Transgenic Res (2012) 21:457–469
DOI 10.1007/s11248-011-9546-2

ORIGINAL PAPER

Muscle-specific growth hormone receptor (GHR) overexpression induces hyperplasia but not hypertrophy in transgenic zebrafish

Marcio Azevedo Figueiredo · Edson A. Mareco · Maeli Dal Pai Silva · Luis Fernando Marins

Received: 18 March 2011 / Accepted: 16 August 2011 / Published online: 24 August 2011

Abstract Even though growth hormone (GH) transgenesis has demonstrated potential for improved growth of commercially important species, the hormone excess may result in undesired collateral effects. In this context, the aim of this work was to develop a new model of transgenic zebrafish (*Danio rerio*) characterized by a muscle-specific overexpression of the GH receptor (GHR) gene, evaluating the effect of transgenesis on growth, muscle structure and expression of growth-related genes. In on line of transgenic zebrafish overexpressing GHR in skeletal muscle, no significant difference in total weight in comparison to non-transgenics was observed. This can be explained by a significant reduction in expression of somatotrophic axis-related genes, in special insulin-like growth factor I (IGF-I). In the same sense, a significant increase in expression of the suppressors of cytokine signaling 1 and 3 (SOCS) was encountered in transgenics. Surprisingly, expression of genes coding for the main myogenic regulatory factors (MRFs) was higher in transgenic than non-transgenic zebrafish. Genes coding for muscle proteins did not follow the MRFs profile, showing a significant decrease in their expression. These results were corroborated by the histological analysis, where a hyperplasic muscle growth was observed in transgenics. In conclusion, our results demonstrated that GHR overexpression does not induce hypertrophic muscle growth in transgenic zebrafish probably because of SOCS impairment of the GHR/IGF-I pathway, culminating in IGF-I and muscle proteins decrease. Therefore, it seems that hypertrophy and hyperplasia follow two different routes for entire muscle growth, both of them triggered by GHR activation, but regulated by different mechanisms.

Keywords Transgenic zebrafish · Growth hormone receptor · Insulin-like growth factor I · Suppressors of cytokine signaling · Hyperplasia · Skeletal muscle

M. A. Figueiredo · L. F. Marins
Programa de Pós-Graduação em Aquicultura, Instituto de Oceanografia, Universidade Federal do Rio Grande—FURG, Rio Grande, RS, Brazil

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

L. F. Marins (✉)
Instituto de Ciências Biológicas, Universidade Federal do Rio Grande—FURG, Av. Itália, Km 8, Rio Grande, RS CEP 96201-900, Brazil
e-mail: dqmluf@furg.br

Introduction

The biotechnological advances over the last decades have provided important tools for gene and chromosome manipulation in organisms. Development of transgenic fish is a high-interest topic in aquaculture

due to the potential that this technology can offer for improving production (Zbikowska 2003; Dunham 2004). The growth hormone (GH) gene has been manipulated in order to raise its plasmatic concentration, inducing an increase in growth performance of commercially important fish. In fact, a higher-level expression of this gene has led to significant results, as reported by Devlin et al. (1994) for coho salmon (*Oncorhynchus kisutch*), Pitkänen et al. (1999) for Arctic charr (*Salvelinus alpinus*) and Nam et al. (2001) for mud loach (*Misgurnus mizolepis*). These studies demonstrated that supraphysiological GH levels can be obtained by genetic manipulation, leading to a significant increment in growth rates through an increase in intracellular signalling of the somatotrophic axis and its consequent biological effects.

GH is an adenohypophysary polypeptide hormone which, besides stimulating somatic growth, is also implied in other physiological mechanisms of fish such as energy mobilization, gonad development, osmoregulation, appetite, social behavior, morphology and immunology (Björnsson et al. 2002; Devlin et al. 2006; Canosa et al. 2007). Free GH in the blood stream can bind to receptors present in the membranes of target cells, promoting intracellular signalling constituted by a series of cascading phosphorylation reactions that actualize the somatotrophic axis. Membrane receptors that recognize GH, called GHR, are members of the cytokine type I receptor family (Butler and LeRoith 2001; Waters et al. 2006) which, when complexed to the hormone, induce phosphorylation of janus kinase (JAK) enzymes normally associated with the intracellular portion of the receptor (Argetsinger et al. 1993; VanderKuur et al. 1994, 1995). Once activated, JAKs phosphorylate specific intracellular regions of the receptor, which function as anchorage sites for cytoplasmic transcription factors of the STATs (signal transducers and activators of transcription) family. Once phosphorylated, STATs form dimers and translocate to the nucleus, activating the transcription of specific genes involved in GH biological responses.

The entire signalling pathway triggered by GH can be controlled in various manners. It is necessary to maintain the hormone at adequate levels, or even absent in some specific physiological or metabolic situations. A highly recognized form of controlling circulating GH levels is the negative feedback mechanism that GH and IGF-I (insulin-like growth factor type I) exert on hormone secretion by the adenohypophysis (Björnsson et al. 2002). This is important due to the fact that GH has a broad action spectrum on various physiological mechanisms, and its excess may provoke adverse collateral effects in the organism (Devlin et al. 2004).

The manner in which GH chooses its target cells can also be considered a regulatory mechanism. It is known that target cells present GHR molecules available in their membranes. Therefore, if cells are able to control the expression of their receptors, they can also control the intracellular signalling levels independently of circulating GH concentrations. In fact, GHR levels apparently vary antagonistically to GH levels, compensating signalling and even blocking the hormone's action in some tissues through a resistance phenomenon (Bartke et al. 2002; Figueiredo et al. 2007a).

Circulating GH levels and the amount of receptors present in membranes of target cells are determinant for intracellular signalling control. However, cells have additional mechanisms that may assist in this process. Recently, the control function of GH intracellular signalling has been attributed to proteins of the SOCS (suppressors of cytokine signaling) family (Croker et al. 2008). These proteins can bind to receptors or JAKs, preventing phosphorylation of STATs (Baker et al. 2009) and, consequently, intracellular signalling promoted by the GH present in the blood stream.

When the somatotrophic axis is not blocked and GH is available in the blood stream, intracellular signalling in target cells culminates with the activation of a series of growth-related genes. Among these is the IGF-I, considered to be the main indirect effecter of GH in growth promotion and tissue differentiation (Daughaday 2000; Butler and LeRoith 2001). Although IGF-I is produced in many cell types targeted by GH, its main production site is the liver, from which it is exported to the blood stream and becomes an important complementary agent for somatic growth (Sjögren et al. 2002; Janssen 2009).

