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ORIGINAL PAPER

GH overexpression causes muscle hypertrophy independent from local IGF-I in a zebrafish transgenic model

Rafael Y. Kuradomi · Márcio A. Figueiredo · Carlos F. C. Lanes · Carlos E. da Rosa · Daniela V. Almeida · Rodrigo Maggioni · Maeli D. P. Silva · Luis F. Marins

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Abstract The aim of the present study was to analyse the morphology of white skeletal muscle in males and females from the GH-transgenic zebrafish (*Danio rerio*) lineage F0104, comparing the expression of genes related to the somatotrophic axis and myogenesis. Histological analysis demonstrated that transgenic fish presented enhanced muscle hypertrophy when compared to non-transgenic fish, with transgenic females being more hypertrophic than transgenic males. The expression of genes related to muscle growth revealed that transgenic hypertrophy is independent from local induction of insulin-like growth factor 1 gene (*igf1*). In addition, transgenic males exhibited significant induction of myogenin gene (*myog*) expression, indicating that myog may mediate hypertrophic growth in zebrafish males overexpressing GH. Induction of the α-actin gene (*acta1*) in males, independently from transgenesis, also was observed. There were no significant differences in total protein content from the muscle. Our results show that muscle hypertrophy is independent from muscle *igf1*, and is likely to be a direct effect of excess circulating GH and/or IGF1 in this transgenic zebrafish lineage.

Keywords Transgenic zebrafish · Growth hormone · Skeletal muscle · Hypertrophy · Myogenic factors

C. E. da Rosa · L. F. Marins (✉)
Instituto de Ciências Biológicas, Universidade Federal do Rio Grande, FURG, Avenida Itália, Km 8, Rio Grande, RS 96201-900, Brazil
e-mail: dqmluf@furg.br

D. V. Almeida · L. F. Marins
Programa de Pós-Graduação em Ciências Fisiológicas, Fisiologia Animal Comparada, Instituto de Ciências Biológicas, FURG, Rio Grande, Brazil

R. Y. Kuradomi · M. A. Figueiredo · L. F. Marins
Programa de Pós-Graduação em Aqüicultura, Instituto de Oceanografia, FURG, Rio Grande, Brazil

C. F. C. Lanes
Faculty of Biosciences and Aquaculture, Bodø University College, 8049 Bodø, Norway

R. Maggioni
Instituto de Ciências do Mar, Universidade Federal do Ceará, UFC, Fortaleza, CE, Brazil

M. D. P. Silva
Departamento de Morfologia, Instituto de Biociências, Universidade Estadual Paulista, UNESP, Botucatu, SP, Brazil

Introduction

Growth hormone (GH) is a pluripotent vertebrate hormone produced by the hypophysis, with the main role of mediating somatic growth. GH acts by binding with specific transmembrane receptors (GHR) located in the target tissues (Reinecke et al. 2005). This

interaction triggers an intracellular signalling cascade that culminates with the transcription of specific genes involved in the development of biological responses to GH (Schindler and Darnell 1995; Ihle 1996; Waters et al. 2006). Physiological response to GH is mainly developed and controlled through a chain known as the somatotrophic axis, where insulin-like growth factor 1 (IGF1) is the main mediator (Moriyama et al. 2000; Butler and Le Roith 2001).

IGF1 integrates tissue-specific regulation and many biological events through interaction with specific receptors (IGF1R) which are present in the target cells (Moriyama et al. 2000; Wood et al. 2005). In fishes, as well as in other vertebrates, the liver is the main IGF1 production site (Yakar et al. 1999; Reinecke et al. 2005; Wood et al. 2005). A positive correlation between plasma IGF1 and growth rate has been demonstrated (Kajimura et al. 2001; Uchida et al. 2003). However, *igf1* gene expression can occur in other tissues, playing both autocrine as well as paracrine roles (for a review, refer to Wood et al. 2005). IGF1 production outside the liver seems to play an important role in growth (Yakar et al. 1999; Butler and Le Roith 2001; Nordgarden et al. 2006; Eppler et al. 2007).

