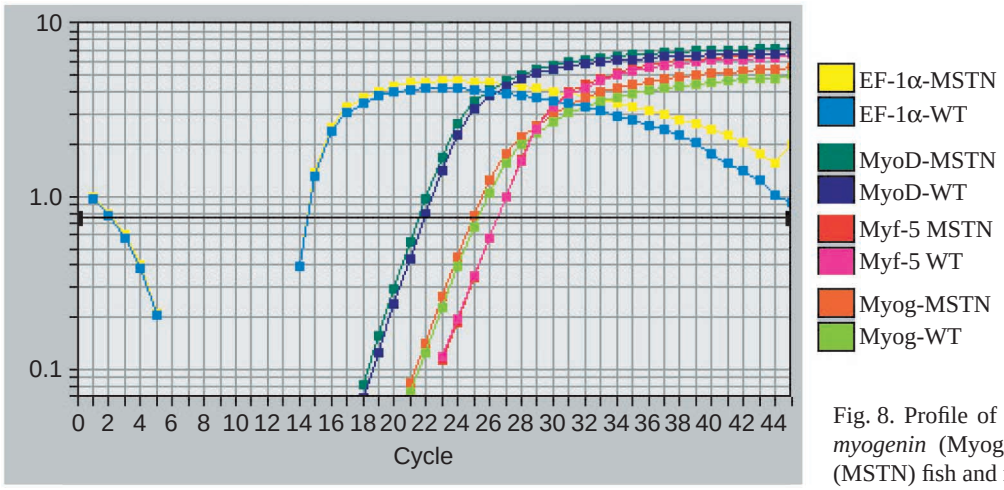

Fig. 8. Profile of real-time PCR showing *MyoD*, *Myf-5*, *myogenin* (Myog) and *Ef-1α* expression in transgenic (MSTN) fish and non-transgenic (WT) control at day 7.

Table 1. *Comparison of myogenic gene expression in transgenic and non-transgenic fish by cycle threshold of real-time polymerase chain reaction*

Age	Fish	*EF-1α*	*MyoD*	*Myf-5*	*myogenin*
Day 2	Non-transgenic	14.01	20.76	25.36	24.16
	Transgenic	14.04	20.4	25.33	24.01
Day 7	Non-transgenic	14.03	21.59	25.22	24.2
	Transgenic	14.02	21.33	25.22	23.54
1.5 months[a]	Non-transgenic	16.0±0.11	21.4±0.77	25.3±0.73	23.5±3.00
	Transgenic	16.9±0.91	20.9±0.32	24.7±0.47	23.8±0.50
8 months[a]	Non-transgenic	14.03±0.06	18.9±0.35	23.5±0.54	20.9±1.54
	Transgenic	14.3±0.24	19.0±0.45	23.5±1.57	22.3±0.64

[a]Threshold values of 1.5 and 8 months fish are mean values of the PCR threshold of 3 individuals ± S.E.M.

months. The results showed that the transgenic fish had approximately 10% more myofibers than the non-transgenic fish that were in the similar body mass group (Table 3). To determine whether inhibiting Myostatin function caused hypertrophic muscle growth, fiber size was compared in transgenic and non-transgenic fish. Because the fiber size varies significantly in different cross section regions, we chose to compare myofibers from a defined region of the epaxial muscles where the fibers appear to be relatively uniform in size. The data revealed that there was no significant difference between fiber size in transgenic and non-transgenic fish (Table 3). Together, these data indicate that Myostatin may

Table 2. *Body mass comparison between transgenic fish and non-transgenic siblings*

		Non-transgenic		Transgenic		
Age (months)	Gender	Number	Mass (g)[d]	Number	Mass (g)[d]	*P* value[e]
2.5[a]	Female	22	0.462±0.092	46	0.531±0.150	<0.001*
	Male	26	0.336±0.112	34	0.361±0.330	>0.200
3[b]	Female	24	0.492±0.123	14	0.441±0.094	>0.100
	Male	28	0.356±0.072	19	0.340±0.060	>0.400
5.5[c]	Female	38	0.830±0.242	18	0.784±0.298	>0.500
	Male	33	0.522±0.060	37	0.535±0.096	>0.400

The three different groups of fish were raised in three separate tanks.

[a]F_2 offspring of a hemizygous transgenic female crossed with a hemizygous transgenic male. It is worth noting that there is a significant increase in mass in female transgenic fish compared with controls at 2.5 months old. Approximately, one third of the transgenic fish represent homozygous transgenic fish.