Besides the liver, one of the main targets of circulating GH is muscle tissue. In fish aquaculture, this tissue is fundamentally important since it generally represents the final commercialized product. It is known that skeletal muscle growth in fish occurs through proliferation and differentiation of myogenic progenitor cells, also known as adult myoblasts or

myosatellite cells. These are responsible for hyperplasic and hypertrophic growth of muscle fibers (Koumans and Akster 1995; Johansen and Overturf 2005). Hyperplasia and hypertrophy are regulated by myogenic regulatory factors (MRFs) that include myod, myogenin (myog) and myf5 (Watabe 1999, 2001). MRFs recognize specific DNA nucleotide sequences present at the promoter regions of most muscle-specific genes, influencing transcription (Lassar et al. 1989; Murre et al. 1989; Blackwell and Weintraub 1990).

Advances in knowledge of GH signalling and regulation mechanisms permit deducting that its excess may result in undesired collateral effects. The development of various intracellular signalling control mechanisms in itself indicates that a rigorous control of its levels is absolutely necessary. In this manner, it seems obvious that maintaining this hormone at supraphysiological levels through genetic manipulation may result in a series of metabolic alterations with unpredictable consequences on other physiological systems in which GH acts (Devlin et al. 2006). Mori et al. (2007) observed alterations in liver, immunological, reproductive and growth-related genes expression in the amago salmon (*Oncorhynchus masou*). As a result of GH overexpression or administration, a significant increase in metabolic rates and oxygen consumption were also observed in Atlantic salmon (Cook et al. 2000; Herbert et al. 2001), tilapia (McKenzie et al. 2000, 2003) and in zebrafish (Rosa et al. 2008, 2011).

A possible alternative to growth manipulation may be the application of transgenesis for increasing GHR levels in a target tissue, instead of raising circulating hormone levels. This new paradigm of genetic manipulation brings forth two interesting possibilities. First, the animal will be able to regulate hormone levels according to its momentary needs, permitting an adequate utilization of available energy. Secondly, the use of tissue-specific promoters may direct the effect of circulating GH to the tissues of interest for increasing productivity of the cultivated organism. In this context, the aim of this work was to develop a new model of genetically modified fish characterized by a muscle-specific overexpression of the GHR gene, evaluating the effect of transgenesis on growth, muscle structure and expression of growth-related genes. Zebrafish (*Danio rerio*) was used as experimental model, which has been reported as a genetic model whose results can be extrapolated to aquaculture species (Dahm and Geisler 2006).

Materials and methods

Production of genetic constructs

For the production of transgenic fish, two genetic constructs were co-injected in recently fertilized eggs at the one-cell stage. These constructs were based on the commercial plasmid pDsRed-Express-DR (Clontech Laboratories, Inc.), which codifies a destabilized variant of the red fluorescent protein of *Discosoma* sp. (DsRed), and permits the insertion of promoters for directing its expression. A 2,582 basepair fragment of the zebrafish's myosin light chain 2 (mylz2) promoter was used, amplified through polymerase chain reaction (PCR) using primers MYO-FOR (5′-CGAATTCGGATATTTTCAAAGCCAATCG-3′) and MYO-REV (5′-CGGATCCGTCGAGACGGTA TGTGTGAAG-3′). For isolation of the GHR gene, reverse transcription PCR (RT-PCR) was performed with primers zfGHR2-FOR (5′-CGGATCCATGGCT CACTCGCTCTCTCTCGA-3′) and zfGHR3-REV (5′-GCGGCCGCAGCTTTTAATAGTCCCCTCAT GG-3′). Both PCRs were conducted in 25 μL reactions containing 2.5 μL 10× PCR buffer, 0.2 μM of each primer, 0.2 mM of each dNTP, 0.75 mM $MgCl_2$, 0.2 units of Platinum Taq DNA Polymerase (Invitrogen, Brazil) and 1 μL of DNA solution. Reactions were incubated at 94°C for 1 min, followed by 35 cycles of 30 s at 94°C, 1 min at 57°C, and 2.5 min at 72°C, with a final extension step at 72°C for 10 min. Fragments were purified from gel with Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Brazil), according to the manufacturer's recommendation.

The mylz2 promoter fragment was inserted in the pDsRed-Express-DR plasmid by adding restriction sites at its 5′ and 3′ extremities for the restriction endonucleases *Eco*R I and *Bam*H I, respectively, producing the construct denominated pMYO-Red (Fig. 1c). For substitution of DsRed by the GHR cDNA, restriction sites were inserted in the 5′ and 3′ extremities of the latter for the restriction endonucleases *Bam*H I and *Not* I, respectively, obtaining the pMYO-GHR construct (Fig. 1b). For linearizing constructs, primers MCS-FOR (5′-GACTCAGATCTCGAGCTCAAGCTT-3′)

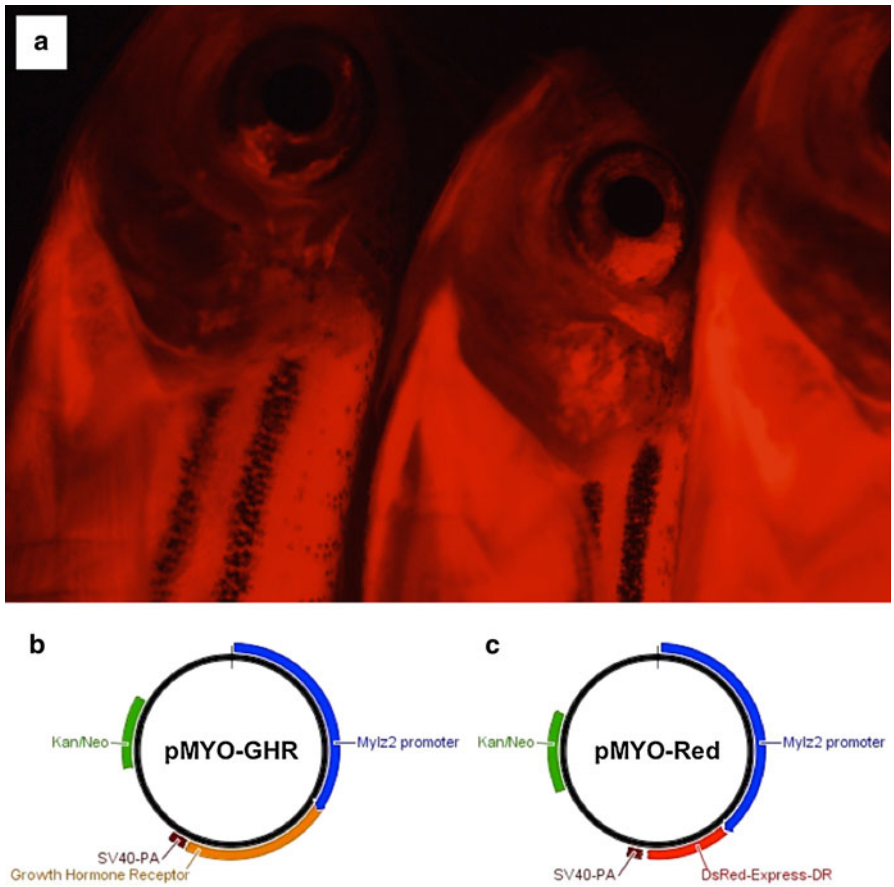

Fig. 1 **a** Muscle-specific expression of the red fluorescent protein (DsRed) driven by mylz2 (myosin light chain 2) promoter in transgenic zebrafish (*Danio rerio*) under UV light. **b** Plasmid pMYO-GHR. **c** Plasmid pMYO-Red

and Bgl II-PA (5′-CAGATCTTGAGTTTGGACAA ACCACAAC-3′) were used. PCR was performed in 25 μL reactions, as described above. Reactions were incubated at 94°C for 1 min, followed by 37 cycles of 30 s at 94°C, 1 min at 60°C and 4 min at 72°C, with a final extension at 72°C for 10 min. Fragments were purified from the gel, as described previously. After this process, constructs were denominated MYO-RED and MYO-GHR.