In skeletal muscle, GH can act upon muscle growth independently from IGF1 (Nordgarden et al. 2006; Sotiropoulos et al. 2006). However, IGF1 maintains its role in the formation, upkeep and regeneration of muscle tissue (Stewart and Rotwein 1996; Benito et al. 1996; Le Roith et al. 2001; Rabinovsky et al. 2002), participating in myoblast proliferation, myogenic differentiation and muscle hypertrophy (Czerwinski et al. 1994; Florini et al. 1996; Le Roith et al. 2001). This diversity of roles suggests that IGF1 acts in synergy with GH in the process of promoting skeletal muscle growth. Skeletal muscle growth in fishes involves the proliferation and differentiation of myogenic cells, also known as adult myoblasts or myosatellite cells, which are responsible for muscle fiber hyperplasic and hypertrophic growth (Koumans and Akster 1995; Johnston 1999; Rowlerson and Veggetti 2001; Johansen and Overturf 2005). Hyperplasia and hypertrophy are regulated by myogenic regulation factors (MRFs) that include myogenic differentiation 1 protein (MYOD1), myogenic factor 4 or myogenin (MYOG), myogenic factor 5 (MYF5), and the MRF4 family (Watabe 1999; Watabe 2001). The MRFs contain a conserved central domain known as the E-box, which is crucial for DNA sequence recognition, allowing the MRFs to locate a promoter motif that is present in most muscle-specific genes (Lassar et al. 1989; Murre et al. 1989; Blackwell and Weintraub 1990). Muscle growth in both mammals and fishes seems to be controlled by myostatin (MSTN), a protein from the TGF-β (transforming growth factor β) family, known as a potent muscle growth mediator (McPherron et al. 1997; Acosta et al. 2005).

Genetic manipulation involving the *gh* gene often has been used to enhance fish growth rate in aquaculture species in experimental level (Devlin et al. 2006). In most cases, *gh* overexpression causes a significant increase in growth (Du et al. 1992; Rahman et al. 1998; Nam et al. 2001; Devlin et al. 2004; Figueiredo et al. 2007a, b). However, growth enhancement by GH injection as well as by gene overexpression may alter muscle tissue structure, increasing the relative amount of fibers with smaller diameter, as observed in rainbow trout, coho salmon and Arctic char (Weatherley and Gill 1982; Fauconneau et al. 1997; Hill et al. 2000; Pitkänen et al. 2001).

Even though there are a number of GH-transgenic fish lineages, the relationship among hormone overexpression, muscle tissue structure, and sex-specific expression of genes involved in the somatotrophic axis and myogenesis is rarely approached. A sex-dependent secretory profile has been reported for pituitary GH in mammals (Jansson et al. 1985; Canosa et al. 2007), but this pattern has not been proven for fishes. We recently have developed and presented a genetically modified lineage of the zebrafish *Danio rerio,* named F0104, which overexpresses a piscine GH gene (Figueiredo et al. 2007a). The F0104 lineage can be used as a model for studies of muscle growth. Different genotypes of this lineage have been studied for growth and growth-related gene expression, including liver *igf1* expression (Figueiredo et al. 2007b), metabolism and the production of reactive oxygen species (Rosa et al. 2008), and intracellular signalling control mechanisms in the somatotrophic axis (Studzinski et al. 2009). Therefore, the objective of the present study was to approach the relationship between white skeletal muscle morphology and the expression of genes involved in the somatotrophic axis and myogenesis in adult fish from the transgenic lineage F0104, considering the influence of sex.

Materials and methods

Transgenic fish

Transgenic and non-transgenic control fish were obtained from crosses between non-transgenic females and hemizygous transgenic males from lineage F0104, following a previously described protocol (Figueiredo et al. 2007a). The F0104 lineage was produced by the co-injection of transgenes cβA/msGH and cβA/GFP. Both transgenes include the carp (*Cyprinus carpio*) β-actin promoter and either marine silverside (*Odontesthes argentinensis*) GH cDNA (Marins et al. 2002) or the green fluorescent protein (GFP) gene. GFP was used as a marker for successful gene transfer, allowing identification through fluorescence analysis (excitation = 485 nm; emission = 520 nm).