[b,c]F_2 offspring of a hemizygous transgenic male crossed with a non-transgenic female.

[d]Mass is shown as mean ± S.E.M.

[e]*P* values are the results of a *t*-test; * indicates a significant difference.

Table 3. Comparison of mass, myofiber number and fiber size between transgenic and non-transgenic fish

	Mass (g)		Fast muscle[a]		Slow muscle[a]		Total fiber[a]		Fiber size (μm)[b]	
Fish	Non-transgenic	Transgenic	Non-transgenic	Transgenic	Non-transgenic	Transgenic	Non-transgenic	Transgenic	Non-transgenic	Transgenic
3 months male										
1	0.435	0.460	NA	NA	NA	NA	2621	3229	36.8±5.5	35.5±2.9
2	0.415	0.413	–	–	–	–	2331	2812	36.5±6.3	39.0±6.2
3	0.351	0.390	–	–	–	–	2355	2537	48.7±5.3	33.6±4.1
4	0.336	0.342	–	–	–	–	2336	2683	43.1±6.4	37.8±5.2
5	0.280	0.270	–	–	–	–	1932	2296	38.6±5.9	38.1±6.6
Average[c]	0.363±0.028	0.375±0.032	–	–	–	–	2315±110	2711±155	40.7±7.5	36.8±5.5
P value[d]	0.793		–		–		0.071		0.174	
5.5 months female										
1	1.302	1.343	2280	2551	425	483	2705	3034	59.8±12.3	65.6±11.6
2	1.056	1.101	2246	2683	394	296	2640	2979	59.5±10.0	64.9±11.6
3	0.908	0.930	2191	2389	325	359	2516	2748	64.2±11.6	59.5±13.0
4	0.866	0.848	2226	2282	371	293	2597	2575	54.0±14.7	58.2±10.9
5	0.751	0.777	1947	2299	424	345	2371	2644	66.9±16.6	60.2±12.0
Average[c]	0.977±0.095	1.000±0.101	2178±60	2441±77	387.8±18.6	355.2±34.5	2566±58	2796±91	61.4±14.2	61.7±12.3
P value[d]	0.871		0.027*		0.43		0.064		0.377	
5.5 months male										
1	0.615	0.626	2210	2386	415	475	2625	2801	72.2±12.5	70.0±12.2
2	0.587	0.582	2196	2438	400	374	2596	2722	61.2±11.0	59.3±13.5
3	0.558	0.561	2153	2248	338	454	2491	2702	58.9±9.5	71.5±8.7
4	0.528	0.526	1879	2166	347	400	2226	2566	64.4±10.5	59.5±8.9
5	0.488	0.490	1849	2127	294	315	2143	2442	57.4±10.7	62.5±10.7
Average[c]	0.555±0.022	0.557±0.023	2057±80	2273±60	358.8±21.9	403.6±28.6	2416±98	2647±64	62.8±12.2	64.6±12.2
P value[d]	0.957		0.063		0.249		0.084		0.65	

[a]Fiber number was counted in one half of the cross section.

[b]Fiber size represents the average diameter of 20 muscle fibers from a defined area of the cross section in the epaxial muscles.

[c]All values are means ± S.E.M.

[d]*P* values are results of a *t*-test; * indicates significant difference ($P<0.05$).

play an inhibitory role in hyperplastic myofiber formation, but has little effect on controlling the size of myofibers in zebrafish. Therefore, inhibiting Myostatin function in zebrafish results in a small but significant increase in muscle hyperplasia, but not in hypertrophy.

Discussion

In this study, we isolated the zebrafish *myostatin* genomic gene and analyzed its expression and function in zebrafish embryos, larvae and adult skeletal muscles. Our data demonstrated that the zebrafish *myostatin* mRNA was weakly expressed in early stage zebrafish embryos. Its expression increased significantly in swimming larvae, juvenile and adult skeletal muscles. Transient expression analysis demonstrated that the zebrafish *myostatin* 5′ flanking sequence contained regulatory elements for muscle expression. To develop a zebrafish model for the functional study of Myostatin in fish, transgenic zebrafish expressing the Myostatin prodomain were generated. The Myostatin prodomain was specifically expressed in skeletal muscle cells using a muscle-specific promoter. The transgenic fish developed normally and showed no defect in muscle development of early stage embryos. The transgenic fish exhibited an increased hyperplastic muscle growth, but no obvious increase in hypertrophic muscle growth compared to non-transgenic siblings. Similar to the results observed in the present report with zebrafish, a significant increase in muscle fiber number (i.e. hyperplasia) has been observed in rainbow trout muscle transfected with a morpholino oligonucleotide directed against myostatin (T. Bradley, personal communication). These data demonstrated that inhibiting Myostatin function in fish had a positive effect in stimulating muscle growth.

