UNIVERSITAT DE BARCELONA

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CONTROL DE LA MIOGÈNESI EN PEIXOS:

FUNCIONS DE LA MIOSINA, L'IGF-II

I ELS FACTORS REGULADORS MIOGÈNICS

Tesi Doctoral

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Memòria presentada per
Marta Codina Potrony
per optar al grau de
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Expressió de Miogenina durant el desenvolupament muscular en embrions i en cèl.lules musculars en cultiu de l'orada (*Sparus aurata*). Relació amb MyoD2, Miostatina i IGF-II

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Resum

El procés de miogènesi està controlat per nombroses senyals extracel·lulars juntament amb factors de transcripció intracel·lulars, l'expressió coordinada dels quals és crítica pel desenvolupament adequat de les fibres musculars. En aquest estudi, hem estudiat el patró d'expressió de diversos d'aquests elements reguladors, com ara els factors de transcripció MyoD2 i Miostatina, o els factors secretats Miostatina i IGF-II, en el procés de diferenciació de mioblasts des de cèl·lules satèl·lit fins a miotubs multinucleats i també en embrions en desenvolupament durant la somitogènesi i fins a l'eclosió en orada (*Sparus aurata*). És important destacar que en aquest estudi també hem validat el Factor d'elongació 1 alfa com a un adequat gen normalitzador per al cultiu primari de cèl·lules musculars d'orada, permetent determinar el patró d'expressió de diferents gens per PCR quantitativa en temps real.

En els embrions, el trànscrit de Miogenina no es va detectar fins el començament de la somitogènesi, i es va trobar la seva màxima expressió a l'estadi corresponent a uns 16 somites (aproximadament 27 hores post fertilització). L'IGF-II es va expressar al llarg de tot el desenvolupament embrionari, tot i que la major expressió es va assolir durant l'eclosió. En cèl·lules musculars en cultiu d'orada, el MyoD2 es va expressar principalment en la fase proliferativa, mentre que els nivells de Miogenina van experimentar un increment durant la diferenciació. L'expressió d'IGF-II va ser màxima en estadis primerencs, disminuint al llarg del cultiu, i no es va trobar una correlació clara entre els seus nivells i els dels MRFs analitzats. Els nivells de miostatina es van mantenir baixos durant tot el procés de diferenciació des de cèl·lules satèl·lit fins a miotubs.

Expression of Myogenin during muscle development in embryos and muscle cells in primary culture of seabream (*Sparus aurata*). Relationship with MyoD2, Myostatin and IGF-II

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ABSTRACT

Myogenesis is controlled by numerous extracellular signals together with intracellular transcriptional factors, whose coordinated expression is critical for the appropriate development of muscle fibres. Here we examine the expression pattern of several of these regulatory elements, such as the transcriptional factors MyoD2 and Myogenin, or the secreted factors Myostatin and IGF-II, in differentiating myoblasts from satellite cells to multinucleated myotubes, as well as in developing embryos during somitogenesis until hatching in seabream (*Sparus aurata*). Importantly, we have validated the elongation Factor 1 alpha as an appropriate house-keeping gene for primary culture of seabream muscle cells, enabling us to determine the expression patterns of the different genes by quantitative Real-Time PCR. In the embryos, Myogenin transcript was not detectable until the beginning of somitogenesis, and we found the maximal expression around the 16-somite stage (approximately 27 hpf). IGF-II was expressed throughout embryo development, although the highest expression was reached at hatching. In seabream muscle cells in culture, Myogenin levels were up-regulated during differentiation, whereas MyoD2 was expressed mostly during the proliferating phase. IGF-II expression was maximal at early stages and decreased along the culture, and we could not find a clear correlation between its levels and those of the analyzed MRFs. Low myostatin levels were found during the differentiation process from satellite cells to multinucleated myotubes. Together, these data help to gain insight into the molecules involved in seabream muscle development *in vitro*.

Short title: MyoD2, Myogenin and IGF-II in developing muscle of seabream

Key words: MyoD2, Myogenin, Myostatin, IGF-II, seabream, muscle, differentiation

INTRODUCTION

In vertebrates, the process of myogenesis involves a series of events in which precursor cells are committed into a myogenic lineage (myoblasts), which proliferate and differentiate by exiting the cell cycle and fusing to form multinucleated myotubes (reviewed by Johnston, 2006).

This process is controlled by numerous extracellular signals together with intracellular factors. Among them, the Myogenic Regulatory Factors (MRFs), whose sequences are highly conserved among amniotes and teleosts, play an essential role for the specification and determination of the muscle cell lineage, acting as transcriptional factors (Holterman and Rudnicki, 2005). This family consists of MyoD, myogenin, myf5 and MRF4, and it belongs to a larger group of proteins that share a highly conserved central region termed the basic helix-loop-helix (bHLH domain), containing a basic DNA-binding motif and a helix-loop-helix dimerization domain. bHLH Dimers specifically bind E-box elements (consensus sequence CANNTG) found in the promoter and enhancers of most, if not all, muscle-specific genes.

During myogenesis, the different MRFs are expressed in a sequential-manner. Thus, Myf5 and MyoD are required for the initial determination of the myogenic lineage, while myogenin and MRF4 are activated during myoblast differentiation and cell fusion. Gene knockout studies in mice show that lack of MyoD and Myf5 results in failure of myoblast formation, and a consequent lack of all head and trunk skeletal muscle (Rudnicki et al., 1993). In zebrafish, using a morpholino for Myf5, defects in myogenesis such as diffused somite boundaries were induced (Chen and Tsai, 2002). On the other hand, in myogenin knockout mice, myoblasts form in the correct place but fail to fuse into muscle fibers (Hasty et al., 1993; Nabeshima et al., 1993; Venuti et al.,

1995). However, other studies showed that the Myogenin itself inefficiently induces skeletal muscle differentiation in culture (Bergstrom and Tapscott, 2001; Gerber et al., 1997; Roy et al., 2002). The function of MRF4 is less clear, as the inactivation of this gene also leads to defects on Myf5 production (Summerbell et al., 2000; Summerbell et al., 2002); more recent studies indicate that MRF4 may also play a key role in the initial myoblast specification (Kassar-Duchossoy et al., 2004).

The four bHLH myogenic genes MyoD, myogenin, myf5 and MRF4 have homologues in all fish species studied, such as zebrafish, trout, carp or seabream among others, and for some of these genes two different isoforms exist. Due to fish genome duplication, in seabream and trout two isoforms of MyoD are present (Chen and Tsai, 2002; Rescan and Gauvry, 1996), with different expression patterns, while a single myogenin gene has been described up to now (Codina et al., 2008a; Rescan et al., 1995). In contrast, zebrafish has a single copy of both MyoD and Myogenin (Weinberg et al., 1996).

The spatial expression pattern among the species may differ. In seabream, MyoD2 accumulates concretely in fast muscle (Tan and Du, 2002) and Myogenin is present in both fast and slow muscles (Codina et al., 2008a), whereas in trout, TMyoD2 and TMyogenin are specifically expressed in slow muscle (Rescan, 2001).

Several studies demonstrate a sequential expression pattern for these genes. In seabream embryos, expression of both MyoD1 and MyoD2 begins before expression of Myogenin (Codina et al., 2008a; Tan and Du, 2002), although it seems Myogenin is expressed earlier than seen in trout, where Myogenin expression also followed that of MyoD, and was expressed only after the formation of the first somites (Delalande and Rescan, 1999; Steinbacher et al., 2007) found that. In zebrafish embryos, MyoD and Myf5

transcripts are detectable 3 hours before Myogenin expression starts (Chen et al., 2000; Weinberg et al., 1996).

A sequential activation of MyoD followed by Myogenin has also been described in differentiating trout myogenic cells in culture (Rescan et al., 1995; Rescan et al., 1994). In this system, Myogenin transcript could be detected after 48h of seeding, and its expression increased significantly after 7 and 11 days in culture (Rescan et al., 1995).

Apart from the MRFs, other factors are involved in the regulation of muscle growth. Myostatin (MSTN) belongs to the transforming growth factor beta (TGF-b) superfamily of signalling molecules, and is a negative regulator of muscle mass. *In vivo*, $MSTN^{-/-}$ mice showed a hypertrophic and hyperplasic muscle phenotype (McPherron et al., 1997), and *in vitro* it has been observed that MSTN is able to inhibit myoblast proliferation in C2C12 cell line (Thomas et al., 2000) and activate differentiation by downregulating the expression of MyoD and Myf5 in chicken embryonic myoblasts (Yang et al., 2005). *In vitro* studies of chicken myoblasts suggest that the highest expression of MSTN occurs during differentiation and fusion (Kocamis et al., 2001). Similar results were found in rat soleus muscle, where MSTN presented low levels in the areas with abundance of satellite cells and actively proliferating myoblasts (Kirk et al., 2000).

Rainbow trout has two different genes for myostatin (Rescan et al., 2001), with different expression patterns (Johnston, 2006). However, recently, and based in a phylogenetic analysis, Garikipati et al. (2007) have suggested that salmonids should possess four distinct genes for MSTN. In seabream, Maccatrozzo and collaborators (2001) found a single copy of MSTN, with a wider expression than the found in mammals.

Hystochemical studies in seabream explants have suggested that the MSTN transcript is present only after 8 days of culture (Funkenstein et al., 2006) .

Other important molecules are the members of the Insulin Growth Factor family, including IGF-I, IGF-II, their receptors and a series of IGF binding proteins (IGFBPs). In mammals it has been seen IGFs regulate muscle mass and fibre size (Barton-Davis et al., 1999; Velloso, 2008); they do so by activating the Akt-mTOR signalling pathway, which has an important role regulating protein synthesis and degradation in fish muscle (Johnston, 2006).