Growth experiments

All fish were reared for 7 months in a system equipped with biological filtration. Each group (transgenic females—TF; transgenic males—TM, non-transgenic females—NTF and non-transgenic males—NTM) was kept at an average density of one fish per litre of water. Water quality was monitored at least once a week. Temperature, pH, nitrogen compounds and photoperiod were kept at the recommended optimum levels for zebrafish (Westerfield 1995). Fish were fed a commercial feed with a high level of total protein (47.5%) twice a day, *ad libidum*. At the end of the experiment, the animals were anesthetised (Tricaine methosulphonate, 0.1 mg mL^{-1}) and photographed with a digital camera. Standard length (L_s) was taken from each image through the open source software IMAGE J (US National Institute of Health, available at http://rsb.info.nih.gov/ij/).

RNA extraction and cDNA synthesis

Total RNA was isolated from white muscle of six individuals from each group, using TRI Reagent Solution (Applied Biosystems, Brazil), following the manufacturer's recommendations. Total RNA was treated with the RNAse-free DNAse I kit (Applied Biosystems, Brazil), and quantified through a Qubit fluorimeter (Invitrogen, Brazil) using Quant-iT RNA Assay kit (Invitrogen, Brazil). RNA integrity was assessed through electrophoresis on 1% agarose gels. cDNA was synthesised using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Brazil), following the manufacturer's instructions.

Gene expression

Gene expression was analysed quantitatively through quantitative Real Time PCR (qRT-PCR). Each sample ($n = 4$) was analysed in triplicate. Gene-specific primers (Table 1) were designed using the software Primer Express 2.0 (Applied Biosystems, Brazil) from sequences available at GenBank. qRT-PCR reactions were performed in a 7,300 Real-Time PCR System (Applied Biosystems, Brazil) using a SYBR green PCR Master Mix kit (Applied Biosystems, Brazil). One-tenth of the cDNA was used for each 20 µL reaction. qRT-PCRs with cDNA serial dilutions were performed for all primers, in order to test reaction efficiency. qRT-PCR conditions were 50°C/3 min, 95°C/10 min, then 40 cycles of 95°C/15 s and 60°C/1 min. The dissociation curve was performed at 95°C/15 s, 60°C/1 min and 95°C/15 s. The expression of target genes (growth hormone receptor a—*ghra*; insulin-like growth factor 1—*igf1*; myogenic differentiation 1—*myod1*; myogenin—*myog*; myostatin b—*mstnb*; eukaryotic translation initiation factor 2, subunit 2 beta—*eif2s2*, myosin heavy chain 4—*myhc4* and α-actin—*acta1*) was normalized to that of the elongation factor 1 alpha gene (*ef1a*), which did not show significant differences among experimental groups (data not shown).

Total protein quantification

In order to quantify total proteins, six muscle tissue samples from each group were weighed (12 mg each) and homogenised in a 1 mg:9 µL mixture of tissue:buffer (Tris–HCl 100 mM; EDTA 2 mM; $MgCl_2 + 6H_2O$ 5 mM, pH 7.75). Homogenised samples were centrifuged for 15 min at 1,257 $rad.s^{-1}$ at 4°C and the supernatant kept on ice. A 1-µL aliquot was used to measure the amount of total protein on a Qubit fluorimeter (Invitrogen, Brazil) using a Quant-iT Protein Assay kit (Invitrogen, Brazil), following the manufacturer's recommendations. The resulting values were normalised by sample weight and used in the following analysis as milligrams of protein per gram of muscle tissue (mg g^{-1}).

Table 1 qRT-PCR gene-specific primers designed using sequences available at GenBank (www.ncbi.nlm.nih.gov)