Fish maintenance and transgenics production

Fish were reared in a closed water circulation system according to Westerfield (1995). Approximately five hundred embryos at the one-cell stage were microinjected according to the general protocol suggested by Vielkind (1992) using an IM-30 (Narishige, Japan) motorized pico-injector for injecting approximately 300 pL of DNA solution, representing a final number of 10^6 copies of each transgene per embryo. Microinjected embryos were incubated at 28°C until hatching, when they were analyzed in an epifluorescence microscope (excitation = 557 nm; emission = 579 nm). Larvae were classified by DsRed expression patterns according to Figueiredo et al. (2007b).

About fifty DsRed positive F0 larvae were obtained, but only four individuals with strong DsRed expression were reared until maturity and separately reproduced with non-transgenic (wild type) fish. In order to confirm presence of the MYO-GHR construct in F1 fish, a small portion of the tail fin of DsRed positive fish was removed for genomic DNA extraction (Sambrook et al. 1989). The MYO-GHR gene was amplified via PCR using primers zfGHR2-FOR and Bgl II-PA and primers MYO-FOR and zfGHR3-REV, as previously described. PCRs were performed in 12.5 μL reactions containing 1.25 μL 10× PCR buffer, 0.2 μM of each primer, 0.2 mM of each dNTP, 0.75 mM $MgCl_2$, 0.1 units of Platinum Taq DNA Polymerase (Invitrogen, Brazil) and 0.5 μL of DNA solution. Reactions were incubated at 94°C for 1 min, followed by 33 cycles of 30 s at 94°C,

1 min at 60°C, and 2 min at 72°C, with a final extension step at 72°C for 10 min. Afterwards, F1 fish were individualized and reproduced with non-transgenic fish. Five positive larvae and five negative (F2) for DsRed, offspring of each reproduced fish were sacrificed and genomic DNA was extracted. This DNA was used as a target for PCR amplification of the MYO-GHR gene, using primers zfGHR2-FOR and Bgl II-PA for verification of its presence.

Growth analysis

Transgenic fish and their non-transgenic F2 siblings were reared until 3 months of age in a closed water circulation system comprised of 15 L aquariums. Eighteen fish from each group were used. Water quality was monitored once a day, and temperature, pH, nitrogen compounds and photoperiod were maintained according to zebrafish requirements (Westerfield 1995). Fish were fed with high-protein (47.5%) ration twice a day, until apparent satiety. At 45, 60, 75 and 90 days fish were anesthetized (Tricaine, 0.1 mg/mL) for performing weighing. Unfortunately, data from 75 days were lost and weight could not be analysed at this time point.

Gene expression

For gene expression analysis, 45-day-old transgenic fish and their non-transgenic siblings were used. For GH expression, total RNA was extracted from the pituitary, and for analysis of remaining genes extraction was from muscle, using TRIzol (Invitrogen, Brazil) method according to the manufacturer's instructions. Extracted RNA was treated with DNAse I Amplification Grade (Invitrogen, Brazil), according to the manufacturer's recommendations, and used as a target for cDNA synthesis through a High Capacity Reverse Transcription kit (Applied Biosystems, Brazil), following the manufacturer's instructions.

Gene expression was analyzed through quantitative Real Time PCR (qPCR). Each sample (n = 5) was analyzed in triplicate. Specific primers for each gene (Table 1) were drawn with software Primer Express 3.0 (Applied Biosystems, Brazil), from sequences available in GenBank (http://www.ncbi.nlm.nih.gov). qPCR reactions were performed in a 7500 Real Time PCR System (Applied Biosystems, Brazil) using Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen, Brazil). Serial dilutions were performed for all primers in order to determine the qPCR reaction efficiency. PCR conditions were 50°C/2 min, 95°C/2 min, followed by 40 cycles of 95°C/15 s and 60°C/30 s. Target gene expression was normalized by the expression of the elongation factor 1 alpha (ef1α) gene, which did not vary significantly among experimental groups (not showed data). It is worth noting that in order to differentiate endogenous and exogenous GHR expression in transgenic animals, two pairs of primers

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

Gene	Forward	Reverse	GenBank
GH	5′-aagatcagtgttcaaagggttcact-3′	5′-ttaaggcaagaatctatcagacagaca-3′	NM001020492.2
GHRa	5′-tgctgtgcgctacaaaatgg-3′	5′-gcttctgcaaaggctgatagaaa-3′	EU649774.1
JAK2	5′-ctacccgccccgaagtg-3′	5′-cgtagtccatgcagctgttga-3′	NM131087.1
STAT5.1	5′-aaattggcggcatcactatagc-3′	5′-cctttcccctgctttgttagg-3′	NM194387.1
IGF-I	5′-caggcaaatctccacgatctc-3′	5′-tttggtgtcctggaatatctgt-3′	NM131825.2
SOCS1	5′-ctccgttttaggatgcaggaat-3′	5′-cattgtgcagtgttcaagtctgata-3′	BC077158.1
SOCS3	5′-ctggtacgatcgctgatcca-3′	5′-ggcaagaatggcgcttca-3′	NM_199950.1
myf5	5′-tccaatgggcctgcaaa-3′	5′-cggcggtccaccgtact-3′	AF270789.1
myod	5′-ggagcgaatttccacagagact-3′	5′-gtgcccctccggtactga-3′	BC114261.1
myog	5′-ggccgctaccttgagagaga-3′	5′-gagcctcaaaggcctcgtt-3′	AF202639.1
Acta1	5′-tctgtccaccttccagcagat-3′	5′-gatggacctgcctcgtcgta-3′	AF180887.1
mylz2	5′-tggaggccatgatcaaggaa-3′	5′-tggtgaggaaaacggtgaagt-3′	BC045520.1
myhc4	5′-gcgcgctgacatttctga-3′	5′-cagcgtcacggcttttgg-3′	AY921650.1
GHR	5′-ttccggtcgcgctagct-3′	5′-gggaagccaagtcttcaggat-3′	EU649774.1
DsRed	5′-ctggacatcacctcccacaac-3′	5′-ctcggcgcgctcgtact-3′	
ef1α	5′-gggcaagggctccttcaa-3′	5′-cgctcggccttcagtttg-3′	NM131263.1

were drawn for this gene, one that amplifies at the non-coding 3′ region (referred to in this work as GHRa) and therefore do not amplify exogenous GHR, and another that amplifies at the coding region of the gene (denominated GHR).