In fish, the implication of IGFs in processes of muscle growth has been widely studied *in vivo*. A positive correlation between expression of IGF-I and IGF-II with the nutritional status has been shown in seabream (Meton et al., 2000; Perez-Sanchez et al., 1995) and trout (Chauvigne et al., 2003; Gentil et al., 1996), and an increase in plasma levels of IGF-I has been observed in periods of increased growth in seabream (Mingarro et al., 2002) and coho salmon (Fukada et al., 2004). More recently, IGF-II has been postulated as a promoter factor throughout the whole life cycle of seabream (Benedito-Palos et al., 2008).

Previous studies in trout and seabream from our group revealed that both IGF-I and IGF-II have a role in muscle cell proliferation and metabolism when cultured *in vitro* (Castillo et al., 2004; Codina et al., 2008b; Montserrat et al., 2007b) and also a role activating signalling pathways such as MAPK and PI3K/Akt (Castillo et al., 2006; Codina et al., 2008b).

Although a volume of information is available on the mechanisms of IGFs, MRFs and MSTN in the activation and control of myogenesis, little is known about the relationship between these molecules and their co-expression during myogenesis in

seabream. In this study we used quantitative Real-Time PCR together with semiquantitative PCR and *in situ* hybridization to determine the expression pattern of several of these genes throughout seabream embryo development and in differentiating muscle cells in primary culture and elucidate possible correlations.

MATERIAL AND METHODS

Animals

Gilthead Seabream (*Sparus aurata*) juveniles were obtained from Aquamar S.L. (San Fernando, Cádiz, Spain) and maintained in the facilities of the Servei d'Estabulari of the Faculty of Biology at the University of Barcelona, in a closed-water flow circuit with water at a temperature of 21 ± 1 ºC. Fish were fed with a commercial diet and fasted for 24h previously to the isolation of muscle cells.

Gilthead Seabream embryos were obtained from IRTA Sant Carles de la Ràpita and kept in the facilities of the Faculty of Biology at the University of Barcelona in aerated water at room temperature (21-23ºC) for 60h.

All animal handling procedures were approved by the Ethics and Animal Care Committee of the University of Barcelona, following the EU, Spanish and Catalan Government-established norms and procedures.

Primary cultures and cell lines

To establish primary cultures of seabream muscle cells, fish (30 to 50 for each culture) with an average weight of 7 g were killed by a blow to the head followed by immersion in 70% ethanol for 30 seconds to sterilize the external surfaces. White muscle was dissected, and cells were isolated, pooled and cultured following the protocol described previously (Montserrat et al., 2007b). Cells were seeded at a density of $2 \cdot 10^5$ per cm^2, in 6 or 12-well plates (NUNC) depending on the experiment, and kept at 22ºC in DMEM medium (SIGMA) supplemented with 1% penicillin-streptomycin and 10% foetal bovine serum (FBS) until further analysis.

The SAF-1 cell line, consisting of seabream fibroblast-like cells that spontaneously immortalized (Béjar et al., 1997), was kindly provided by Dr J.J.Borrego, from the

University of Málaga (Spain). Cells were grown at 22ºC in Leibovitz (L-15) medium (SIGMA) supplemented with 2% L-glutamine, 1% penicillin–streptomycin and 10% FBS until further analysis.

Primary cultures treatments

To check the effect of IGF-II or serum restriction on the expression pattern of myogenin, satellite cells on day 1 after seeding were washed and incubated with DMEM 10% FBS supplemented with human IGF-II (BACHEM UK) at concentrations of 50 and 100 nM, or with DMEM 2% FBS. Control cells were kept in DMEM 10% FBS. Medium of each well was changed every day until sampling.

RNA extraction and gene expression

RNA was extracted from cells at different days of culture or embryos at different stages using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. Quantity and purity of RNA samples were determined by measure of absorbance at 260 nm, and its integrity was tested by electrophoresis in 1.5% agarose gels.

cDNA was generated starting from 3 µg of total RNA using the Transcriptor Reverse Transcriptase and oligo$(dT)_{15}$ (ROCHE) as anchor primer. Briefly, 3 µg of RNA were incubated with 10µM oligo$(dT)_{15}$ at 65ºC for 5 minutes, followed by incubation in 20 µl of a RT reaction mixture (1× RT buffer, 1mM of each dNTP, 20 U RNasin (RNase inhibitor), and 10 U Reverse Transcriptase) at 55 °C for 30 min and then at 85 °C for 10 minutes to inactivate the reverse transcriptase.

Real–time PCR was performed to quantify MyoD2, Myogenin, Myostatin, IGF-II and Elongation Factor 1-alpha (eF1α) transcripts. Specific primers for IGF-II were made as described by Saera-Vila and col. (2007). Primers for MyoD2, Myogenin, Myostatin and eF1α were designed flanking an intron to prevent genomic amplification (see table 1)

Real-time PCR was carried out using the iCycler iQ^{TM} and the iQ^{TM} SYBR Green Supermix (Bio-Rad). Reactions were performed as shown in table 1 in triplicate and fluorescence data were analyzed by interpolation of the cycle threshold (Ct) value. Each transcript level was normalized to the housekeeping gene eF1α using the delta-delta method (Livak and Schmittgen, 2001)

Specificity of reactions was verified by the analysis of melting curves and by electrophoresis and sequencing of PCR amplified products. The efficiency of the PCR was determined using standard curves generated by serial dilutions of a pool of RT reactions. It ranged from 90% to 110% ($E=10^{(-1/slope)}-1$).

Real-time PCR data for eF1α were exported and analyzed using a reference gene stability software package (GeNorm), which generate a measure of gene stability (Vandesompele et al., 2002).

Semiquantitative PCR was performed to verify the results for Myostatin. Primers used were the same as for the Real-time PCR and protocol is shown in table 1. Reaction was stopped either at cycles 30, 35 or 40 for the housekeeping eF1a, and at cycles 35, 40 or 45 for the myostatin reaction, and products were analysed by electrophoresis in a 1% agarose gel.

Conventional PCR. In our aim of finding a molecule which helps us to confirm the absence of fibroblasts in our primary culture and also to define the stage of muscle cells

in culture, we evaluated the expression of desmin, a well-known marker for cells of myogenic lineage. Specific forward primer and degenerated reverse primer were designed to amplify a 232bp region, flanking an intron to avoid genomic amplification. PCR protocol is detailed in table 1. Amplified fragments were analysed in a 1% agarose gel. Specificity of reaction was verified by sequencing the amplified fragment.

Gene (amplicon size)	Primers (5′→3′)	PCR Conditions
MYOD2 (149pb)	F: CACTACAGCGGGGATTCAGAC R: CGTTTGCTTCTCCTGGACTC	Real time PCR 95 °C 7 min (1 cycle) 95 °C 15s, 60 °C 35s (40 cycles) 84 °C 5s (1 cycle)
MYOGENIN (182pb)	F: CAGAGGACTGCCCAAGGTGGAG R: CAGGTGCTGCCCGAACTGGGCTC	Real time PCR 95 °C 7 min (1 cycle) 95 °C 15s, 68 °C 35s (40 cycles) 84 °C 5s (1 cycle)
MYOSTATIN (180pb)	F: GTACGACGTGCTGGGAGACG R: CGTACGATTCGATTGGCTTG	Real time PCR 95 °C 7 min (1 cycle) 95 °C 15s, 60 °C 35s (40 cycles) 84 °C 5s (1 cycle)
		Semiquantitative PCR 94 °C 2 min (1 cycle) 94 °C 15s, 60 °C 45s, 72 45s (45 cycles) 72 °C 7 min (1 cycle)
eF1α (152pb)	F: TCAAGGGATGGAAGGTTGAG R: AGTTCCAATACCGCCGAT	Real time PCR 95 °C 7 min (1 cycle) 95 °C 15s, 60 °C 35s (40 cycles) 84 °C 5s (1 cycle)
		Semiquantitative PCR 94 °C 2 min (1 cycle) 94 °C 15s, 60 °C 45s, 72 45s (45 cycles) 72 °C 7 min (1 cycle)
IGF-II (109pb)	F: TGGGATCGTAGAGGAGTGTTGT R: CTGTAGAGAGGTGGCCGACA	Real time PCR 95 °C 7 min (1 cycle) 95 °C 15s, 60 °C 35s (40 cycles) 81 °C 5s (1 cycle)
DESMIN (232pb)	F: CCAGTCCTACACCTGCGAGATTG R: GTAGGTGGCATCTCCACATCC	Conventional PCR 94 °C 5 min (1 cycle) 94 °C 30s, 60 °C 30s, 72ºC 2min (35cycles) 72 °C 7min (1 cycle)

Table 1. Nucleotide sequences of primers and PCR conditions used to analyze gene expression.

Flow cytometry

SAF-1 cells and myocytes at different days of culture were washed with PBS and collected. Cells were then fixed with paraformaldeheid 4% in PBS for 20 min at 4ºC, followed by treatment with permeabilization solution (1x PBS, 5% FBS, 0.05% Saponin).

Cells were stained using an anti-Desmin monoclonal antibody (SIGMA # D1033) at 1/100 dilution in permeabilization solution, for 40 min at 4ºC, followed by an Alexa Fluor 488 anti-mouse (INVITROGEN #A11017) at 1/300 dilution. For negative control, the staining was performed in the absence of primary antibody. Cells were then washed and analyzed on a FACScan flow cytometer, using CELLQuest software (Becton Dickinson). Further analysis was performed using FlowJo software (Tree Star, Inc. Oregon).