Gene	Forward primer	Reverse primer	GenBank
ef1a	5′-CAAAATTGGAGGTATTGGAACTGTAC-3′	5′-TCAACAGACTTGACCTCAGTGGTT-3′	L47669
ghra	5′-TGCTGTGCGCTACAAAATGG-3′	5′-GCTTCTGCAAAGGCTGATAGAAA-3′	BC134903
igf1	5′-ACTTTGTGGGCACATGCAAA-3′	5′-CATGATCTCATTGCGAATTCCTT-3′	BC114262
myod1	5′-GGAGCGAATTTCCACAGAGACT-3′	5′-GTGCCCCTCCGGTACTGA-3′	BC114261
myog	5′-GGCCGCTACCTTGAGAGAGA-3′	5′-GAGCCTCAAAGGCCTCGTT-3′	AF202639
mstnb	5′-TGCTTTCCGCAAGACACTGT-3′	5′-GAAGCGGTGCCCAGAGAGT-3′	AF540956
eif2s2	5′-GCGCTCGTGGGTTTGTTG-3′	5′-CCTCAGGCTTTCGGTTTCC-3′	BC066706
myhc4	5′-GCGCGCTGACATTTCTGA-3′	5′-CAGCGTCACGGCTTTTGG-3′	AY921650
acta1	5′-TCTGTCCACCTTCCAGCAGAT-3′	5′-GATGGACCTGCCTCGTCGTA-3′	AF180887

Histological analysis

Six adult zebrafish per treatment at 7 months of age were anesthetised (Tricaine methosulfonate, 0.1 mg mL^{-1}) and euthanized on ice for collection of tissue samples. Samples were immediately fixed in Karnovsky solution (2.5% glutaraldehyde, 2% paraformaldehyde, 0.1 M phosphate buffer, pH 7.2) and preserved in 70% ethanol. Tissue samples then were dehydrated in an ethanol series (80, 95 and 100%) and embedded in resin (Historesin—Leica Instruments GmbH, Germany), according to the manufacturer's recommendations. Transverse histological sections (2 μm) from muscle fibers were obtained with a glass blade microtome. The sections were stained by hematoxilin-eosin (HE). Permanent slides were observed under an optical microscope (400×) equipped with a digital camera. From the images, the minor diameter was measured for at least 100 white muscle fibers per animal. All measures were taken using the open source software IMAGE J (US National Institute of Health, available at http://rsb.info.nih.gov/ij/).

Statistical analysis

Student's t test was used to compare average values of standard length (L_s) and total protein content (P_T) between transgenic and non-transgenic fish. Analysis of variance (ANOVA) was used to compare groups TF, TM, NTF and NTM, following Tukey's HSD test at a 5% significance level (Sokal and Rohlf 1995). Variance of muscle fiber proportions was analysed through a $r \times c$ table at a 5% significance level. When a significant difference was found, Marascuillo's method for multiple proportions comparison was applied (National Institute of Statistics—NIST, http://www.itl.nist.gov/div898/handbook). For gene expression analysis, the relative quantification method from the REST software (Pfaffl et al. 2002) was used, making pairwise comparisons among T × NT, TF × NTF, TM × NTM, TM × TF and NTM × NTF. The reference sample in each pairwise comparison was either the non-transgenic or the female.

Results

In the present study, transgenic fish were longer ($L_s = 3.40 \pm 0.35$ cm; $n = 20$) than the non-transgenic fish ($L_s = 2.82 \pm 0.19$ cm; $n = 20$). Comparing sexes within the same group, TF ($L_s = 3.56 \pm 0.32$ cm; $n = 10$) were significantly longer than TM ($L_s = 3.22 \pm 0.30$ cm; $n = 10$). However, among non-transgenic fish, lengths of NTF ($L_s = 2.93 \pm 0.18$ cm; $n = 10$) were not significantly different from those of NTM ($L_s = 2.72 \pm 0.13$ cm; $n = 10$). Comparing animals of the same sex, TM and TF were significantly longer than NTM and NTF, respectively.

In the muscle fiber analysis, the diameter frequency distributions revealed profiles that were significantly different between transgenic and non-transgenic fish (Fig. 1). The largest difference among the groups was observed regarding 40 μm diameter fibers (Fig. 1). In the present study, fibers at or below 40 μm diameter were called "thin" fibers, while fibers above this value were called "thick" fibers. Figure 2 shows that the relative proportion of thick fibers was significantly higher in transgenic (75%) than non-transgenic fish (39%). Comparing sexes

