Interaction between the GH-IGF axis and myostatin in regulating muscle growth in *Sparus aurata*

IS-3703-05

Final scientific report
Interaction between the GH-IGF axis and myostatin in regulating muscle growth in *Sparus aurata*

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Cover Page

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Project Title: Interaction between the GH-IGF axis and myostatin in regulating muscle growth in *Sparus aurata*

Investigators

Principal Investigator (PI):
Bruria Funkenstein

Co-Principal Investigator (Co-PI):
Shaojun (Jim) Du

Institutions

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COMB, UMBI, Baltimore, Maryland, USA

Collaborating Investigators:

Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.
aquaculture, sea bream, promoter, reporter gene, polymorphism, MyoD, myogenin

Abbreviations *commonly* used in the report, in alphabetical order: FBS (fetal bovine serum), GH (growth hormone), IGF (insulin-like growth factor), IB (inclusion bodies), Myostatin (MSTN), TF (transcription factor).

Budget: IS: $191,000 US: $109,000 Total: $300,000

Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution
**Publication Summary** (numbers)

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**Postdoctoral Training:** List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

**Cooperation Summary (numbers)**

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**Description Cooperation:**

This research project was based on a close collaboration between the research groups at IOLR, Israel and at COMB, UMBI, Baltimore. *Sparus aurata* MSTN promoters was cloned in BF’s lab and then ligated to a GFP containing vector that was provided by JD. Large quantities of DNA constructs containing saMSTN-2 promoter and GFP were prepared at IOLR and sent to JD for analysis using transient expression in zebrafish embryos. In addition, as planned, JD’s lab cloned saMyoD2 in an expression vector and sent the constructs to BF, to be used in co-transfection experiments in Haifa.

The two partners communicated with each other on a regular basis by e-mail and the reports were prepared together. During the third year of the project, the Israeli PI visited JD’s lab at COMB. The two partners met also as part of their UMBI-BARD project in Israel and in Baltimore which strengthened their collaboration. Materials and information were exchanged often, when needed. The final report submitted hereby was prepared by the two partners from IOLR and from COMB.

**Patent Summary** (numbers)

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Abstract

Growth rate of cultured fish from hatching to commercial size is a major factor in the success of aquaculture. The normal stimulus for muscle growth in growing fish is not well understood and understanding the regulation of muscle growth in fish is of particular importance for aquaculture. Fish meat constitutes mostly of skeletal muscles and provides high value proteins in most people’s diet. Unlike mammals, fish continue to grow throughout their lives, although the size fish attain, as adults, is species-specific.

Evidence indicates that muscle growth is regulated positively and negatively by a variety of growth and transcription factors that control both muscle cell proliferation and differentiation. In particular, growth hormone (GH), fibroblast growth factors (FGFs), insulin-like growth factors (IGFs) and transforming growth factor-β (TGF-β) play critical roles in myogenesis during animal growth.

An important advance in our understanding of muscle growth was provided by the recent discovery of the crucial functions of myostatin (MSTN) in controlling muscle growth. MSTN is a member of the TGF-β superfamily and functions as a negative regulator of skeletal muscle growth in mammals. Studies in mammals also provided evidence for possible interactions between GH, IGFs, MSTN and the muscle-specific transcription factor MyoD with regards to muscle development and growth.

The goal of our project was to try to clarify the role of MSTNs in Sparus aurata muscle growth and in particular determine the possible interaction between the GH-IGF axis and MSTN in regulating muscle growth in fish. The steps to achieve this goal included: i) Determining possible relationship between changes in the expression of growth-related genes, MSTN and MyoD in muscle from slow and fast growing sea bream progeny of full-sib families and that of growth rate; ii) Testing the possible effect of over-expressing GH, IGF-I and IGF-II on the expression of MSTN and MyoD in skeletal muscle both in vivo and in vitro; iii) Studying the regulation of the two S. aurata MSTN promoters and investigating the possible role of MyoD in this regulation.