***In situ* hybridization in myocytes in culture**

Isolated cells from seabream muscle were seeded on cover-slips pre-treated with poly-L-lysine and laminin and cultured as above.

At different days of culture, cover-slips were collected and cells were washed and fixed with 4% paraformaldeheid over night at 4ºC. After several washes with PBS, cells were kept in methanol at -20ºC until further analysis.

Cells were washed 3 times with PBST (0.1% Tween-20 in 1 x PBS) for 5 min, and then permeabilized with 0.1% Triton X-100 during 15 min at room temperature. Cells were then washed several times and acetylated with 0.25% acetic anhydride in 100mM Triethano/amine-saline buffer at RT for 10 min. After washing with PBST followed by a 5 min wash in 2 x SSC and rinced with 50% formamide in 1 x SSC, cells were prehybridized in 200 µl prehybridization solution (50 % formamide, 1 x Denhardt's, 10

% dextran sulphate, 250µg/ml yeast tRNA, 500µg/l calf thymus DNA in 1 x SSPE) for 2 h at 37°C. Hybridization was carried out with 50 ng DIG-labeled Myogenin probe (Codina et al., 2008a) in 200 µl hybridization solution at 37°C overnight in a humid chamber. The cells were then washed with 50 % formamide in 1 x SSC at RT for 5 min, twice with 0.2 x SSC containing 0.1 % CHAPS at RT for 15 min, and once with blocking buffer (10 % goat serum, 2 % BMB blocking reaction in 1 x MAB) for 15 min. Cells were blocked for 30 min at RT, then incubated in preabsorbed anti-DIG-POD conjugated antibody diluted 1:1000 in blocking buffer for 1 h at RT. The cells were washed 4 times with PBST, followed by incubation with TSA Cy3 substrate (Perkin Elmer # NEL.741) for 30 min at RT and protected from light. After washing extensively, nuclei were stained with Hoechst solution diluted 1:2000, for 10 min at RT. Samples were prepared with Vectashield mounting medium (VECTOR), observed under fluorescence microscope (Axioplan2, Zeiss) and photographed using an digital camera (DP70, Olympus). Images where merged with Photoshop software (Microsoft).

Statistics

Each experiment was performed at least in triplicate. Data were plotted using Prism software (Graphpad, Inc.), and represented as means ± the standard error. Statistical differences between conditions were analyzed by one-way analysis of variance, followed by the Kruskal-wallis test when possible.

RESULTS

Myogenin and IGF-II expression in seabream embryos

Expression of Myogenin and IGF-II was analysed in embryos from 19 hours post fertilization (hpf) to 58 hpf. Results showed a huge variation on the myogenin levels along the embryo development, with a significant increase between 22 and 40 hpf, after which its expression dropped again. The maximum expression level was found between 27 and 28 hpf, when the expression was almost 3000-fold higher than at 19 hpf (figure 1A).

IGF-II transcript was present in embryos from 19 to 58 hpf, with the maximum expression between 38 and 48 hpf, when expression was between 8 and 10-folds higher than at the starting point. At 58 hpf the expression of IGF-II decreased again (figure 1B).

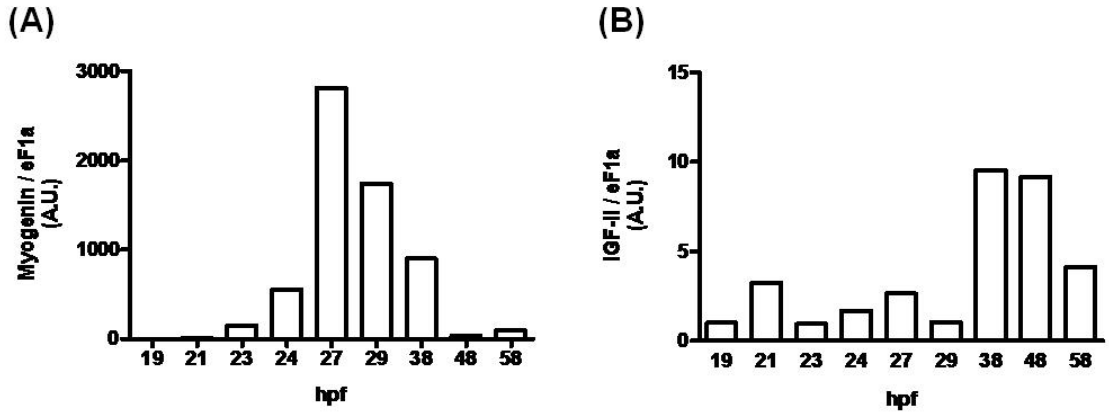

Figure 1. ***Expression of Myogenin and IGF-II in seabream embryos at different development stages***. cDNA from embryos from 19 to 58 hours post fertilization was synthesised and expression of Myogenin (A) and IGF-II (B) was analysed by real-time quantitative PCR. Pools of around 300 embryos were used for quantification. Results were normalized with the house-keeping gene eF1α. One representative experiment of three is shown.

Desmin expression in myocytes in culture

In order to evaluate the expression of desmin, we first designed primers to amplify about 232bp of the sequence and check if the transcript was present in muscle cells in culture. We used cDNA from SAF-1 cells as a negative control. We found expression in myocytes in culture at different days after seeding, but not in the SAF-1 (figure 2A). To confirm the specificity of the PCR reaction, we sequenced the amplified fragment, finding a high homology with the desmin RNA from zebrafish.

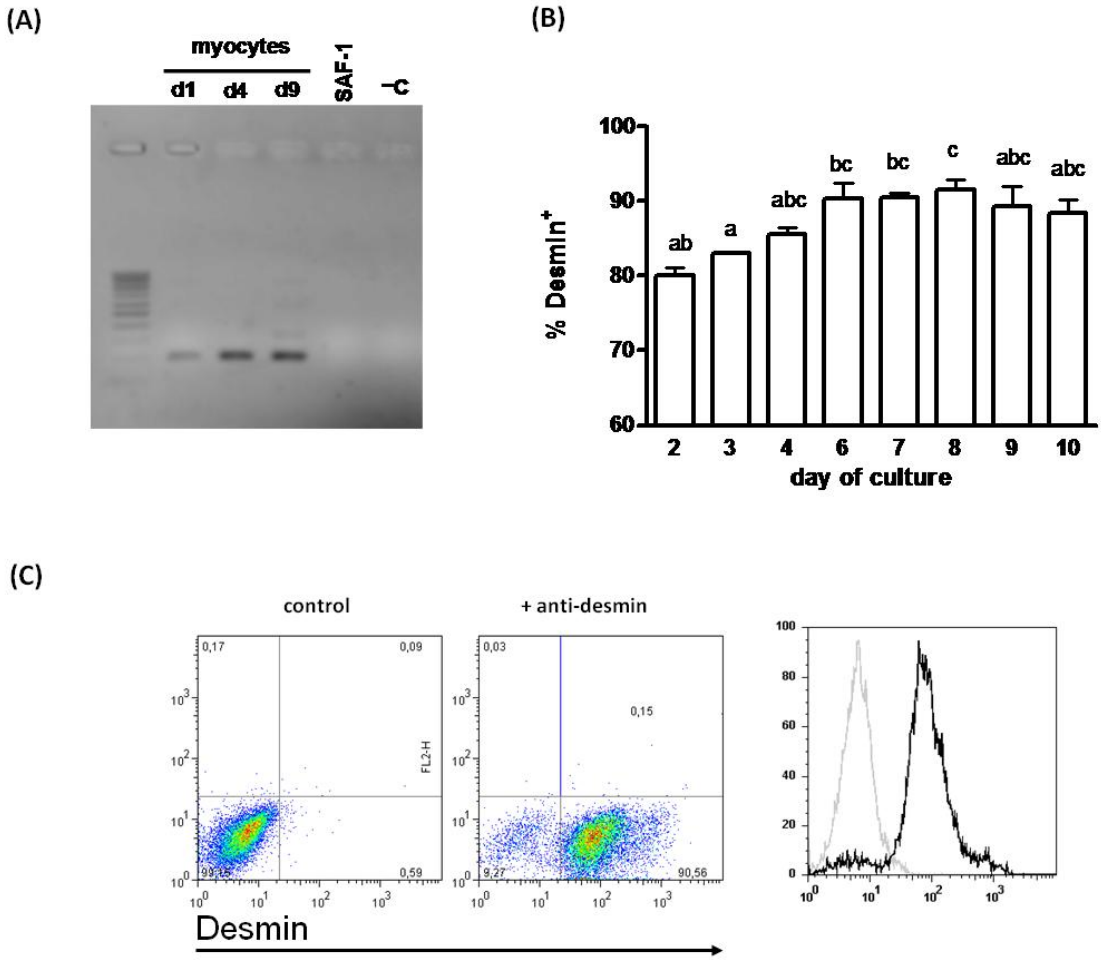

Figure 2. ***Expression of desmin in seabream muscle cells in primary culture***. Desmin expression was detected in muscle cells in culture from day 1 to day 10 after seeding, and the percentage of cells desmin-positive increased in parallel to differentiation of cells. (A) 1% agarose gel showing the single 232bp product amplified by RT-PCR with the primers designed for desmin. Fibroblast-like cells (SAF-1 cell line) were used as a negative control. (B) Graphic representing the percentage of cells desmin-positive at different days of culture after incubation with an anti-desmin monoclonal antibody and FACS analysis. Values are means ± SE (n = 3) experiments Values not sharing a common letter are significantly different ($P < 0.05$). (C) *Dot Plot* and *Histogram* of a representative experiment. Cells in the right-down quadrant are desmin-positive. A 90% of the cells are desmin-positive (black) compared to negative control (light grey).