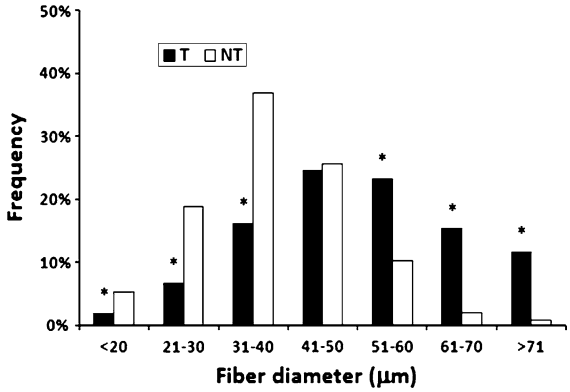

Fig. 1 Frequency distribution of muscle fiber diameters from transgenic (*T*) and non transgenic (*NT*) zebrafish (*Danio rerio*). *Asterisks* represent statistically significant differences ($P < 0.05$)

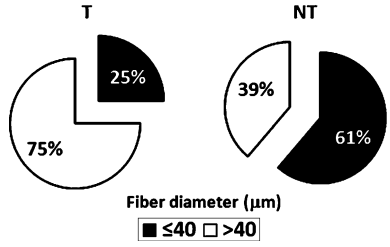

Fig. 2 Proportion of *thin* (≤40 µm) and *thick* (>40 µm) muscle fibers from transgenic (*T*) and non transgenic (*NT*) zebrafish (*Danio rerio*). Differences were significantly different at $P < 0.05$

(Fig. 3), the diameter frequency distribution was not different between NTM and NTF, but the difference was significant for TM and TF. The proportion of thick and thin fibers was similar among non-transgenic fish, while TF fish presented a significantly higher ($P < 0.05$) proportion of thick fibers than TM fish (Fig. 4). Both TM and TF fish showed a significant increase in thick fiber proportion than NTM and NTF, respectively (Fig. 4).

In the gene expression analysis, a significant ($P < 0.05$) 60% increase in *myog* expression was observed among transgenic fish (Fig. 5). Among non-transgenic fish, only *acta1* showed higher induction in males (133%) than in females (Fig. 6a). For TM fish, higher expression of *acta1* (47%) as well as *myog* (53%) was observed, in comparison to TF fish (Fig. 6b). When comparing females, no significant differences were observed in the expression of the analysed genes (Fig. 7a). Among males, however, *myog* was found at a significantly higher level (124%)

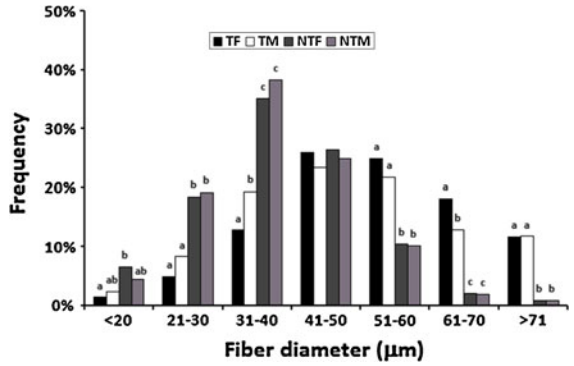

Fig. 3 Frequency distribution of muscle fiber diameters from transgenic and non transgenic zebrafish (*Danio rerio*), considering sex. *TF* transgenic females; *TM* transgenic males; *NTF* non transgenic females; *NTM* non transgenic females. *Different letters* represent statistically significant differences ($P < 0.05$)

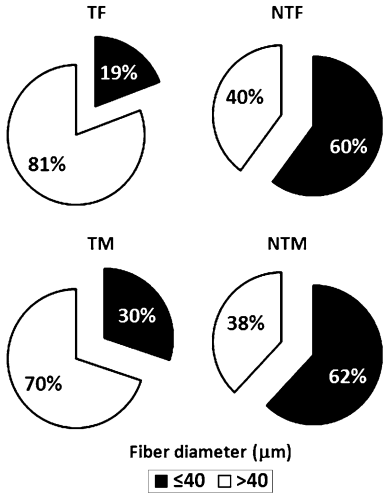

Fig. 4 Proportion of *thin* (≤40 µm) and *thick* (>40 µm) muscle fibers from transgenic (*T*) and non transgenic (*NT*) zebrafish (*Danio rerio*), considering sex. *TF* transgenic females; *TM* transgenic males; *NTF* non transgenic females; *NTM* non transgenic females. Differences were significantly different at $P < 0.05$