Histological analysis

Six 45-day-old transgenic and non-transgenic fish were anesthetized (Tricaine, 0.1 mg/mL) and sacrificed in ice for skeletal muscle tissue collection. Samples were immediately fixed in Karnovsky solution (glutaraldehyde 2.5%, paraformaldehyde 2%, 0.1 M phosphate buffer, pH 7.2) and preserved in 70% ethanol. Samples were dehydrated with a series of ethanol concentrations (80, 95 and 100%) and soaked in resin (Historesin—Leica Instruments GmbH, Germany), according to the manufacturer's recommendations. Transverse histological sections (4 μm) of muscle fibers were obtained through a glass-razor microtome. Sections were stained through the hematoxylin-eosin (HE) method. Fiber diameter was determined using a microscope coupled to an image analyzer. The area of white muscle fibers was measured and fiber diameter determined by the formula $D = 2\,A^{0.5}\,pi^{-0.5}$ (Valente et al. 1999). Fibers were classified according to their diameter as follows: <16 μm (thin fibers) and >16 μm (thick fibers).

Statistical analysis

Growth data was analyzed with one-way ANOVA followed by Tukey's post-hoc test. Variation in proportion of muscle fibers was analyzed through $r \times c$ (2×2) table at a 5% significance level, with a Chi-square test using Yates correction. For gene expression analysis, the relative quantification method was used in software REST (Pfaffl et al. 2002), performing paired comparisons between transgenic and non-transgenic fish. Reference samples in each paired comparison were always the non-transgenics. The adopted alpha was 0.05 and results were expressed as median ± standard error (SE).

Results

Transgenic zebrafish were produced by co-injection in recently fertilized eggs at the one-cell stage, using MYO-RED and MYO-GHR constructs at an equimolar ratio. From four DsRed positive F0 individuals reproduced, only one male was transmitting both constructs to descendants in a Mendelian manner, probably integrated in the same chromosome. This fish was considered the transgenic line founder. Expression of the red fluorescent protein in skeletal muscle was observed under UV light (Fig. 1a). Presence of the MYO-GHR construct in F1 was confirmed by PCR. After reproduction of F1 fish with non-transgenic animals, it was confirmed that the transgenes were being transmitted together to F2, since every animal that presented expression for DsRed was PCR-positive for the MYO-GHR transgene. Animals without fluorescence did not possess the MYO-GHR transgene, indicating that segregation of the two transgenes was not occurring.

Growth analysis of F2 transgenic animals revealed no significant difference in relation to non-transgenics (Fig. 2). However, in the expression analysis it was verified that the GHR of transgenic fish was over 100 times more expressed in muscle when compared to non-transgenic animals ($n = 5$). No significant difference in GH expression was observed between transgenic and non-transgenic fish (Fig. 3a). On the other hand, a significant induction of over 2.5 times in GHRa expression was observed in transgenics when compared to non-transgenics (Fig. 3a). Also, a significant reduction in STAT5.1 and IGF-I gene expression of over 70 and 60%,

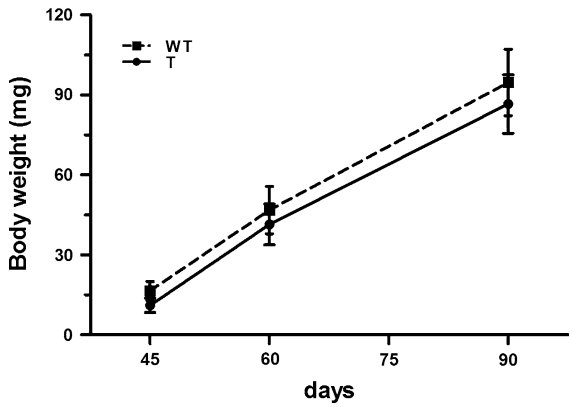

Fig. 2 Growth of transgenic (*T*) and wild type (*WT*) zebrafish (*Danio rerio*)

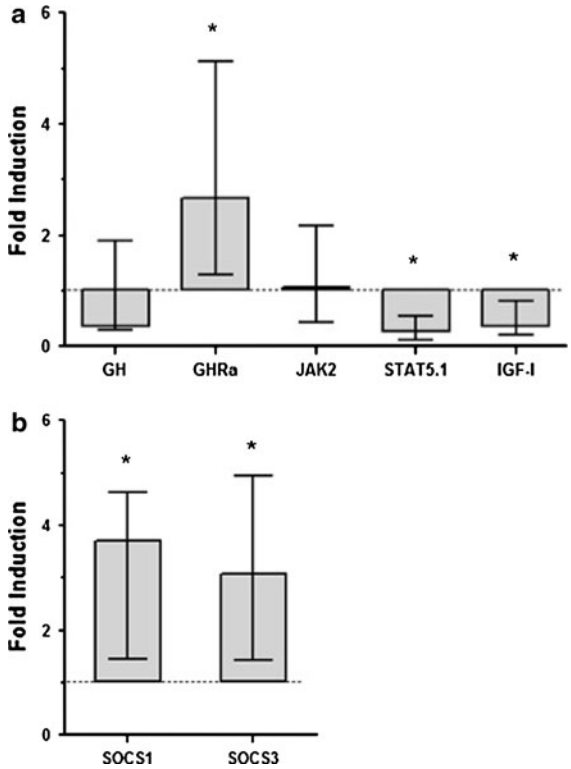

Fig. 3 Relative gene expression comparing transgenic and wild type zebrafish (*Danio rerio*). **a** Somatotrophic axis-related genes. **b** SOCS1 and SOCS3 genes. Wild type were considered controls, where gene expression = 1 (*dashed line*). *Asterisks* represent statistically significant differences ($P < 0.05$). In all cases n = 5