The major findings of our research can be summarized as follows: 1) Two MSTN promoters (saMSTN-1 and saMSTN-2) were isolated and characterized from S. aurata and were found to direct reporter gene activity in A204 cells. Studies were initiated to decipher the regulation of fish MSTN expression in vitro using the cloned promoters; 2) The gene coding for saMSTN-2 was cloned. Both the promoter and the first intron were found to be polymorphic. The first intron zygosity appears to be associated with growth rate; 3) Full length cDNA coding for S. aurata growth differentiation factor-11 (GDF-11), a closely related growth factor to MSTN, was cloned from S. aurata brain, and the mature peptide (C-terminal) was found to be highly conserved throughout evolution. GDF-11 transcript was detected by RT-PCR analysis throughout development in S. aurata embryos and larvae, suggesting that this mRNA is the product of the embryonic genome. Transcripts for GDF-11 were detected by RT-PCR in brain, eye and spleen with highest level found in brain; 4) A novel member of the TGF-β superfamily was partially cloned from S. aurata. It is highly homologous to an unidentified protein (TGF-β-like) from Tetraodon nigroviridis and is expressed in various tissues, including muscle; 5) Recombinant S. aurata GH was produced in bacteria, refolded and purified and was used in in vitro and in vivo experiments. Generally, the results of gene expression in response to GH administration in vivo depended on the nutritional state (starvation or feeding) and the time at which the fish were sacrificed after GH administration. In vitro, recombinant saGH activated signal transduction in two fish cell lines: RTH149 and SAF1; 6) A fibroblastic-like cell line from S. aurata (SAF-1) was characterized for its gene expression and was found to be a suitable experimental system for studies on GH-IGF and MSTN interactions; 7) The gene of the muscle-specific transcription factor Myogenin was cloned from S. aurata, its expression and promoter activity were characterized; 8) Three genes important to myofibrillogenesis were cloned from zebrafish: SmyD1, Hsp90a1 and skNAC.

Our data suggests the existence of an interaction between the GH-IGF axis and MSTN. This project yielded a great number of experimental tools, both DNA constructs and in vitro systems that will enable further studies on the regulation of MSTN expression and on the interactions between members of the GH-IGF axis and MSTN in regulating muscle growth in S. aurata.
**Achievements**

Acheivements: The goal of the original research proposal was to try to clarify the role of MSTNs in *Sparus aurata* muscle growth and in particular determine the interaction between the GH-IGF system and MSTN in regulating muscle growth.

During the three years of this project, we fulfilled part of this general objective and also investigated aspects that were not included in the original project in light of the results that were obtained during the project.

The achievements and their significance are described below:

1. Several genomic fragments of *S. aurata* Myostatin-1 (saMSTN-1) promoter were cloned. MSTN promoter activity was determined, for the first time in a fish species, by a quantitative reporter gene assay using the A204 rhabdomyosarcoma cell line and the luciferase reporter gene. This quantitative *in vitro* system allowed us to determine the possible regulation of MSTN promoter activity by glucocorticoids, cAMP and MyoD and will enable us in the future to continue our search for other regulatory factors that might affect fish MSTN expression in fish. The *in vitro* system will also be useful in testing factors that inhibit MSTN promoter activity.

2. Several genomic fragments of the second MSTN gene promoter (saMSTN-2) were also cloned. As for saMSTN-1 promoter, the promoter activity was determined by a quantitative reporter gene assay, using the A204 cells and luciferase reporter gene and was found to be several folds more active in A204 cells than saMSTN-1 promoter. Promoter activity was tested in two additional cell lines: Chinese hamster ovary, CHO K1 cells, and a neuronal cell line PC12, which is a cancer cell line derived from a pheochromocytoma of the rat adrenal medulla. The latter cell line was included since MSTN-2 is highly expressed in *S. aurata* brain. Comparison between the two promoters revealed differences in their sequence and structure organization. As for saMSTN-1 promoter, the quantitative *in vitro* assay provided us with a system in which we could test the effects of glucocorticoids and MyoD on promoter activity and will be useful in future studies of searching for other regulatory factors.