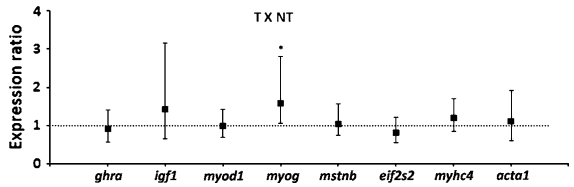

Fig. 5 Relative gene expression comparing transgenic (*T*) and non transgenic (*NT*) zebrafish (*Danio rerio*). NT were considered controls, where gene expression = 1 (*dashed line*). *Asterisks* represent statistically significant differences ($P < 0.05$)

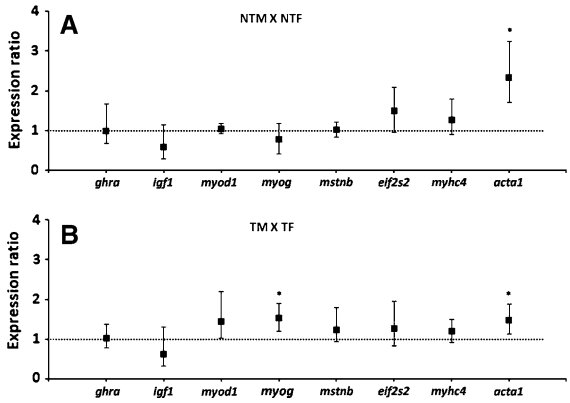

Fig. 6 Relative gene expression comparing zebrafish (*Danio rerio*) males and females, considering transgenesis (transgenic and non transgenic). **a** Comparison between non transgenic males (*NT*) and females (*NTF*). **b** Comparison between transgenic males (*TM*) and females (*TF*). In all comparisons females were considered controls, where gene expression = 1 (*dashed line*). *Asterisks* represent statistically significant differences ($P < 0.05$)

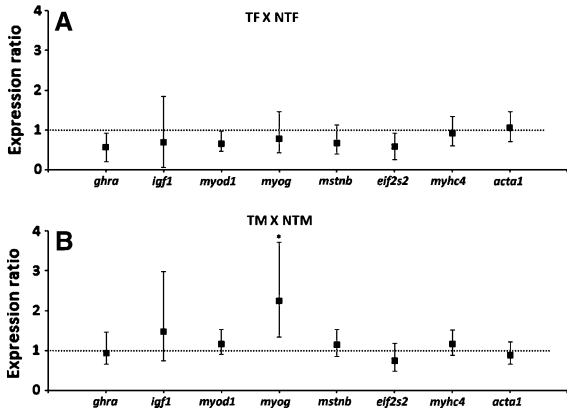

Fig. 7 Relative gene expression comparing transgenic and non transgenic zebrafish (*Danio rerio*), considering sex. **a** Comparison between transgenic females (*TF*) and non transgenic females (*NTF*). **b** Comparison between transgenic males (*TM*) and non transgenic males (*NTM*). In all comparisons non transgenics were considered controls, where gene expression = 1 (*dashed line*). *Asterisks* represent statistically significant differences ($P < 0.05$)

in TM fish (Fig. 7b). No significant difference in total protein content was observed between transgenic and non-transgenic fish or between males and females.

Discussion

Myogenesis involves a series of processes which culminate with the development and growth of muscle tissue. Among mammals, post-natal muscle growth occurs mainly through hypertrophy (increase in myotube size) and very little through hyperplasia (increase in myotube numbers and cellular proliferation). However, in most fish species, muscle growth by both hypertrophy and hyperplasia are observed during the whole life cycle (Rowlerson and Veggetti 2001). Among species that present limited muscle growth, such as zebrafish, this process involves a short hyperplastic growth period at the early development stages, followed by hypertrophy of the fibers formed (Weatherley et al. 1988). Considering that transgenic mice overexpressing *gh* show a marked hypertrophy (Dudley and Portanova 1987; Hikida et al. 1995; Clark et al. 2006), mainly hypertrophic muscle growth could be predicted for GH-transgenic zebrafish. Indeed, the present study showed a change in the muscle fiber diameter distribution in GH-transgenic zebrafish (Fig. 1). The transgenic profile was characterized by a 36% higher frequency of thick fibers (>40 μm) (Fig. 2). It is possible that these differences are related to the fusion of myoblasts to pre-existing fibers, which characterizes hypertrophy (Valente et al. 1999; Rowlerson and Veggetti 2001).

