**論 文 要 旨**

**Background**

Myostatin, also known as growth differentiation factor 8 (GDF-8), is a member of the transforming growth factor-β (TGF-β) family that functions as a negative regulator of skeletal muscle development and growth in mammals. A model for the biological activity of myostatin has been proposed in that myostatin inactivates a specific group of genes, and a negative correlation between myostatin expression and growth/number of muscle fibers has been observed. It is widely known that myostatin-knockout mice exhibit a dramatic increase of skeletal muscle mass that results from a combination of hyperplasia and hypertrophy. Natural mutation of the myostatin gene has been observed in the double muscle breeds of Belgian Blue and Piedmontese cattle, which have significantly more muscle mass than normal breeds do. Myostatin is found almost exclusively in the skeletal muscle in mammals, but appears more ubiquitously in fish, suggesting more diverse functions in their growth and development. A single encoding gene in mammals expresses myostatin, whereas teleost fish possess at least two myostatin genes that are differentially expressed in both muscular and non-muscular tissues. Recently, it was reported that silencing of the myostatin genes resulted in a giant phenotype in zebrafish, and a dominant-negative form of myostatin resulted in the doubling of muscle-fiber number in transgenic medaka.

Myostatin genes have been characterized in several commercially important fishes such as striped

bass, white perch, Mozambique tilapia, white bass, Atlantic salmon, rainbow trout, gilthead sea bream, shi drum, catfish species, European sea bass, Croceine croaker, orange spotted grouper, and Japanese sea perch. Worldwide fish consumption has been steadily increasing, and strategies for enhancing skeletal muscle growth of aquaculture species can help to meet the increasing demand for this protein source. It is quite important to determine how myostatin gene expression is regulated and to uncover the protocol that controls the action of myostatin. To gain better understanding of the mechanisms regulating myostatin gene expression, we have analyzed the genomic structures of the myostatin gene in a sea bass, *Lateolabrax japonicus*, spanning primary DNA sequences to higher-ordered structures (chromatin and DNA methylation). We have also examined the transient expression of green fluorescent protein in the embryos of medaka (*Oryzias latipes*) driven by a myostatin promoter isolated from a sea bream (*Acanthopagrus latus*). This trial will assist us in evaluating the environmental effect on myostatin expression in future studies using transgenic medaka fish.

## Results and Discussion
### (1) Methylation status of myostatin gene promoter region in *Lateolabrax japonicus*

DNA methylation at cytosine residues is involved in epigenetic regulation to suppress gene expression through heterochromatinization. However, the methylation status in fish genomes remains unclear. To examine the methylation status in myostatin promoter regions of *Lateolabrax japonicus*, total genomic DNA was isolated from tissues of the brain, kidney, spleen, liver, heart, eye, muscle, intestine, and gill. The genomic DNA samples were digested with a restriction enzyme and subjected to bisulfite modification. The bisulfite treatment introduces specific changes in the DNA sequence that depend on the methylation status of individual cytosine residues, yielding information on the DNA segments' methylation status. DNA fragments were treated with sodium bisulfite to convert unpaired cytosine residues to uracil under conditions whereby methylated cytosine remained essentially intact. The modified DNA was used as the PCR template to specifically amplify the sense and antisense strands of bisulfite-treated DNA with their respective PCR primer pairs. The PCR products from each reaction were subjected to a second PCR amplification with nested primers to obtain the DNA fragments that originated from each strand. They were cloned into a plasmid vector and the nucleotide sequences were determined. The efficiency of the bisulfite modification (conversion of C to T) was estimated by counting the numbers of cytosine residues remaining at CpN sites (in the case of N ≠ G), and the conversion frequency was found to be 99% or higher. The number of cytosine residues remaining at CpG sites appeared to be slightly higher than that in the background level in sense strands from the eye and heart, but were identical to that in the background level in complementary strands from the same tissues. The frequency of methylated cytosine was very low in the tissues examined (within the error range of random sampling) regardless of the level of myostatin gene expression. The results may mean that the methylation at CpG is not involved in regulation of the myostatin gene in *L. japonicus*.

**(2) Probing the chromatin structures in *L. japonicus***

Gene expression also reflects the status of the chromatin (euchromatin or heterochromatin) in the region where the gene is situated. The status of the chromatin in particular gene regions is dynamically modulated to control gene expression and other fundamental cellular processes such as proliferation and differentiation. Active chromatin can be distinguished from bulk chromatin by its increased susceptibility to endonuclease. Micrococcal nuclease (MNase) preferentially cleaves chromatin between nucleosomes (naked DNA regions). To examine tissue-specific chromatin structures in myostatin gene regions by MNase as an enzymatic probe, nuclei were isolated from some *L. japonicus* organs (the liver, eye, kidney, brain, and heart), purified, and treated with MNase. The mixtures were incubated at 37°C for 15, 30, and 60 min, and one-third of the original reaction volumes were collected at each time point. The DNA was isolated from each reaction mixture and subjected to Southern blot hybridization analysis after restriction digestion. A fragment of the *L. japonicus* myostatin promoter region was used as a probe to detect an approximately 2 kb *Taq*I restriction fragment. Myostatin gene promoter regions in the brain and eye were highly susceptible to MNase while those in the heart were less susceptible, and those in the kidney and liver were even more resistant to MNase. The results suggest that the myostatin gene is compacted into heterochromatin in tissues where it is not expressed, such as in the liver and kidney, whereas it is susceptible to MNase in the eye and brain, which do express myostatin. According to the results of methylation analysis and MNase probing, DNA methylation may not be involved in regulating heterochromatinization and thus, expression of myostatin, in *L. japonicus*.