To further characterize the expression pattern of desmin along the culture, we stained cells with anti-desmin antibody at different days after seeding. We found that the number of cells desmin^{+} increased in parallel to differentiation, reaching the maximum at day 8, when a 91.5 ± 1.3 % of cells presented desmin (figure 2B,C), indicating they were cells from muscle lineage. No desmin binding was observed in SAF-1 cells (data not shown).

Validation of eF1α as a house-keeping for seabream myocytes in culture

Standard curves were designed using serial dilutions of cDNA pools. The reaction gave a single product (figure 3A) and analysis of sequence confirmed its specificity. The efficiency of the reaction ranged between 97 and 100% (figure 3B)

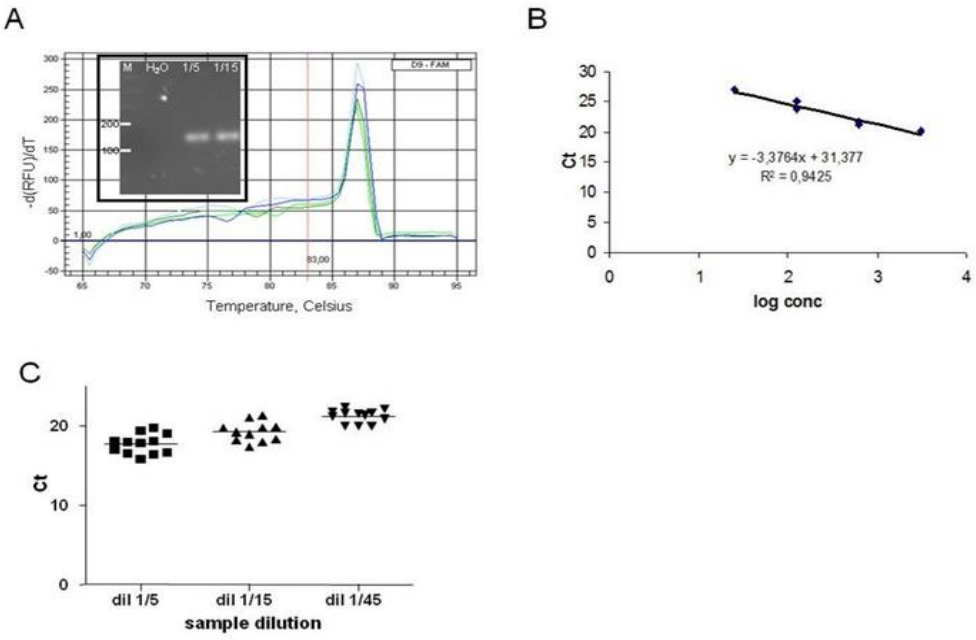

Figure 3. ***Elongation Factor 1 alpha is an accurate housekeeping gene for seabream muscle cells in primary culture***. cDNA from cells at six different stages was obtained and expression of eF1α was analysed by real-time PCR using serial dilutions of each sample. (A) Melting curve from one representative sample at 1 to 5 and 1 to 15 dilution showing a single product corresponding with the 152pb band that appears in the gel inserted in the graph. (B) Linear regression representation from one representative experiment. Formula shown in the graph was used to calculate primers efficiency (100%) and relative expresion of eF1α. (C) Same dilution samples from different days of culture are represented. Very little variations in the Ct were seen between samples at the same dilution

In order to confirm the elongation Factor 1 alpha as an accurate housekeeping gene for our model, we used 6 different samples, corresponding to cells at days 3, 5, 6, 7, 9 and 11 of culture. The exact same quantity of RNA from each sample was transcribed to cDNA, and then a serial dilution for each cDNA was established. Each sample was tested in triplicate.

The Ct values of the same dilution samples varied very little, with an average Ct of 17.7±0.37 for the 1/5 dilution; 19.2±0.38 for the 1/15 dilution and 21.1±0.24 for the 1/45 dilution (figure 3C).

Further analyses were performed using the GeNorm Software, which generate a measure of gene stability (M value). The values obtained in all dilutions were below 0.53.

Expression of MyoD2 and Myogenin in seabream muscle cells in culture

MyoD2 expression was evaluated in cells from day 1 to day 12 of culture. A peak of expression was found at day 3 after seeding, when the expression was between 3 and 5-folds higher than the expression at day 1. After that, expression dropped until day 12, when the transcript was barely detectable (figure 4).

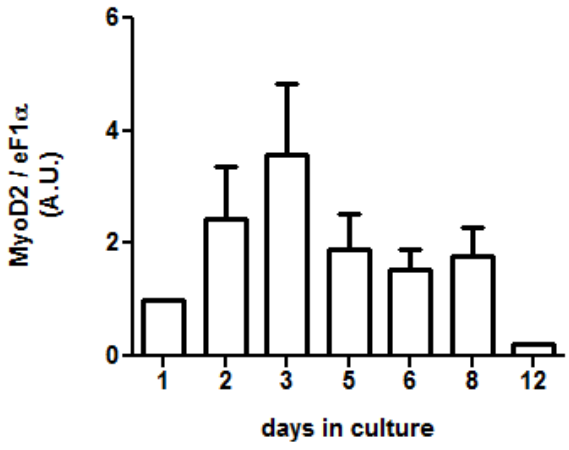

Figure 4. ***MyoD2 expression in seabream muscle cells in culture.*** cDNA from cells at different days of culture was synthesised and expression of MyoD2 was analysed by real-time quantitative PCR. Results were normalized with the house-keeping gene eF1α. Values are means±SE of 4 independent experiments. Values not sharing a common letter are significantly different ($P < 0.05$).

Myogenin expression were kept steady from day 1 to day 7 of culture and there is a peak at day 8, after which its expression decreased again. The levels of the transcript at day 8 were between 3 and 7 folds higher than the levels at day 2 (figure 5A). Expression at day one was extremely low.

The data from the *in situ* hybridization showed similar results (figure 5B). As the culture progressed, we could see how mononucleated cells expressing no or very small quantity of myogenin transcript, proliferated and differentiated in multinucleated cells, increasing the expression of Myogenin.

Incubations with IGF-II had no effect on myogenin expression throughout the culture. Likewise, serum restriction did not affect the expression pattern of myogenin (data not shown).

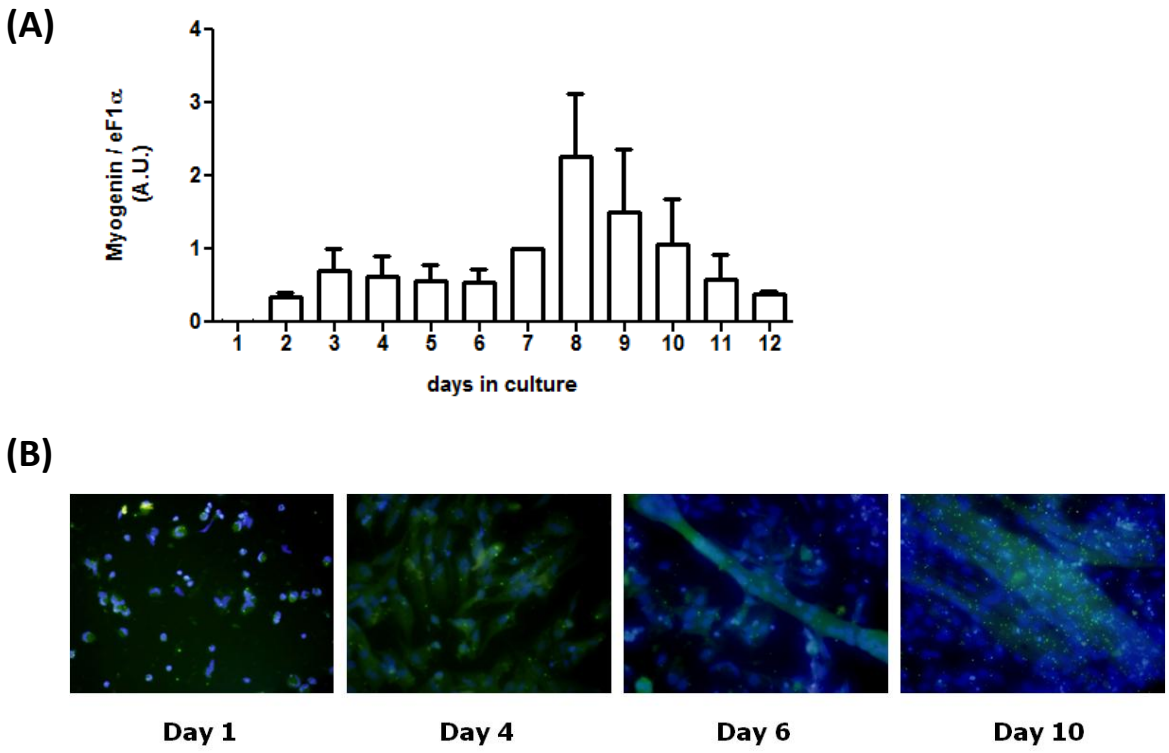

Figure 5. ***Myogenin expression in seabream muscle cells in culture.*** (A) cDNA from cells at different days of culture was synthesised and expression of Myogenin was analysed by real-time quantitative PCR. Results were normalized with the house-keeping gene eF1α. Values are means ± SE of 6 independent experiments. Values not sharing a common letter are significantly different ($P < 0.05$). (B) Myogenin transcript was detected in cultured muscle cells by *in situ* hybridisation using a specific probe. One experiment representative of three with similar results is shown.