Characterization of fish myostatin genes

Although fish *myostatin* genes share high sequence identity with their mammalian counterparts, *myostatin* mRNA expression in fish is different compared with that in mammals. In mice, *myostatin* mRNA is strongly expressed in developing somite and skeletal muscles, and weakly expressed in cardiomyocytes, mammary glands and adipose tissue (McPherron et al., 1997; Ji et al., 1998; Sharma et al., 1999). In fish, several studies have demonstrated that, in addition to muscle expression, *myostatin* mRNA was expressed in eyes, spleen, gill filaments, ovaries, gut and brain and, to a lesser extent, in testes (Rodgers et al., 2001; Maccatrozzo et al., 2001a; Roberts and Goetz, 2001; Ostbye et al., 2001; Radaelli et al., 2003). Moreover, in contrast to strong expression in developing somites in mouse embryos, little or no *myostatin* mRNA expression could be detected in developing somites of fish embryos by whole-mount *in situ* hybridization. *Myostatin* mRNA expression in early stage zebrafish embryos could only be detected by RT-PCR. This is consistent with a recent report in seabream by Maccatrozzo et al. (2001a), and in zebrafish by Vianello et al. (2003).

The different pattern of *myostatin* expression in fish and mice raised the question of whether the *myostatin* that we focused on in this study was the mammalian homologue, and whether or not additional *myostatin* genes are present in zebrafish, given that zebrafish have more duplicated genes compared to mammals (Wittbrodt et al., 1998; Robinson-Rechavi et al., 2001). Our BLAST search found two additional *myostatin*-related genes in the zebrafish genome sequence. They share approximately 60–70% identity with the zebrafish *myostatin* gene used in this study. These two *myostatin* related genes, however, did not show any expression in developing somites when examined by *in situ* hybridization (data not shown) and their DNA sequences share less identity with the mouse *myostatin* gene compared with the *myostatin* gene characterized in this study.

Recently, a closely related gene GDF-11/BMP-11 was identified in human, mouse and frog. GDF-11 is expressed in many tissues other than the skeletal muscles, such as brain and eye (Nakashima et al., 1999; McPherron et al., 1999; Gamer et al., 1999). GDF-11 and *myostatin* are thought to be derived from the same ancestral gene through gene duplication. The *myostatin*-like genes in zebrafish could represent the zebrafish GDF-11. These data suggest that the duplication event that generated *myostatin* and GDF-11 might occur before the divergence of the fish species. It is unknown at present whether the functions of Myostatin and GDF-11 are highly specific, as in mammals. Further studies, especially the characterization of fish GDF-11 expression and function, will provide more insight into the possible function of these two highly related genes in fish.

Myostatin functions in regulating fish muscle formation

Histological analysis revealed that the transgenic fish exhibited stratified hyperplasia (data not shown). Stratified hyperplasia generates new fibers along a distinct germinal layer. This type of hyperplasia is found in all fish species. In addition to stratified hyperplasia, another type of hyperplasia, mosaic hyperplasia, results in a large increase in total fiber number during juvenile growth, and is therefore very common in commercially important aquatic species that grow to a large size. Mosaic hyperplasia is greatly reduced or entirely lacking in species such as zebrafish, guppies and other fish that remain small (Van Raamsdonk et al., 1983; Weatherley and Gill, 1984, 1985; Weatherley et al., 1988). The lack of a dramatic effect on enhancing muscle growth in zebrafish could be due to the lack of mosaic hyperplasia in zebrafish. Nevertheless, inhibiting Myostatin function resulted in a small but significant increase in fiber numbers in the transgenic fish. This is consistent with the recent finding that growth hormone transgenic zebrafish only grow 20% faster than non-transgenic control (Morales et al., 2001). It will be interesting to determine if blocking Myostatin function in large aquatic species has a more dramatic effect in stimulating muscle growth.