3. The gene coding for saMSTN-2 was cloned. The gene consists of three exons and two introns. Sequence analysis of the promoter and of the first intron revealed the presence of polymorphism of both the promoter and the first intron. Analysis of two groups of fish, small and large, of a population from a fish farm that underwent several round of selection for body mass, revealed an association between the zygosity and body weight. This novel finding suggests that MSTN-2 gene might be useful as a genetic marker in growth selection program of *S. aurata*.

4. Recombinant *S. aurata* growth hormone (saGH) was produced in bacteria, refolded and purified and was used for *in vivo* and *in vitro* studies.
5. The SAF-1 cell line, which is derived from the caudal fin tissue of *S. aurata* and displays fibroblast-like morphology, was found suitable for studying the interactions between the growth hormone-insulin-like growth factor (GH-IGF) axis and myostatin (MSTN) as gene expression analysis showed that these cells express IGF-II, IGF type 1 receptor (IGF-1R), GH receptor (GHR) and MSTN-1. In particular, the discovery that SAF-1 express both receptors GHR and IGF-1R, provides a unique homologous *in vitro* system to test effects of GH, IGF-I and IGF-II on cell proliferation and gene expression. Accordingly, we have initiated studies on the effect of recombinant saGH on gene expression in these cells.

6. *In vivo* studies on the effect of GH on GH-IGF axis and MSTN gene expression in muscles and livers from juvenile *S. aurata*. Expression and response to GH depended on the experimental protocol used. Few genes appear to be elevated after GH treatment (MSTN and IGF-1R in muscle), decreased (MSTN in red muscle) or unchanged. Nutritional status (starvation or feeding) prior to injection, and time of sacrifice after GH administration, should be taken into account when testing the effect of GH *in vivo* on gene expression.

7. MyoD2, a myogenic transcription factor, was cloned in an expression vector. Co-transfection experiments together with saMSTN-1 and saMSTN-2 promoter constructs suggest a minor effect of this transcription factor on MSTN expression in the *in vitro* system utilized by us. It is possible the protein produced is not biologically active and further studies should be carried out to determine the role of MyoD in regulating fish MSTN promoter activity.

8. Isolation and characterization of myogenin gene from *S. aurata*. Myogenesis of skeletal muscles in vertebrates is controlled by members of the myogenic regulatory family, including MyoD, Myf5 and Myogenin. To characterize the gene structure and expression of fish myogenin, we have isolated the myogenin genomic gene and cDNA from *S. aurata* and analyzed the genomic structure, pattern of expression and the regulation of muscle-specific expression.

9. Isolation and characterization of SmyD1, a key regulator of myofibrillogenesis in skeletal muscles of zebrafish. SmyD1 is a recently identified protein that is specifically expressed in skeletal and cardiac muscles. Its biological function in muscle formation is unclear. We demonstrated for the first time that SmyD1 is a histone methyltransferase that controls muscle development by histone methylation. Knockdown of SmyD1 resulted in paralyzed fish without muscle contraction in skeletal and cardiac muscles.

10. Functional analysis of heat shock protein 90a1 (Hsp90a1) in myofibrillogenesis of skeletal muscles in zebrafish. Hsp90a1 is specifically expressed in skeletal and cardiac muscles. Its biological function in muscle development is unknown. By using the knockdown approach, we demonstrated that Hsp90a1 is required for assembly of myosin thick filaments in skeletal muscles. To our knowledge, this is the first report of Hsp90a1 function in skeletal muscles *in vivo*.

11. Cloning and characterization of *sknac* (Skeletal *naca*, nascent polypeptide-associated complex alpha) gene from zebrafish. The *sknac* knockdown embryos showed a paralyzed phenotype with little muscle
contraction and contained disorganized thick and thin filaments. Myosin protein levels were significantly reduced. These results demonstrate that skNAC plays a vital role in myofibril assembly and function during muscle cell differentiation.