Myostatin expression in cultured muscle cells from seabream

Myostatin transcript was detected in cells from day 1 to day 12 but at very low levels. Thus, we were unable to detect the transcript by real-time PCR even if the reaction gave a single fragment of the expected size. These low levels were corroborated by semiquantitative PCR, where more than 35 cycles were necessary to visualize the amplified product (see figure 6). Sequence analysis demonstrated that the PCR product corresponded to seabream myostatin (see supplementary data).

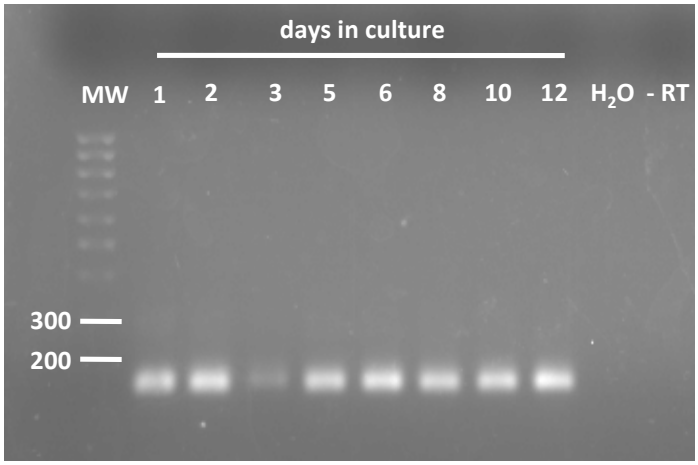

Figure 6. ***Myostatin expression in seabream muscle cells from day 1 to day 12 in culture.*** Myostatin transcript was detected in muscle cells samples at different days after seeding by semiquantitative PCR (40 cycles). A single product at very low concentration was detected.

IGF2 expression in seabream myocytes in culture

IGF-II expression was evaluated in cells from day 2 to day 12 of culture. Figure 7 shows how IGF-II levels decreased throughout the culture, being the expression at day 2 between 2 and 4-folds higher than the expression at day 13. It is interesting to point out that a double wave was suggested with a small peak at day 6.

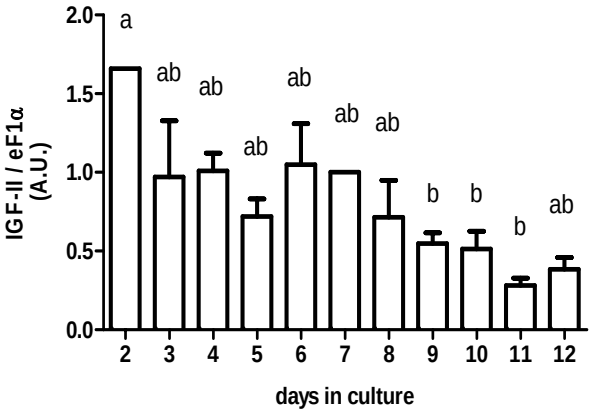

Figure 7. ***Expression of IGF-II in seabream muscle cells in primary culture***. cDNA from cells at different days of culture was synthesised and expression of IGF-II was analysed by real-time quantitative PCR. Results were normalized with the house-keeping gene eF1α.Values are means ± SE of 4 independent experiments. Values not sharing a common letter are significantly different ($P < 0.05$).

DISCUSSION

The present study reports the changes in the expression of several regulatory molecules throughout seabream muscle development at two different levels: during embryonic myogenesis, and during differentiation of muscle cells from satellite cells to multinucleated fibers.

Expression of myogenin and IGF-II was tested in embryos from the beginning of somitogenesis until few hours post-hatching. Results indicated that Myogenin transcript did not accumulated in seabream embryos before the appearance of somites (around 20 hpf). The highest levels of Myogenin were found 27 hpf, when the embryo had around 16 somites. This would confirm previous studies using *in situ* hybridization in which myogenin mRNA levels were highest between 28 and 30hpf (Codina et al., 2008a), when the medial somites are differentiating. Similar results were found in atlantic halibut (*Hippoglossus hippoglossus*) (Galloway et al., 2006), carp (Cole et al., 2004) or flounder (Zhang et al., 2006) using *in situ* hybridization approaches. As far as we know, no studies using quantitative approaches have been published up to now. IGF-II mRNA levels were detected at all points analyzed, from 19 hpf and until few hours post hatching, with its maximum expression between 38 hpf (when some of the embryos were already undergoing hatching) and 50 hpf (when the vast majority of embryos were already swimming). Radaelli and collaborators (2003) and Tiago and collaborators (2008) found expression of IGF-II in seabream larvae, but levels in embryos were not analyzed. Similar approaches led to similar results in larvae of seabass (*Dicentrarchus labrax*) (Radaelli et al., 2008) and redbanded seabream (*Pagrus auriga*) (Ponce et al., 2008). These results would indicate that myogenin is important during somitogenesis in seabream embryos, specially during the formation of the medial somites, whereas IGF-II is kept at relatively steady levels during embryonic development, and is up-regulated

when the hatching starts, suggesting a more relevant function throughout larvae development.

We also analyzed the expression of several factors involved in muscle cell proliferation and differentiation. When using real-time quantitative PCR, one of the important points to obtain reliable results is to find a good house-keeping gene. We chose elongation Factor 1 alpha (eF1α), which has proved to be the most stable gene in studies of atlantic salmon (*Salmo salar*) (Olsvik et al., 2005) and one of the best in studies on coho salmon (*Oncorhynchus Kisutch*) (Luckenbach et al., 2008), zebrafish (*Danio rerio*) (McCurley and Callard, 2008) and flatfish (Infante et al., 2008). In seabream, Nowell and col. (2000) demonstrated that the eF1 α was stable in different tissues and in embryos, its expression remained steady from 15 hpf onwards. In order to validate the eF1α as a normalizer gene for our model system, we designed several standard curves which gave an efficiency between 97 and 100%. To test the stability of this gene throughout primary cell culture, three different dilutions of 6 samples from different days of culture were tested, showing very low variations, indicating that eF1α could be a good housekeeping. The M values below 0.53 demonstrated a very high stability of the gene throughout the culture, confirming the eF1α as a perfect house-keeping gene for our system.

Desmin is known to be a good marker cells commited to muscle lineage (Conboy and Rando, 2002; Loh et al., 2000). We found cells expressing desmin from day 2 of culture (proliferating myoblasts) to day 10 (mostly differentiated myotubes), indicating they are veritable muscle cells. In our system, the percentage of cells immunoreactive for desmin increased from 80% at day 2 up to 90% after 6 days in culture. The rest of the cells could be either remaining quiescent satellite cells, which are known to be desmin negative (Conboy and Rando, 2002) or most likely fibroblasts. Similar or lower

percentages of desmin positive cells were found in other primary cultures systems from mammals (Grohmann et al., 2005; Machida et al., 2004; van der Ven et al., 1992). In fish, desmin is expressed in cultured post-mitotic myoblasts of carp (Koumans et al., 1991). In seabream muscle explants, desmin positive cells were found after 2 days in culture (Funkenstein et al., 2006).

In order to determine a possible relationship between MRFs expression, IGF system and Myostatin expression, we analyzed the mRNA expression of MyoD2, Myogenin, IGF-II and MSTN in seabream muscle cells in primary culture, from satellite cells to differentiated myotubes. Figure 8 summarizes the variation in expression of the four genes along the cell culture.

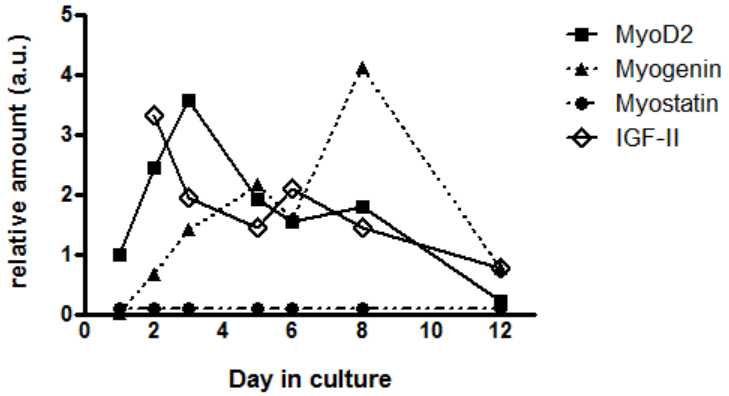

Figure 8. Variations in the expression of MyoD2, Myogenin, Myostatin and IGF-II in seabream muscle cells in culture. Data are means of different experiments and arranged to fit in a single figure.

MyoD2 was chosen because of its specificity for fast muscle in seabream (Tan and Du, 2002). We found expression of MyoD2 in all the stages evaluated, indicating that even cells at day one are already committed to muscle lineage, as quiescent cells would not express MRFs (Olguin and Olwin, 2004). MyoD2 presented higher levels at the beginning of the culture, when the cells were mostly proliferating, while Myogenin expression was kept relatively low until 6 days after seeding and then increased significantly, coinciding to the active differentiation process described previously in our group (Montserrat et al., 2007a). This is in agreement with findings in trout muscle cells

in primary culture, where TMyoD was expressed before TMyogenin, and TMyogenin presented higher levels at day 7 and 11 of culture than at day 2 (Rescan et al., 1995; Rescan et al., 1994). Studies in mammalian cells showed that MyoD was necessary for myoblast progression (Wilson et al., 2003), and Myogenin expression increased upon differentiation (Rotwein et al., 1995; Shefer et al., 2006). In seabream muscle explants, Funkenstein and collaborators (2006) found MyoD expression after 4 days in culture, corresponding to the moment when cells started to actively proliferate.