The lack of a significant effect on hypertrophic growth in transgenic fish differs from previous findings in mice. In the *myostatin* knockout mice, the marked increase in muscle

mass was attributed to both hypertrophy and hyperplasia (McPherron et al., 1997), while the transgenic mice expressing the Myostatin prodomain or the dominant negative form of Myostatin exhibited primarily hypertrophy (Zhu, 2000; Yang et al., 2001). The different response to Myostatin in fish and mice could be due to the different types of muscle growth in postnatal or post-larval stages. Postnatal muscle growth in mammals is largely contributed by hypertrophy. In contrast, in most fish species, muscle growth in post-embryonic life is attributed to continuous hyperplastic and hypertrophic growth (reviewed by Rowlerson and Veggetti, 2001). The less dramatic effect in fish could also be due to other reasons. Several studies have demonstrated that there is no correlation between changes in *myostatin* mRNA and Myostatin protein levels. Moreover, in zebrafish, it has been shown that most of the Myostatin proteins exist as precursor protein (Vianello et al., 2003). Therefore, overexpression of the Myostatin prodomain may not have a dramatic effect in inhibiting Myostatin activity in zebrafish.

The possibility that *myostatin* related genes may be also involved in the process should not be overlooked. Recently, Lee and McPherron (2001) demonstrated that overexpression of Follistatin, a TGF-β/BMPs inhibitor, in skeletal muscles of transgenic mice induced hyperplasia and hypertrophy. Interestingly, the muscle phenotype is more dramatic than that obtained from the *myostatin* knockout, suggesting that Follistatin may have an additional function than simply blocking Myostatin activity in skeletal muscles (Lee and McPherron, 2001). Follistatin has been cloned in zebrafish and was found to be expressed in developing somite and skeletal muscles (Bauer et al., 1998). It remains to be determined if overexpressing Follistatin in fish skeletal muscles has a more dramatic effect in stimulating fish muscle growth.

Myostatin inhibits myoblast specification and differentiation by downregulating *Pax3*, *Myf-5* and *MyoD* expression in myoblasts (Langley et al., 2002). In this study, we demonstrated that expression of *MyoD*, *Myf-5* and *myogenin* was not significantly affected in transgenic zebrafish expressing the Myostatin prodomain. This result is not surprising considering that only a 10% increase in hyperplasia was found in transgenic fish.

Regulation of myostatin gene expression

Although *myostatin* mRNA is predominantly expressed in skeletal muscles, several studies have demonstrated that it is also expressed in other tissues. To understand its regulation of expression in muscle cells, we analyzed the activity of the zebrafish *myostatin* 5′ flanking sequence in zebrafish embryos. We found that the zebrafish *myostatin* 5′ flanking sequence could drive GFP expression in muscle fibers of zebrafish embryos. We also noticed that GFP expression in myofibers driven by the *myostatin* flanking sequence was much stronger than *myostatin* mRNA expression observed by *in situ* hybridization. The discrepancy could be due to the fact that the fluorescent signal from GFP is more sensitive than staining from the whole-mount *in situ* hybridization. In addition, for microinjection, a large number of copies (10^6) of the transgene were injected into zebrafish embryos. Compared with the *myostatin* mRNA expression from the two copies of the endogenous gene, GFP expression from the injected transgene is expected to be significantly stronger. Moreover, it cannot be ruled out that the *myostatin* 5′ flanking sequence used in this study lacks some of the inhibitory elements that restrict high levels of *myostatin* expression in early stage embryos.

Myostatin expression is restricted to specific types of muscle fibers **(**Kambadur et al., 1997; Ji et al., 1998; Kocamis et al., 1999; Roberts and Goetz, 2001; Rescan et al., 2001**)**. Because of the mosaic nature of the transient expression assay, it was difficult to determine if the expression of the *myostatin-GFP* transgene was restricted to certain types of myofibers in zebrafish embryos. Future studies are required to generate the *myostatin-GFP* transgenic model that could be used to study the expression and regulation of *myostatin* expression in zebrafish and to clarify whether its expression in zebrafish is restricted to certain types of myofibers during development and growth.

We would like to thank Bosheng Zhang and Tracy Robinson for assistance in screening the transgenic fish, Se-Jin Lee (Johns Hopkins University), Phil Hamilton and Bob Curtis (Cape Aquaculture Technologies) for helpful discussions during the execution of this project, and John Stubblefield for editorial assistance. We would also like to thank the two anonymous reviewers for their very constructive comments on the manuscript. This work was supported in part by a NIH grant to SJD (RO1GM58537) and a research contract from Cape Aquaculture Technologies. This publication is contribution number 04-606 from the Center of Marine Biotechnology, University of Maryland Biotechnology Institute.

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