Considering that GH excess in transgenic adult zebrafish from lineage F0104 triggers hepatic *igf1* transcription (Figueiredo et al. 2007b), in association with the muscle hypertrophy reported in the present study, a new question arises: is increased muscle growth an effect from GH directly, from hepatic IGF1, or from both? In order to approach this question, *ghra* and *igf1* expression were measured, comparing transgenic and non-transgenic fish (Fig. 5). The results clearly show that GH excess is not associated with increased transcription for either *ghra* or *igf1* in transgenic zebrafish muscle. Sotiropoulos et al. (2006) provided evidence that GH has a hypertrophic effect on muscle, stimulating myoblast fusion into mammalian myotubes, suggesting that GH and IGF1 present independent and additive hypertrophic effects through distinct signalling pathways. Since IGF1 is considered a critical myogenic agent (Florini et al. 1996; Shavlakadze et al. 2005), the lack of muscle *igf1* induction in transgenic zebrafish observed here may indicate that circulating IGF1 is already at a high level. Even though liver *igf1* was not evaluated in the present study, Figueiredo et al. (2007b) already reported its induction in the same zebrafish model.

The expression analysis of other myogenesis regulation factors (*myod*, *myog* and *mstnb*) showed that only *myog* was significantly induced among transgenics (Fig. 5). Many studies have demonstrated that myogenin is involved in the control of myotube fusion and growth (Hasty et al. 1993; Nabeshima et al. 1993; Black and Olson 1998; Pownall et al. 2002), and that this transcription factor is likely to be a hypertrophy indicator (Johansen and Overturf 2005). The increased *myog* expression observed here among transgenic fish represents further evidence of its role in support of hypertrophy. However, a significant increase in *myog* expression was observed only among transgenic males. It is possible that hypertrophic females had reached their growth limit before the time of sampling. The largest standard length recorded for wild zebrafish is 3.5 cm (Spence et al. 2007). Transgenic females in the present study averaged 3.56 (±0.32) cm standard length, with hypertrophy significantly higher than males. Even though these data suggest that females had reached the maximum size, this hypothesis remains to be tested.

The analysis of *acta1* showed sex-dependent expression which is independent of transgenic status. In this case, males from both groups showed higher expression levels than females. Recent studies on mammals suggested the involvement of androgenic hormones and their receptors with the promoter region of the ACTA1 gene, affecting its expression (Vlahopoulos et al. 2005; Hong et al. 2008). It is well known that ACTA1 in is an important skeletal muscle protein, and that the androgens are likely to produce an anabolic effect through increased expression (Hong et al. 2008). This is the first time that differential sex-specific *acta1* expression is reported for fish muscle tissue.

No differences were found in the total protein content between transgenic and non-transgenic fish or between males and females from both groups. Even though *acta1* expression clearly was induced in males from both groups, this induction does not seem enough to provoke a significant increase in total muscle protein content. This result, however, is consistent with unaltered *eif2s2* expression, since this gene is an indicator of general protein synthesis, recycling other initiation factors (Price et al. 1996). Studies on GH-transgenic common carp have reported a higher level of total protein among transgenics (Chatakondi et al. 1995; Fu et al. 1998; Dunham et al. 2002). However, lower total protein levels have been recorded for transgenic Atlantic salmon (Cook et al. 2000) and Nile tilapia (Rahman et al. 2001). Hence, the effect of GH on total protein levels seems to vary across species and transgenic lines.

The results presented here showed that GH-transgenic zebrafish reach a more hypertrophic muscle structure than non-transgenic fish at the same age. This structure likely resulted from direct GH action in association with circulating hepatic IGF1. Muscle hypertrophy is likely to be mediated to some extent by myogenin as well. Even though commercial transgenic species, such as salmonids, present hyperplasic growth (Hill et al. 2000; Pitkänen et al. 2001), in the present study, only hypertrophic growth was observed among transgenic zebrafish.

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