Myostatin levels were low throughout the culture, from myoblasts at day 1 to differentiated myotubes at day 12. Real-time PCR was not sensitive enough to evaluate changes in its expression, although the specificity of reaction was confirmed. Myostatin is known to arrest muscle growth in vertebrates, but controversial data is found when its expression is analyzed *in vitro*. In seabream, cultured muscle explants present an increase in MSTN protein levels as the culture progresses (Funkenstein et al., 2006); similar results were found in chicken myoblasts or C2C12 myoblasts, where MSTN was upregulated during differentiation and fusion (Kocamis et al., 2001; Mendler et al., 2000). Contrarily, Kirk and collaborators (2000) described low expression of MSTN in areas rich in satellite cells and regenerating fibers, and in chicken (*Gallus domesticus*), MSTN was found to suppress satellite cell activation, as well as myoblast proliferation and differentiation (McFarland et al., 2007), suggesting that low levels are required for satellite cells to progress.

It is known that expression of IGF-II in fish is higher than the expression of IGF-I (Gabillard et al., 2003; Peterson et al., 2004), suggesting a relevant role of this hormone in muscle development. Previous data from our group indicate that, in trout and seabream muscle cells, IGF-I and IGF-II have both mitogenic and metabolic actions, activating the MAPK and the PI3K/Akt pathways, presumably through the IGF-I

receptor (Castillo et al., 2006; Castillo et al., 2004; Codina et al., 2008b; Montserrat et al., 2007a). Here we found detectable levels of IGF-II in both myoblasts and myotubes, suggesting a role of IGFs in satellite cells progression, stimulating both proliferation and differentiation, similarly to what happened for IGF-I in humans (Barton et al., 2002; McKay et al., 2008). In our system, levels of IGF-II decreased as the cultured progressed, with a small peak between day 6 and 7 of culture, coinciding with the differentiation stage, and suggesting a double-wave expression. Studies in seabream muscle explants, using immunostaining techniques, showed that IGF-II transcript levels increased with differentiation (Funkenstein et al., 2006). This discrepancy can be due to the different model and experimental approaches used.

The functional relationship between MRFs and IGF-II is not well-understood. Our results indicated a peak of IGF-II just before the MyoD expression peak was found, and suggested a second peak of IGF-II expression just before the peak of Myogenin. This led us to think that IGF-II could modulate MRFs expression in seabream muscle cells in culture, specially Myogenin, which in mammals is known to be regulated by activation of PI3K/Akt pathway through the IGF-I receptor (Mourkioti and Rosenthal, 2005; Tsuchiya et al., 2007; Wilson et al., 2003; Xu and Wu, 2000). However, when incubating the cells with IGF-II, no changes in Myogenin levels were found, contrarily of what happens in other systems using stem cells or muscle cell lines, where IGF-II was clearly involved in the Myogenin expression (Prelle et al., 2000; Ren et al., 2008; Wilson and Rotwein, 2006). Other studies using IGF-I reported different results. Engert and col. (1996) using L6E9 cells, found that IGF-I inhibited Myogenin expression, and Florini and col. (1991) determined a positive correlation between IGF-I and Myogenin but only at low doses of the peptide. A recent study in primary culture of human

satellite cell showed no correlation between IGF-I and Myogenin expression (McKay et al., 2008).

Serum privation did not affect Myogenin expression either, contrarily of what was expected from what is known in mammals and fish. In C2C12, myogenin expression was increased after serum withdrawal (Tsuchiya et al., 2007), and *in vivo* studies in fish indicated non consistent results, since Myogenin expression was enhanced after nutrient restriction in atlantic salmon (Bower et al., 2008) whereas it decreased in trout (Montserrat et al., 2007a).

In summary, the present study describes the variations in mRNA levels of different genes involved in seabream muscle development and growth. Low Myostatin levels during the primary cell culture would be necessary for correct activation, proliferation and differentiation of satellite cells to become multinucleated myotubes in seabream. We have shown that Myogenin levels are clearly increased during the formation of medial somites in seabream embryos, and that IGF-II expression is kept relatively low during embryogenesis and is up-regulated when hatching. The validation of the elongation Factor 1 alpha as a good house-keeping gene enabled us to evaluate MyoD2, Myogenin and IGF-II transcript levels in seabream muscle cells in primary culture, finding that MyoD2 presents a peak of expression at early stages when cells have high proliferation taxes, whereas Myogenin is up-regulated when differentiation process starts. No clear relationship between MRFs and IGF-II levels has been found in our model, although expression of IGF-II throughout the whole culture would indicate it has a relevant role in satellite cell progression. Further studies are needed to elucidate the mechanisms through which IGF-II and nutritional factors regulate muscle cell differentiation in seabream muscle cells in culture, and how they are related to Myogenic Regulatory Factors.

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REFERENCES

Barton-Davis, E. R., Shoturma, D. I. and Sweeney, H. L. 1999. *Contribution of satellite cells to IGF-I induced hypertrophy of skeletal muscle*. Acta Physiol Scand. 167. 301-5

Barton, E. R., Morris, L., Musaro, A., Rosenthal, N. and Sweeney, H. L. 2002. *Muscle-specific expression of insulin-like growth factor I counters muscle decline in mdx mice*. J Cell Biol. 157. 137-48

Béjar, J., Borrego, J. J. and Alvarez, M. C. 1997. *A continuous cell line from the cultured marine fish gilthead seabream (Sparus aurata L.).* Aquaculture. 150. 143-53

Benedito-Palos, L., Navarro, J. C., Sitja-Bobadilla, A., Bell, J. G., Kaushik, S. and Perez-Sanchez, J. 2008. *High levels of vegetable oils in plant protein-rich diets fed to gilthead sea bream (Sparus aurata L.): growth performance, muscle fatty acid profiles and histological alterations of target tissues*. Br J Nutr. 100. 992-1003

Bergstrom, D. A. and Tapscott, S. J. 2001. *Molecular distinction between specification and differentiation in the myogenic basic helix-loop-helix transcription factor family*. Mol Cell Biol. 21. 2404-12

Bower, N. I., Li, X., Taylor, R. and Johnston, I. A. 2008. *Switching to fast growth: the insulin-like growth factor (IGF) system in skeletal muscle of Atlantic salmon*. J Exp Biol. 211. 3859-70

Castillo, J., Ammendrup-Johnsen, I., Codina, M., Navarro, I. and Gutierrez, J. 2006. *IGF-I and insulin receptor signal transduction in trout muscle cells*. Am J Physiol Regul Integr Comp Physiol. 290. R1683-90

Castillo, J., Codina, M., Martinez, M. L., Navarro, I. and Gutierrez, J. 2004. *Metabolic and mitogenic effects of IGF-I and insulin on muscle cells of rainbow trout*. Am J Physiol Regul Integr Comp Physiol. 286. R935-41

Codina, M., Bian, Y. H., Gutierrez, J. and Du, S. J. 2008a. *Cloning and characterization of myogenin from seabream (Sparus aurata) and analysis of promoter muscle specificity*. Comp Biochem Physiol D Genomics and Proteomics. 3. 128-39

Codina, M., Garcia de la serrana, D., Sanchez-Gurmaches, J., Montserrat, N., Chistyakova, O., Navarro, I. and Gutierrez, J. 2008b. *Metabolic and mitogenic effects of IGF-II in rainbow trout (Oncorhynchus mykiss) myocytes in culture and the role of IGF-II in the PI3K/Akt and MAPK signalling pathways*. Gen Comp Endocrinol. 157. 116-24

Cole, N. J., Hall, T. E., Martin, C. I., Chapman, M. A., Kobiyama, A., Nihei, Y., Watabe, S. and Johnston, I. A. 2004. *Temperature and the expression of myogenic regulatory factors (MRFs) and myosin heavy chain isoforms during embryogenesis in the common carp Cyprinus carpio L*. J Exp Biol. 207. 4239-48

Conboy, I. M. and Rando, T. A. 2002. *The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis*. Dev Cell. 3. 397-409

Chauvigne, F., Gabillard, J. C., Weil, C. and Rescan, P. Y. 2003. *Effect of refeeding on IGFI, IGFII, IGF receptors, FGF2, FGF6, and myostatin mRNA expression in rainbow trout myotomal muscle*. Gen Comp Endocrinol. 132. 209-15

Chen, Y. H., Lee, W. C., Cheng, C. H. and Tsai, H. J. 2000. *Muscle regulatory factor gene: zebrafish (Danio rerio) myogenin cDNA*. Comp Biochem Physiol B Biochem Mol Biol. 127. 97-103

Chen, Y. H. and Tsai, H. J. 2002. *Treatment with Myf5-morpholino results in somite patterning and brain formation defects in zebrafish*. Differentiation. 70. 447-56

Delalande, J. M. and Rescan, P. Y. 1999. *Differential expression of two nonallelic MyoD genes in developing and adult myotomal musculature of the trout (Oncorhynchus mykiss)*. Dev Genes Evol. 209. 432-7

Engert, J. C., Berglund, E. B. and Rosenthal, N. 1996. *Proliferation precedes differentiation in IGF-I-stimulated myogenesis*. J Cell Biol. 135. 431-40

Florini, J. R., Ewton, D. Z. and Roof, S. L. 1991. *Insulin-like growth factor-I stimulates terminal myogenic differentiation by induction of myogenin gene expression*. Mol Endocrinol. 5. 718-24