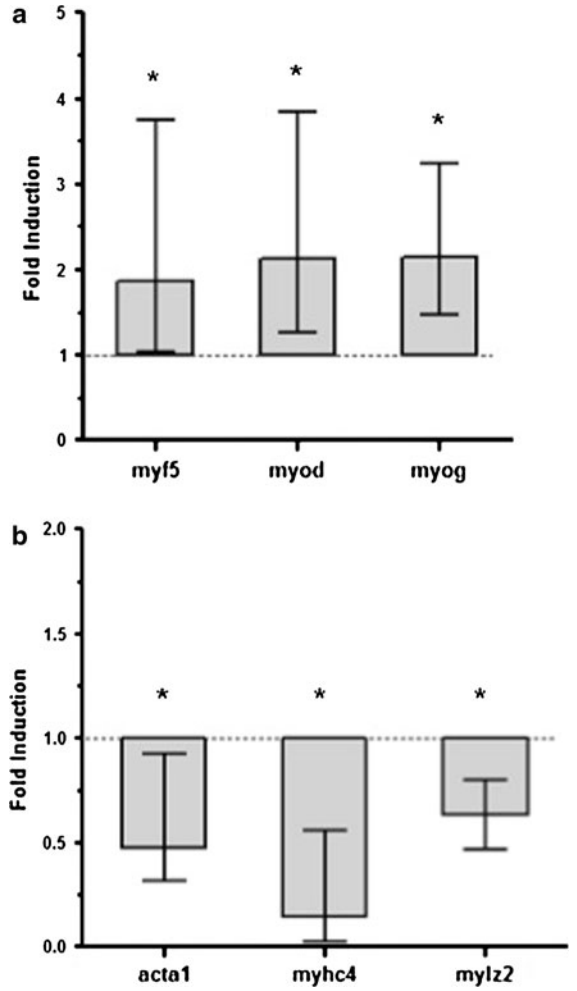

Fig. 4 Relative gene expression comparing transgenic and wild type zebrafish (*Danio rerio*). **a** Myogenic regulator factor genes. **b** Genes coding for structural muscle proteins. Wild type were considered controls, where gene expression = 1 (*dashed line*). *Asterisks* represent statistically significant differences ($P < 0.05$). In all cases n = 5

respectively, was observed in the transgenic animals (Fig. 3a).

Regarding GHR regulator genes, a significant increase of approximately three times in SOCS1 and SOCS3 expression was encountered in transgenic fish when compared to non-transgenics (Fig. 3b). Additionally, the expression of all MRF genes analysed (myod, myf5 and myog) was two times higher in transgenic fish than in non-transgenic animals (Fig. 4a). However, expression of genes coding for muscle proteins was significantly reduced in transgenic animals (Fig. 4b).

Histological analysis of the mean diameter of muscle fibers showed that the number of fibers with diameter inferior to 16 μm significantly increased in transgenic animals in relation to non-transgenics (Fig. 5), which apparently evidences hyperplasic muscle growth in these fish.

Discussion

The production of genetically modified fish models, especially for GH, is an important tool for growth studies. Recently, our group produced a lineage of transgenic zebrafish overexpressing the GH gene, which presented accelerated growth (Figueiredo et al. 2007a, b). However, many studies performed on this lineage show important physiological and biochemical alterations, demonstrating that accelerated growth comes at a high cost (Rosa et al. 2008, 2010, 2011). This has also been observed in other

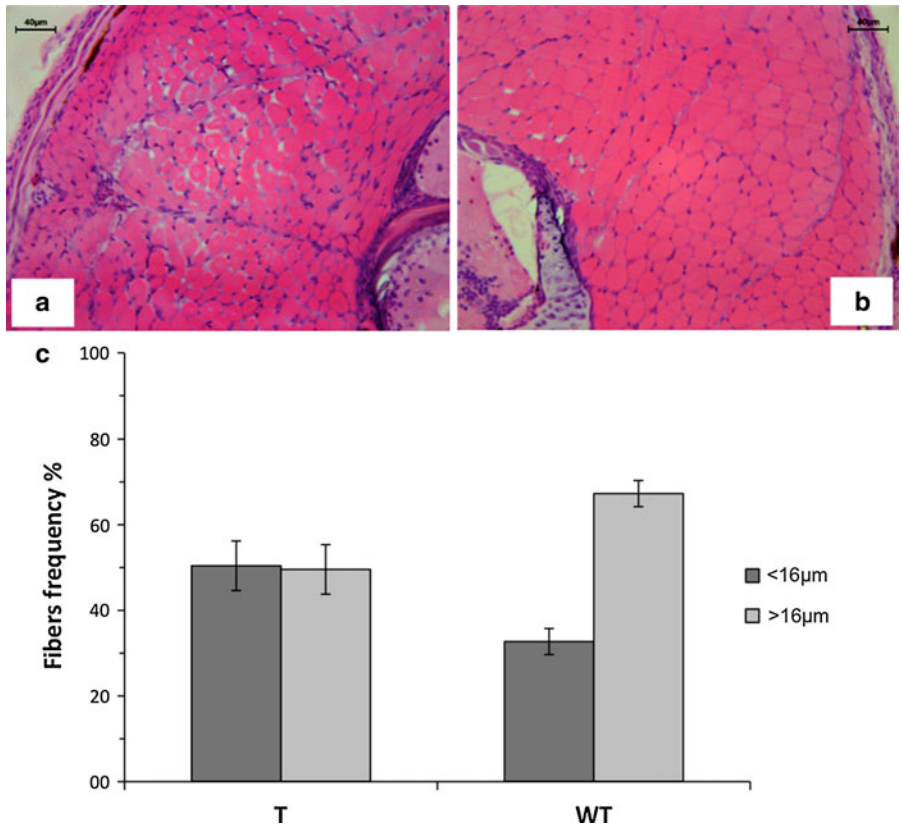

Fig. 5 **a** Muscle histological section from transgenic zebrafish. **b** Muscle histological section from wild type zebrafish. **c** Proportion of thin (<16 µm) and thick (>16 µm) muscle fibers from transgenic (*T*) and wild type (*WT*) zebrafish (*Danio rerio*). Chi-square with Yates correction = 12.62, df = 1 ($P < 0.01$)

transgenic animals overexpressing GH (Cook et al. 2000; Herbert et al. 2001; Bartke et al. 2002; McKenzie et al. 2003). Although a higher growth can be obtained by GH overexpression, these evidences suggest that it is necessary to pursue alternative methods to obtain the desired growth without the collateral effects of excessive circulating hormones.

The increase in knowledge on GH intracellular signalling has permitted the development of new paradigms in genetic manipulation with the purpose of growth acceleration. With the objective of restraining actions of the somatotrophic axis signalling to a tissue of interest and therefore decrease its collateral effects, in the present work we developed a transgenic fish lineage overexpressing GHR in a muscle-specific manner. For such, we used a genetic construct consisting of the mylz2 promoter directing GHR expression in zebrafish. Additionally, we co-injected a second genetic construct that induced expression of a reporter gene (DsRed) under control of the same promoter. This strategy permitted in vivo identification of the muscle-specific effect of the mylz2 promoter (Fig. 1a). It has been demonstrated in previous works that this promoter is efficient in directing gene expression specifically to zebrafish skeletal muscle (Xu et al. 1999; Ju et al. 2003; Funkenstein et al. 2007).