**(3) Genomic sequences of myostatin gene promoter regions in Sparidae fishes**

The 5′-flanking regions of the myostatin gene were isolated from two sea breams, *Acanthopagrus latus* and *Acanthopagrus schlegelii*, by inverse PCR. Genomic DNA of *A. latus* and *A. schlegelii* were digested with restriction enzymes and the fragments were self-circularized under low DNA concentration. The closed circular DNA molecules were used as the template for PCR with a set of DNA primers for the first-round PCR, after which nested PCR was performed with another set of DNA primers. The PCR products were cloned into a plasmid vector and sequenced by the primer-walking strategy with internal primers. After the entire sequence was determined, a genomic DNA fragment was amplified with 5′- and 3′-end primers to confirm the genomic sequences. The partial-coding regions of cytochrome oxidase subunit I (COI) and 18S rRNA from the specimens were also amplified and sequenced to confirm identity of species.

Two alleles of the myostatin gene were identified for both *A. latus* and *A. schlegelii*. The nucleotide sequences were aligned with the promoter region of the *Sparus aurata* myostatin gene, showing that the conserved regions spanned approximately 1 kb. The potential *cis*-acting elements were observed in highly conserved regions between these two Sparidae species. They contain two putative TATA-boxes, one CAAT box, and seven putative E-boxes. Comparative analysis of

myostatin gene regulatory regions with those of other Percoidei fishes revealed the occurrence of highly conserved regions approximately 300 bp upstream of the translation start site.

**(4) Evaluation of promoter activity in transient expression of GFP in medaka embryos**
The genomic fragment was connected to the AcGFP coding sequence to evaluate the activity of the promoters isolated from *A. latus*. The recombinant fragments were cloned into a plasmid vector. Six different myostatin fragments in total, truncated at their 5′ ends, were constructed. Some were used for microinjection into the cytoplasm of fertilized medaka fish at the one-cell stage. Embryos were observed under a fluorescence microscope to visualize GFP expression. The expression of the AcGFP driven by the *A. latus* myostatin promoter in the medaka embryos was weak but distinctive, with a series of truncated fragments. Based on the current available results, we believe that the genomic fragment obtained from *A. latus* has minimal promoter activity in medaka embryos. The *A. latus* promoter might not have worked well in medaka because medaka (Beloniformes) is distantly related to sea bream (Perciformes) evolutionarily, or the *A. latus* DNA fragment used may have been not enough to express full promoter activity.

**Conclusions**

**It is challenging to clarify the regulatory mechanisms of the myostatin gene in fish. Myostatin expression is suppressed parallel to heterochromatinization but regardless of DNA methylation in *L. japonicus*. Sequencing of 5′-flanking DNA fragments of the myostatin-coding region in *A. latus* identified several potential *cis*-elements and higher similarity to the myostatin gene promoter in other fish species. Weak promoter activity of DNA fragments isolated from *A. latus* was observed in medaka embryos in a transient expression assay. This assay system may allow us to evaluate the role of promoter elements *in vivo*. Establishing a transgenic medaka carrying AcGFP driven by an *A. latus* myostatin promoter will be extremely valuable to efforts to examine expression profiles in response to environmental stimuli under culture conditions in the future.**

# 審査結果の要旨

　ミオスタチンは、骨格筋の成長増殖を抑制する因子のひとつである。主として骨格筋のみでこれを発現する哺乳類と異なり、魚類では広範な組織においてその発現が認められることから、多様な機能が予想されている。ゼブラフィッシュやメダカ等の小型魚類モデル生物を用いた先行研究では、ミオスタチン機能の抑制による個体の大型化や筋繊維数の増大などの効果が報告されている。

　本研究では、**Chapter 1** において、スズキ *Lateolabrax japonicus* のミオスタチン遺伝子領域の **DNA** のメチル化とクロマチン構造について解析した。**DNA** のメチル化は、哺乳類（**CpG**

部位の C のメチル化）、および高等植物（CpNpG 部位の C のメチル化）において、遺伝子の不活化等との関連が調べられているが、エピジェネティックな発現調節機構との関わりを直接示す報告は魚類ではほとんどない。そこで、スズキのミオスタチン遺伝子転写調節領域のうち近縁種間で高度に保存されている領域のメチル化状態を調べたところ、有意な CpG 部位のメチル化は検出されなかった。一方、MNase 限定分解によるクロマチン構造解析を行ったところ、ミオスタチン発現組織において MNase 感受性が高く、非発現組織においては分解抵抗性を示した。これらのことから、スズキにおけるミオスタチンの組織特異的発現には、ヘテロクロマチン化による発現制御がなされており、DNA メチル化の関与はないことが示唆された。

Chapter 2 において。キチヌ *Acanthopagrus latus* およびクロダイ *A. schlegelii* のミオスタチン遺伝子転写調節領域を単離し、その一次構造を決定した。近縁種のヨーロッパヘダイ *Sparus aurata* における既知のミオスタチン遺伝子構造との比較により、翻訳開始点から上流約 1kb にわたる領域が保存されており、また、様々な転写因子に対する相互作用部位の存在が示唆された。続いて、キチヌから得た DNA 断片を緑色蛍光タンパク質遺伝子へとつないでメダカ胚における発現実験を行ったところ、転写活性が観察された。今後の遺伝子導入メダカ個体の作出と、それを用いることによる養殖研究への応用が期待される。

本研究対象の魚種は、日本のみならず世界中で重要な食用魚および遊漁対象魚種として経済的にも注目されている。本研究は、ミオスタチン遺伝子発現制御機構の一端を解明する重要な成果とともに、優良な形質をもった系統の育種や養殖・畜養法の開発に資する基礎研究としても有用な成果を含むものであり、当審査委員会は、本論文が博士（理学）の学位を授与するにふさわしいと判断した。