Fukada, H., Ozaki, Y., Pierce, A. L., Adachi, S., Yamauchi, K., Hara, A., Swanson, P. and Dickhoff, W. W. 2004. *Salmon growth hormone receptor: molecular cloning, ligand specificity, and response to fasting*. Gen Comp Endocrinol. 139. 61-71

Funkenstein, B., Balas, V., Skopal, T., Radaelli, G. and Rowlerson, A. 2006. *Long-term culture of muscle explants from Sparus aurata*. Tissue Cell. 38. 399-415

Gabillard, J. C., Weil, C., Rescan, P. Y., Navarro, I., Gutierrez, J. and Le Bail, P. Y. 2003. *Effects of environmental temperature on IGF1, IGF2, and IGF type I receptor expression in rainbow trout (Oncorhynchus mykiss)*. Gen Comp Endocrinol. 133. 233-42

Galloway, T. F., Bardal, T., Kvam, S. N., Dahle, S. W., Nesse, G., Randol, M., Kjorsvik, E. and Andersen, O. 2006. *Somite formation and expression of MyoD, myogenin and myosin in Atlantic halibut (Hippoglossus hippoglossus L.) embryos incubated at different temperatures: transient asymmetric expression of MyoD*. J Exp Biol. 209. 2432-41

Garikipati, D. K., Gahr, S. A., Roalson, E. H. and Rodgers, B. D. 2007. *Characterization of rainbow trout myostatin-2 genes (rtMSTN-2a and -2b): genomic organization, differential expression, and pseudogenization*. Endocrinology. 148. 2106-15

Gentil, V., Martin, P., Smal, J. and Le Bail, P. Y. 1996. *Production of recombinant insulin-like growth factor-II in the development of a radioimmunoassay in rainbow trout (Oncorhynchus mykiss)*. Gen Comp Endocrinol. 104. 156-67

Gerber, A. N., Klesert, T. R., Bergstrom, D. A. and Tapscott, S. J. 1997. *Two domains of MyoD mediate transcriptional activation of genes in repressive chromatin: a mechanism for lineage determination in myogenesis*. Genes Dev. 11. 436-50

Grohmann, M., Foulstone, E., Welsh, G., Holly, J., Shield, J., Crowne, E. and Stewart, C. 2005. *Isolation and validation of human prepubertal skeletal muscle cells: maturation and metabolic effects of IGF-I, IGFBP-3 and TNFalpha*. J Physiol. 568. 229-42

Hasty, P., Bradley, A., Morris, J. H., Edmondson, D. G., Venuti, J. M., Olson, E. N. and Klein, W. H. 1993. *Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene*. Nature. 364. 501-6

Holterman, C. E. and Rudnicki, M. A. 2005. *Molecular regulation of satellite cell function.* Semin Cell Dev Biol. 16. 575-84

Infante, C., Matsuoka, M. P., Asensio, E., Canavate, J. P., Reith, M. and Manchado, M. 2008. *Selection of housekeeping genes for gene expression studies in larvae from flatfish using real-time PCR*. BMC Mol Biol. 9. 28

Johnston, I. A. 2006. *Environment and plasticity of myogenesis in teleost fish*. J Exp Biol. 209. 2249-64

Kassar-Duchossoy, L., Gayraud-Morel, B., Gomes, D., Rocancourt, D., Buckingham, M., Shinin, V. and Tajbakhsh, S. 2004. *Mrf4 determines skeletal muscle identity in Myf5 : Myod double-mutant mice*. Nature. 431. 466-71

Kirk, S., Oldham, J., Kambadur, R., Sharma, M., Dobbie, P. and Bass, J. 2000. *Myostatin regulation during skeletal muscle regeneration*. J Cell Physiol. 184. 356-63

Kocamis, H., McFarland, D. C. and Killefer, J. 2001. *Temporal expression of growth factor genes during myogenesis of satellite cells derived from the biceps femoris and pectoralis major muscles of the chicken*. J Cell Physiol. 186. 146-52

Koumans, J. T., Akster, H. A., Booms, G. H., Lemmens, C. J. and Osse, J. W. 1991. *Numbers of myosatellite cells in white axial muscle of growing fish: Cyprinus carpio L. (Teleostei)*. Am J Anat. 192. 418-24

Livak, K. J. and Schmittgen, T. D. 2001. *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method*. Methods. 25. 402-8

Loh, S. H., Chan, W. T., Gong, Z., Lim, T. M. and Chua, K. L. 2000. *Characterization of a zebrafish (Danio rerio) desmin cDNA: an early molecular marker of myogenesis*. Differentiation. 65. 247-54

Luckenbach, J. A., Iliev, D. B., Goetz, F. W. and Swanson, P. 2008. *Identification of differentially expressed ovarian genes during primary and early secondary oocyte growth in coho salmon, Oncorhynchus kisutch*. Reprod Biol Endocrinol. 6. 2

Maccatrozzo, L., Bargelloni, L., Radaelli, G., Mascarello, F. and Patarnello, T. 2001. *Characterization of the myostatin gene in the gilthead seabream (Sparus aurata): sequence, genomic structure, and expression pattern*. Mar Biotechnol (NY). 3. 224-30

Machida, S., Spangenburg, E. E. and Booth, F. W. 2004. *Primary rat muscle progenitor cells have decreased proliferation and myotube formation during passages*. Cell Prolif. 37. 267-77

McCurley, A. T. and Callard, G. V. 2008. *Characterization of housekeeping genes in zebrafish: male-female differences and effects of tissue type, developmental stage and chemical treatment*. BMC Mol Biol. 9. 102

McFarland, D. C., Velleman, S. G., Pesall, J. E. and Liu, C. 2007. *The role of myostatin in chicken (Gallus domesticus) myogenic satellite cell proliferation and differentiation*. Gen Comp Endocrinol. 151. 351-7

McKay, B. R., O'Reilly, C. E., Phillips, S. M., Tarnopolsky, M. A. and Parise, G. 2008. *Co-expression of IGF-1 family members with myogenic regulatory factors following acute damaging muscle-lengthening contractions in humans*. J Physiol. 586. 5549-60

McPherron, A. C., Lawler, A. M. and Lee, S. J. 1997. *Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member*. Nature. 387. 83-90

Mendler, L., Zador, E., Ver Heyen, M., Dux, L. and Wuytack, F. 2000. *Myostatin levels in regenerating rat muscles and in myogenic cell cultures*. J Muscle Res Cell Motil. 21. 551-63

Meton, I., Caseras, A., Canto, E., Fernandez, F. and Baanante, I. V. 2000. *Liver insulin-like growth factor-I mRNA is not affected by diet composition or ration size but shows diurnal variations in regularly-fed gilthead sea bream (Sparus aurata)*. J Nutr. 130. 757-60

Mingarro, M., Vega-Rubin de Celis, S., Astola, A., Pendon, C., Valdivia, M. M. and Perez-Sanchez, J. 2002. *Endocrine mediators of seasonal growth in gilthead sea bream (Sparus aurata): the growth hormone and somatolactin paradigm*. Gen Comp Endocrinol. 128. 102-11

Montserrat, N., Gabillard, J. C., Capilla, E., Navarro, M. I. and Gutierrez, J. 2007a. *Role of insulin, insulin-like growth factors, and muscle regulatory factors in the compensatory growth of the trout (Oncorhynchus mykiss)*. Gen Comp Endocrinol. 150. 462-72

Montserrat, N., Sanchez-Gurmaches, J., Garcia de la Serrana, D., Navarro, M. I. and Gutierrez, J. 2007b. *IGF-I binding and receptor signal transduction in primary cell culture of muscle cells of gilthead sea bream: changes throughout in vitro development*. Cell Tissue Res. 330. 503-13

Mourkioti, F. and Rosenthal, N. 2005. *IGF-1, inflammation and stem cells: interactions during muscle regeneration*. Trends Immunol. 26. 535-42

Nabeshima, Y., Hanaoka, K., Hayasaka, M., Esumi, E., Li, S., Nonaka, I. and Nabeshima, Y. 1993. *Myogenin gene disruption results in perinatal lethality because of severe muscle defect*. Nature. 364. 532-5

Nowell, M. A., Power, D. M., Guerreiro, P. M., Llewellyn, L., Ramsurn, V. V., Wigham, T. and Sweeney, G. E. 2000. *Cloning and Expression of an Elongation Factor-1alpha in Sea Bream (Sparus aurata) Larvae and Adult Tissue*. Mar Biotechnol (NY). 2. 173-9

Olguin, H. C. and Olwin, B. B. 2004. *Pax-7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: a potential mechanism for self-renewal*. Dev Biol. 275. 375-88

Olsvik, P. A., Lie, K. K., Jordal, A. E., Nilsen, T. O. and Hordvik, I. 2005. *Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon*. BMC Mol Biol. 6. 21

Perez-Sanchez, J., Marti-Palanca, H. and Kaushik, S. J. 1995. *Ration size and protein intake affect circulating growth hormone concentration, hepatic growth hormone binding and plasma insulin-like growth factor-I immunoreactivity in a marine teleost, the gilthead sea bream (Sparus aurata)*. J Nutr. 125. 546-52

Peterson, B. C., Waldbieser, G. C. and Bilodeau, L. 2004. *IGF-I and IGF-II mRNA expression in slow and fast growing families of USDA103 channel catfish (Ictalurus punctatus)*. Comp Biochem Physiol A Mol Integr Physiol. 139. 317-23

Ponce, M., Infante, C., Funes, V. and Manchado, M. 2008. *Molecular characterization and gene expression analysis of insulin-like growth factors I and II in the redbanded seabream, Pagrus auriga: transcriptional regulation by growth hormone*. Comp Biochem Physiol B Biochem Mol Biol. 150. 418-26