Skeletal muscle growth in fish occurs through proliferation and differentiation of myogenic progenitor cells, responsible for hyperplasic and hypertrophic growth of muscle fibers (Johnston 1999; Rowlerson and Veggetti 2001), being this process regulated by myogenic regulatory factors (MRFs) (Sabourin and Rudnicki 2000). Hypertrophy (increase in myofiber size) and hyperplasia (increase in number of myofibers) are the two main mechanisms that promote augment in muscle mass. These mechanisms are apparently regulated by GH and IGF-I (Velloso 2008), with the effects of GH growth promotion regulated mainly by IGF-I (LeRoith et al. 2001).

The effects of endocrine and/or autocrine/paracrine GH and IGF-I can have distinct actions in the regulation of muscle mass (Velloso 2008). It was demonstrated in mice that knocking out the GHR

gene provokes not only a reduction in absolute weight of muscle tissue but also in the muscle weight/body weight relation, when compared to non-transgenic animals (Sotiropoulos et al. 2006). In another study, Kim et al. (2005) demonstrated that mice with the IGF-I receptor (IGF-IR) knocked out specifically in muscle tissue presented myofibers with reduced diameter and smaller muscles than non-transgenic animals, suggesting that GH may not be directly involved with increase in muscle mass.

In the present study, GHR transgenic zebrafish were compared to their non-transgenic siblings in terms of growth, skeletal muscle structure and expression of genes related to the somatotrophic axis and myogenesis. Growth analysis did not reveal significant differences between experimental groups (Fig. 2), despite the fact that transgenics expressed the GHR gene approximately 100 times more than non-transgenic animals. In a similar study, Ishtiaq Ahmed et al. (2011) observed accelerated growth in transgenic zebrafish overexpressing GHR that had been constitutively activated due to the introduction of leucine zippers in the molecule's structure. In this case, the somatotrophic axis remained activated in a GH-independent manner in all tissues due to the ubiquitous CMV promoter used. Therefore, it is probable that the liver of these animals is producing a higher amount of IGF-I, as observed in the expression analysis of this gene in transgenic embryos. Differently, in our model the muscle-specific expression of GHR probably did not alter plasmatic IGF-I concentration, maintaining body growth at normal levels.

In order to evaluate the intracellular signalling level of GH in muscle tissue of our transgenic model, we analyzed the expression of the main genes of the somatotrophic axis. Results shown in Fig. 3a indicate that the muscle-specific overexpression of GHR caused, by contraries, a significant decrease in signalling of the somatotrophic axis, evidenced by the reduction in IGF-I and Stat5.1. In addition, it was observed an increase in GHRa (endogenous) gene expression, which can be attributed to a tissue response to the signalling decrease. The question that arises here is: if receptor expression increases, in what manner is the somatotrophic axis signalling affected?

It has been demonstrated that proteins of the SOCS family have an important function in the regulation of GH intracellular signalling (Herrington and Carter-Su 2001; Zhu et al. 2001; Greenhalgh and Alexander 2004; Croker et al. 2008; Walters and Griffiths 2009). Recently, Studzinski et al. (2009) verified that SOCS1 and SOCS3 are the main modulators of the somatotrophic axis in the liver of transgenic homozygous zebrafish of the F0104 lineage. In this manner, the present work analyzed SOCS1 and SOCS3 expression in the muscle tissue of GHR-transgenic fish. Results showed a significant increase in the expression of both genes (Fig. 3b), indicating that these proteins may also have a modulatory function in the GH intracellular signalling pathway in muscle. The hypothesis raised by Studzinski et al. (2009) for explaining the increase of SOCS1 and 3 proteins in transgenic homozygotes of the F0104 lineage was that since these animals express twice the amount of exogenous GH, they activated the signalling pathway above the limit supported by their energetic budget. This could be occurring to the transgenic lineage developed here, in the muscle of fish overexpressing GHR.

IGF-I has been recognized as a hypertrophic agent (Glass 2003; Clemmons 2009). DeVol et al. (1990) demonstrated that IGF-I expression increases during compensatory hypertrophy provoked experimentally in rats. IGF-I increases muscle mass by stimulus of the phosphatidylinositol-3 kinase (PI3K)/kinase B protein (Akt) pathway, resulting in activation of protein synthesis (Bodine et al. 2001; Rommel et al. 2001; Singleton and Feldman 2001) associated with hypertrophy (Glass 2005; Clemmons 2009). In order to verify if this pathway was blocked we analyzed the expression of genes that codify for some of the main structural muscle proteins. The results obtained showed that the alpha actin 1 (Acta1), myosin heavy chain 4 (myhc4) and mylz2 genes presented significantly reduced expression (Fig. 4b). For evaluating if the degree of hypertrophy was actually altered in the transgenic fish produced due to the low expression of the IGF-I gene, we performed histological analyses of muscle tissue. Figure 5 shows that transgenic fish presented a significantly higher percentage of smaller diameter fibers in relation to non-transgenics. Skeletal muscle consists of a mixture of smaller and larger diameter fibers, with smaller fibers being initial stages of the larger fibers, and utilized as a diagnosis of hyperplasic growth when present in elevated numbers (Weatherly and Gill 1987). The statistical analysis of fiber proportion by Chi-square

test showed a significant difference between transgenics and non-transgenics ($P < 0.01$), indicating that muscle growth of the transgenic fish produced in this work was altered towards hyperplasia.

As mentioned beforehand, muscle growth in fish is regulated by MRFs (Hawke and Garry 2001). Myod and myf5, primary MRFs, are responsible for proliferation and differentiation of myoblasts (Emerson 1990; Watabe 1999; Sabourin and Rudnicki 2000). On the other hand myog, considered a secondary MRF, controls muscle differentiation at a later stage through regulation of myoblast fusion and consequent formation of myotubes (Megeney and Rudnicki 1995; Rudnicki and Jaenish 1995). Due to these reasons and the tendency towards hyperplasic growth observed in histological sections, the expression of genes that codify for myf5, myod and myog was analyzed. As a result, a significant increase in the expression of these genes in transgenic animals was observed (Fig. 4a). This indicates that in some way, even without activation of the somatotrophic axis, overexpression of muscle GHR led to an increased expression of MRF genes. It is worth highlighting that, even GHR-overexpressing transgenics have the somatotrophic axis signalling decreased in muscle, it is probably that these animals preserve a normal production of hepatic IGF-I. Thus, a new question arises: could endocrine IGF-I be acting in muscle and therefore be compensating for decrease in IGF-I produced in an autocrine/paracrine manner?