12. GDF-11 (growth differentiation factor-11), a closely related peptide to MSTN, was cloned from *S. aurata* brain. Its sequence analysis revealed high conservation throughout vertebrate evolution with only 3 amino acids difference between fish and mammalian mature GDF-11. It is highly expressed in the brain (as is saMSTN-2) and is found during early embryonic stages. This new cDNA will enable us to compare the functions of GDF-11 and MSTN-2 in fish development. It will also enable us to produce this growth factor and investigate the role of its prodomain in refolding mature GDF-11.

13. A novel TGF-β (TGF-β6) was partially cloned from *S. aurata* larvae. The novel cDNA shows high homology with a putative TGF-β from *Tetraodon nigroviridis* and with one of the two TGF-β2-like peptides reported in Genbank for zebrafish. TGF-β6 is expressed in various tissues including muscle and SAF-1 cells. Since both TGF-β2 and TGF-β3 are involved in myogenesis in mammals, the new clone will provide us with a new potential regulatory factor in myogenesis also in fish.

**Agricultural and economic impacts:** The research activities during this project provided us with molecular tools and an *in vitro* system that enabled us to start dissecting the regulation of MSTN expression in fish using the genomic fragments of the promoters of the two MSTN genes which are expressed in *S. aurata* fish. To gain insight into the regulation of MSTN gene expression in fish and determine if MSTN can serve as a genetic marker in marker-assisted selection of cultured *S. aurata* fish, we also cloned and characterized the MSTN-2 gene. PCR amplification of genomic DNA from cultured *S. aurata* populations combined with sequence analysis, revealed the existence of at least three variants of the promoter, although one is the most represented. Intron-targeted PCR of genomic DNA from two groups of fish, large and small fish, from a cultured population, revealed differences in the distribution of the intron alleles. The two most common alleles were found at the same frequency in both populations; however, their zygosity state was different. In the large fish a similar proportion of heterozygotes and homozygotes were found, while in the small fish 3-fold more homozygotes were found compared to heterozygotes. The recombinant saGH injected *in vivo* enabled us to test *in vivo* the regulation of MSTN by GH. A second homologous *in vitro* system (SAF-1 cells) that was characterized by us during this project showed its usefulness for analysis of the inter-relation between the GH-IGF and MSTN since we showed that these cells express the receptor for IGFs (IGF type 1 receptor) and GH receptor. The sequence information provided us with the possibility to assess the degree of conservation of 1) Proximal promoter of the MSTN-1 gene in Perciformes; 2) the conservation of a MSTN-related peptide, GDF-11, along the evolution of vertebrates in general, and fish in particular. In addition, our cloning of a novel TGFβ which is expressed also in skeletal muscle and in the SAF-1 cells provide new
targets for manipulating gene function to stimulate fish muscle growth. Finally, our findings of SmyD1, Hsp90a1 and skNAC functions in skeletal muscles provide new target for manipulating gene function to stimulate fish muscle growth.

**Cooperation:** This research project was based on a close collaboration between the research groups at IOLR, Israel and at COMB, UMBI, Baltimore.

*Sparus aurata* MSTN promoters was cloned in BF’s lab and then ligated to a GFP containing vector that was provided by JD. Large quantities of DNA constructs containing saMSTN-2 promoter and GFP were prepared at IOLR and sent to JD for analysis using transient expression in zebrafish embryos. In addition, as planned, JD cloned saMyoD2 in an expression vector and sent the constructs to BF’s lab, where it was used in the co-transfection *in vitro* experiments together with the MSTN constructs.

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**List of publications:**


Papers presented in conferences, meetings and seminars (national and international):


3. **Du, S. J.** Zebrafish as a model for uncovering gene functions involved in muscle and bone development. 8th IMBC meeting, March 11-16, Eilat, Israel 2007.