Prelle, K., Wobus, A. M., Krebs, O., Blum, W. F. and Wolf, E. 2000. *Overexpression of insulin-like growth factor-II in mouse embryonic stem cells promotes myogenic differentiation*. Biochem Biophys Res Commun. 277. 631-8

Radaelli, G., Patruno, M., Maccatrozzo, L. and Funkenstein, B. 2003. *Expression and cellular localization of insulin-like growth factor-II protein and mRNA in Sparus aurata during development*. J Endocrinol. 178. 285-99

Radaelli, G., Poltronieri, C., Bertotto, D., Funkenstein, B. and Simontacchi, C. 2008. *Cellular localization of insulin-like growth factor-II protein in the sea bass (Dicentrarchus labrax) from hatching to adult*. Histol Histopathol. 23. 523-30

Ren, H., Yin, P. and Duan, C. 2008. *IGFBP-5 regulates muscle cell differentiation by binding to IGF-II and switching on the IGF-II auto-regulation loop*. J Cell Biol. 182. 979-91

Rescan, P. Y. 2001. *Regulation and functions of myogenic regulatory factors in lower vertebrates*. Comp Biochem Physiol B Biochem Mol Biol. 130. 1-12

Rescan, P. Y. and Gauvry, L. 1996. *Genome of the rainbow trout (Oncorhynchus mykiss) encodes two distinct muscle regulatory factors with homology to myoD*. Comp Biochem Physiol B Biochem Mol Biol. 113. 711-5

Rescan, P. Y., Gauvry, L. and Paboeuf, G. 1995. *A gene with homology to myogenin is expressed in developing myotomal musculature of the rainbow trout and in vitro during the conversion of myosatellite cells to myotubes*. FEBS Lett. 362. 89-92

Rescan, P. Y., Gauvry, L., Paboeuf, G. and Fauconneau, B. 1994. *Identification of a muscle factor related to MyoD in a fish species*. Biochim Biophys Acta. 1218. 202-4

Rescan, P. Y., Jutel, I. and Ralliere, C. 2001. *Two myostatin genes are differentially expressed in myotomal muscles of the trout (Oncorhynchus mykiss)*. J Exp Biol. 204. 3523-9

Rotwein, P., James, P. L. and Kou, K. 1995. *Rapid activation of insulin-like growth factor binding protein-5 gene transcription during myoblast differentiation*. Mol Endocrinol. 9. 913-23

Roy, K., de la Serna, I. L. and Imbalzano, A. N. 2002. *The myogenic basic helix-loop-helix family of transcription factors shows similar requirements for SWI/SNF chromatin remodeling enzymes during muscle differentiation in culture*. J Biol Chem. 277. 33818-24

Rudnicki, M. A., Schnegelsberg, P. N., Stead, R. H., Braun, T., Arnold, H. H. and Jaenisch, R. 1993. *MyoD or Myf-5 is required for the formation of skeletal muscle*. Cell. 75. 1351-9

Saera-Vila, A., Calduch-Giner, J. A. and Perez-Sanchez, J. 2007. *Co-expression of IGFs and GH receptors (GHRs) in gilthead sea bream (Sparus aurata L.): sequence analysis of the GHR-flanking region*. J Endocrinol. 194. 361-72

Shefer, G., Van de Mark, D. P., Richardson, J. B. and Yablonka-Reuveni, Z. 2006. *Satellite-cell pool size does matter: defining the myogenic potency of aging skeletal muscle*. Dev Biol. 294. 50-66

Steinbacher, P., Haslett, J. R., Obermayer, A., Marschallinger, J., Bauer, H. C., Sanger, A. M. and Stoiber, W. 2007. *MyoD and Myogenin expression during myogenic phases in brown trout: a precocious onset of mosaic hyperplasia is a prerequisite for fast somatic growth*. Dev Dyn. 236. 1106-14

Summerbell, D., Ashby, P. R., Coutelle, O., Cox, D., Yee, S. and Rigby, P. W. 2000. *The expression of Myf5 in the developing mouse embryo is controlled by discrete and dispersed enhancers specific for particular populations of skeletal muscle precursors*. Development. 127. 3745-57

Summerbell, D., Halai, C. and Rigby, P. W. 2002. *Expression of the myogenic regulatory factor Mrf4 precedes or is contemporaneous with that of Myf5 in the somitic bud*. Mech Dev. 117. 331-5

Tan, X. and Du, S. J. 2002. *Differential expression of two MyoD genes in fast and slow muscles of gilthead seabream (Sparus aurata)*. Dev Genes Evol. 212. 207-17

Thomas, M., Langley, B., Berry, C., Sharma, M., Kirk, S., Bass, J. and Kambadur, R. 2000. *Myostatin, a negative regulator of muscle growth, functions by inhibiting myoblast proliferation*. J Biol Chem. 275. 40235-43

Tiago, D. M., Laize, V. and Cancela, M. L. 2008. *Alternatively spliced transcripts of Sparus aurata insulin-like growth factor 1 are differentially expressed in adult tissues and during early development*. Gen Comp Endocrinol. 157. 107-15

Tsuchiya, K., Hosoi, H., Misawa-Furihata, A., Houghton, P. J. and Sugimoto, T. 2007. *Insulin-like growth factor-I has different effects on myogenin induction and cell cycle progression in human alveolar and embryonal rhabdomyosarcoma cells*. Int J Oncol. 31. 41-7

van der Ven, P. F., Schaart, G., Jap, P. H., Sengers, R. C., Stadhouders, A. M. and Ramaekers, F. C. 1992. *Differentiation of human skeletal muscle cells in culture: maturation as indicated by titin and desmin striation*. Cell Tissue Res. 270. 189-98

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. 2002. *Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes*. Genome Biol. 3. RESEARCH0034

Velloso, C. P. 2008. *Regulation of muscle mass by growth hormone and IGF-I*. Br J Pharmacol. 154. 557-68

Venuti, J. M., Morris, J. H., Vivian, J. L., Olson, E. N. and Klein, W. H. 1995. *Myogenin is required for late but not early aspects of myogenesis during mouse development*. J Cell Biol. 128. 563-76

Weinberg, E. S., Allende, M. L., Kelly, C. S., Abdelhamid, A., Murakami, T., Andermann, P., Doerre, O. G., Grunwald, D. J. and Riggleman, B. 1996. *Developmental regulation of zebrafish MyoD in wild-type, no tail and spadetail embryos*. Development. 122. 271-80

Wilson, E. M., Hsieh, M. M. and Rotwein, P. 2003. *Autocrine growth factor signaling by insulin-like growth factor-II mediates MyoD-stimulated myocyte maturation*. J Biol Chem. 278. 41109-13

Wilson, E. M. and Rotwein, P. 2006. *Control of MyoD function during initiation of muscle differentiation by an autocrine signaling pathway activated by insulin-like growth factor-II*. J Biol Chem. 281. 29962-71

Xu, Q. and Wu, Z. 2000. *The insulin-like growth factor-phosphatidylinositol 3-kinase-Akt signaling pathway regulates myogenin expression in normal myogenic cells but not in rhabdomyosarcoma-derived RD cells*. J Biol Chem. 275. 36750-7

Yang, W., Zhang, Y., Ma, G., Zhao, X., Chen, Y. and Zhu, D. 2005. *Identification of gene expression modifications in myostatin-stimulated myoblasts*. Biochem Biophys Res Commun. 326. 660-6

Zhang, Y., Tan, X., Zhang, P. J. and Xu, Y. 2006. *Characterization of muscle-regulatory gene, MyoD, from flounder (Paralichthys olivaceus) and analysis of its expression patterns during embryogenesis*. Mar Biotechnol (NY). 8. 139-48

SUPPLEMENTARY DATA

(A)

GCGNTGGGAGCTCTCCCATATGGTCGACCTGCAGGCGGCCGCGAATTCACTAGTGACC**CGTACGA**
TTCGATTGGCTTGAATCTTTTGAGTGAAGGAGAAAAAGCAGCACCTCGGCTCCCCATCCACCTGGA
CGACGGGCTCAGGTTCAGTGGCCATCATCATAATCGTCTCCGTGATGGCGTGCTCATCGTCCTCCT
CCATAACCACATCCCTGTTGT**CGTCTCCCAGCACGTCGTAC**AATCGAATTCCCGCGGCCGCCATGG
CGGCCGGAGCTGCANCGC

(B)

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Query      59 CGTACGATTCGATTGGCTTGAATCTTTTGAGTGAAGGAGAAAAAGCAGCACCTCGGCTCC  118
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AF258448  491 CGTACGATTCGATTGGCTTGAATCTTTTGAGTGAAGGAGAAAAAGCAGCACCTCGGCTCC  432

Query     119 CCATCCACCTGGACGACGGGCTCAGGTTCAGTGGCCATCATCATAATCGTCTCCGTGATG  178
              || ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AF258448  431 CCGTCCACCTGGACGACGGGCTCAGGTTCAGTGGCCATCATCATAATCGTCTCCGTGATG  372

Query     179 GCGTGCTCATCGTCCTCCTCCATAACCACATCCCTGTTGTCGTCTCCCAGCACGTCGTAC  238
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AF258448  371 GCGTGCTCATCGTCCTCCTCCATAACCACATCCCTGTTGTCGTCTCCCAGCACGTCGTAC  312
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Supplementary figure 1. (A) Sequence obtained by cloning the Myostatin purified band in pGEM-Teasy vector and sequencing using SP6 primer and BigDye mix. (B) A 177bp fragment with a 100% homology to the Myogenin from seabream (AF258448) was amplified.