It is known that besides the PI3K/Akt intracellular signalling pathway, which is more related to the differentiation process and protein synthesis (Coolican et al. 1997; Clemmons 2009), IGF-I also activates the mitogen- and extracellular signal-regulated kinase (MEK)/extracellular-regulated kinase (ERK) pathway, related to the proliferative process (Clemmons 2009). This hormone also activates the STAT3 pathway, associated to the negative feedback mechanism of IGF-I (Himpe and Kooijman 2009). The MEK/ERK pathway is also an alternative GH route (Herrington and Carter-Su 2001), being also related to the proliferative process. There are various evidences that GH intracellular signalling utilizes this pathway independently of JAK2 (Brooks et al. 2008), and therefore is not regulated by SOCS proteins. It has been demonstrated that SOCS1 and 3 proteins, besides modulating GH intracellular signalling by inhibiting JAK2, can also regulate the signalling of IGF-I and insulin through the action of these proteins on the insulin receptor substrate (IRS) (Dominici et al. 2005) and on JAK2, controlling the PI3K/Akt and the JAK/STAT pathways, respectively (Himpe and Kooijman 2009). On the other hand, SOCS proteins do not regulate the proliferative MEK/ERK signalling pathway for GH, as well as for IGF-I (Himpe and Kooijman 2009). In our model, it is likely that the MEK/ERK pathway presents an increased activation level due to the probable blockage of the PI3K/Akt and JAK/STAT pathways caused by SOCS1 and 3 proteins. Coolican et al. (1997), utilizing inhibitors for the PI3K/Akt and MEK/ERK pathways in rat myoblasts, demonstrated that when one pathway is blocked, activation increases in another. Since the MEK/ERK pathway is proliferative, it is likely that the increase in MRF genes expression observed in transgenics of the present work is related to a possible increase in the activation of this pathway.

Based on our results on one line of transgenic fish, we can conclude that the increase in GHR expression in muscle tissue does not necessarily implies an elevation of autocrine/paracrine IGF-I production. The PI3K/Akt pathway is apparently regulated by SOCS proteins, which most likely also regulate intracellular signalling induced by circulating IGF-I. Furthermore, the decrease in IGF-I signalling led to a reduction in the synthesis of some of the main muscle proteins, causing a diminution in muscle fiber diameter that characterized hyperplasic growth. Therefore, it is evident that GHR represents a key point in the muscle growth process, being partially used to activate the IGF-I pathway that culminates in protein synthesis and hypertrophic growth (PI3K), and partially used to activate the MEK/ERK pathway, which culminates in the activation of the MRFs responsible for myoblast proliferation. In this sense, hypertrophy and hyperplasia seems to follow two different pathways, both of them triggered by GHR activation, but regulated by different mechanisms.

In order to obtain a balanced hypertrophic/hyperplasic muscle growth, it seems to be necessary that both the PI3K/Akt and MEK/ERK pathways be activated in an equivalent manner. Perhaps, as proposed by Ishtiaq Ahmed et al. (2011), an interesting alternative could be to associate transgenics to a molecular design of constitutively activated proteins in specific tissues. In the case of muscle

tissue, the design of GH or IGF-I receptors resistant to the modulatory action of SOCS proteins could favour the proliferation of myoblasts accompanied by an adequate protein synthesis in order to sustain elevated hypertrophic growth.

Acknowledgments This work was supported by Brazilian CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, proc. no. 471437/2009-3) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior).

References

Argetsinger LS, Campbell GS, Yang X, Witthuhn BA, Silvennoinen O, Ihle JN, Carter-Su C (1993) Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. Cell 74:237–244

Baker BJ, Akhtar LN, Benveniste EN (2009) SOCS1 and SOCS3 in the control of CNS immunity. Trends Immunol 30:392–400

Bartke A, Chandrashekar V, Bailey B, Zaczek D, Turyn D (2002) Consequences of growth hormone (GH) overexpression and GH resistance. Neuropeptides 36:201–208

Björnsson BT, Johansson V, Benedet S, Einarsdottir IE, Hildahl J, Agustsson T, Jönsson E (2002) Growth hormone endocrinology of salmonids: regulatory mechanisms and mode of action. Fish Physiol Biochem 27:227–242

Blackwell T, Weintraub H (1990) Differences and similarities in DNA-binding preferences of MyoD and E2A protein complexes revealed by binding site selection. Science 250:1104–1110

Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, Zlotchenko E, Scrimgeour A, Lawrence JC, Glass DJ, Yancopoulos GD (2001) Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. Nat Cell Biol 3:1014–1019

Brooks AJ, Wooh JW, Tunny KA, Waters MJ (2008) Growth hormone receptor; mechanism of action. Int J Biochem Cell Biol 40:1984–1989

Butler AA, LeRoith DL (2001) Control of growth by the somatotrophic axis: growth hormone and the insulin-like growth factors have related and independent roles. Annu Rev Physiol 63:141–164

Canosa LF, Chang JP, Peter RE (2007) Neuroendocrine control of growth hormone in fish. Gen Comp Endocrinol 151:1–26

Clemmons DR (2009) Role of IGF-I in skeletal muscle mass maintenance. Trends Endocrinol Metab 20(7):349–356

Cook JT, McNiven MA, Richardson GF, Sutterlin AM (2000) Growth rate, body composition and feed digestibility/conversion of growth-enhanced transgenic Atlantic salmon (*Salmo salar*). Aquaculture 188:15–32

Coolican SA, Samuel DS, Ewton DZ, McWade FJ, Florini JR (1997) The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signaling pathways. J Biol Chem 272(10):6653–6662

Croker BA, Kiu H, Nicholson SE (2008) SOCS regulation of the JAK/STAT signaling pathway. Semin Cell Dev Biol 19:414–422

Dahm R, Geisler R (2006) Learning from small fry: the zebrafish as a genetic model organism for aquaculture fish species. Mar Biotechnol 8:329–345

Daughaday WH (2000) Growth hormone axis overview-somatomedin hypothesis. Pediatr Nephrol 14:537–540

Devlin RH, Yesaki TY, Biagi CA, Donaldson EM, Swanson P, Chan WK (1994) Extraordinary salmon growth. Nature 371:209–210

Devlin RH, Biagi CA, Yesaki TY (2004) Growth, viability and genetic characteristics of GH transgenic coho salmon strains. Aquaculture 236:607–632

Devlin RH, Sundstro LF, Muir WM (2006) Interface of biotechnology and ecology for environmental risk assessments of transgenic fish. Trends Biotechnol 24:89–97

DeVol DL, Rotwein P, Sadow JL, Novakofski J, Bechtel PJ (1990) Activation of insulin-like growth factor gene expression during work-induced skeletal muscle growth. Am J Physiol 259:E89–E95

Dominici FP, Argentino DP, Muñoz MC, Miquet JG, Sotelo AI, Turyn D (2005) Influence of the crosstalk between growth hormone and insulin signalling on the modulation of insulin sensitivity. Growth Horm IGF Res 15:324–336

Dunham RA (2004) Aquaculture and fisheries biotechnology: genetic approaches. CABI Publishing, Oxford

Emerson CP (1990) Myogenesis and developmental control genes. Curr Opin Cell Biol 2:1065–1075

Figueiredo MA, Lanes CFC, Almeida DV, Proietti MC, Marins LF (2007a) The effect of GH overexpression on GHR and IGF-I gene regulation in different genotypes of GH-transgenic zebrafish. Comp Biochem Physiol Part D Genomics Proteomics 2:228–233

Figueiredo MA, Lanes CFC, Almeida DV, Marins LF (2007b) Improving the production of transgenic fish germline: in vivo mosaicism evaluation by GFP transgene co-injection strategy. Genet Mol Biol 30:31–36

Funkenstein B, Skopal T, Rapoport B, Rebhan Y, Du SJ, Radaelli G (2007) Characterization and functional analysis of the 5′ flanking region of myosin light chain-2 gene expressed in white muscle of the gilthead sea bream (*Sparus aurata*). Comp Biochem Physiol Part D Genomics Proteomics 2:187–199

Glass DJ (2003) Signalling pathways that mediate skeletal muscle hypertrophy and atrophy. Nat Cell Biol 5:87–90

Glass DJ (2005) Skeletal muscle hypertrophy and atrophy signaling pathways. Int J Biochem Cell Biol 37:1974–1984

Greenhalgh CJ, Alexander WS (2004) Suppressors of cytokine signalling and regulation of growth hormone action. Growth Horm IGF Res 14:200–206

Hawke TJ, Garry DJ (2001) Myogenic satellite cells: physiology to molecular biology. J Appl Physiol 91:534–551

Herbert NA, Armstrong JD, Björnsson BT (2001) Evidence that growth hormone-induced elevation in rotine metabolism of juvenile Atlantic salmon is a result of increased spontaneous activity. J Fish Biol 59:754–757

Herrington J, Carter-Su C (2001) Signaling pathways activated by the growth hormone receptor. Trends Endocrinol Metab 12(6):252–257

Himpe E, Kooijman R (2009) Insulin-like growth factor-I receptor signal transduction and the Janus Kinase/Signal Transducer and Activator of Transcription (JAK-STAT) pathway. Biofactors 35(1):76–81

Ishtiaq Ahmed AS, Xiong F, Pang S-C, He M-D, Waters MJ, Zhu Z-Y, Sun Y-H (2011) Activation of GH signaling and GH-independent stimulation of growth in zebrafish by introduction of a constitutively activated GHR construct. Transgenic Res 20(3):557–567

Janssen JAMJL (2009) Advantages and disadvantages of GH/IGF-I combination treatment. Rev Endocr Metab Disord 10:157–162

Johansen KA, Overturf K (2005) Quantitative expression analysis of genes affecting muscle growth during development of rainbow trout (*Oncorhynchus mykiss*). Mar Biotechnol 7:576–587

Johnston IA (1999) Muscle development and growth: potential implication for flesh quality in fish. Aquaculture 177:99–115

Ju B, Chong SW, He J, Wang X, Xu Y, Wan H, Tong Y, Yan T, Korzh V, Gong Z (2003) Recapitulation of fast skeletal muscle development in zebrafish by transgenic expression of GFP under the mylz2 promoter. Dev Dyn 227:14–26

Kim H, Barton E, Muja N, Yakar S, Pennisi P, LeRoith D (2005) Intact insulin and insulin-like growth factor-I receptor signaling is required for growth hormone effects on skeletal muscle growth and function in vivo. Endocrinology 146(4):1772–1779

Koumans JTM, Akster HA (1995) Myogenic cells in development and growth of fish. Comp Biochem Physiol A Mol Integr Physiol 110:3–20

Lassar A, Buskin JN, Lockshon D, Davis RL, Apone S, Hauschka SD, Weintraub H (1989) MyoD is a sequence-specific DNA binding protein requiring a region of myc homology to bind to the muscle creatine kinase enhancer. Cell 58:823–831

LeRoith D, Bondy C, Yakar S, Liu JL, Butler A (2001) The somatomedin hypothesis: 2001. Endocr Rev 22(1):53–74

McKenzie DJ, Martinez IR, Morales A, Acosta J, Taylor EW, Steffensen JF, Estrada MP (2000) Metabolic rate, exercise performance and hypoxia tolerance of growth hormone transgenic tilapia (*Oreochromis sp*.). Comp Biochem Physiol 126:S66

McKenzie DJ, Martínez R, Morales A, Acosta J, Morales R, Taylor EW, Steffensen JF, Estrada MP (2003) Effects of growth hormone transgenesis on metabolic rate, exercise performance and hypoxia tolerance in tilapia hybrids. J Fish Biol 63:398–409

Megeney LA, Rudnicki MA (1995) Determination versus differentiation and the MyoD family of transcription factors. Biochem Cell Biol 73:723–732

Mori T, Hiraka I, Kurata Y, Kawachi H, Mano N, Devlin RH, Nagoya H, Araki K (2007) Changes in hepatic gene expression related to innate immunity, growth and iron metabolism in GH-transgenic amago salmon (*Oncorhynchus masou*) by cDNA subtraction and microarray analysis, and serum lysozyme activity. Gen Comp Endocrinol 151:42–54

Murre C, McCaw PS, Vaessin H, Caudy M, Jan LY, Jan YN, Cabrera CV, Buskin JN, Hauschka SD, Lassar AB (1989) Interactions between heterologous helix–loop–helix proteins generate complexes that bind specifically to a common DNA sequence. Cell 58:537–544

Nam YK, Noh JK, Cho YS, Cho HJ, Cho KN, Kim CG, Kim DS (2001) Dramatically accelerated growth and extraordinary gigantism of transgenic mud loach *Misgurnus mizolepis*. Transgenic Res 10:353–362

Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30:e36

Pitkänen TI, Krasnov A, Teerijoki H, Mölsä H (1999) Transfer of growth hormone (GH) transgenes into Arctic charr (*Salvelinus alpinus* L.). I. Growth response to various GH constructs. Genet Anal 15:91–98

Rommel C, Bodine SC, Clarke BA, Rossman R, Nunez L, Stitt TN, Yancopoulos GD, Glass DJ (2001) Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. Nat Cell Biol 3:1009–1013

Rosa CE, Figueiredo MA, Lanes CFC, Almeida DV, Monserrat JM, Marins LF (2008) Metabolic rate and reactive oxygen species production in different genotypes of GH-transgenic zebrafish. Comp Biochem Physiol B Biochem Mol Biol 149:209–214

Rosa CE, Kuradomi RY, Almeida DV, Lanes CFC, Figueiredo MA, Dytz AG, Fonseca DB, Marins LF (2010) GH overexpression modifies muscle expression of anti-oxidant enzymes and increases spinal curvature of old zebrafish. Exp Gerontol 45:449–456

Rosa CE, Figueiredo MA, Lanes CFC, Almeida DV, Marins LF (2011) Genotype-dependent gene expression profile of the antioxidant defense system (ADS) in the liver of a GH-transgenic zebrafish model. Transgenic Res 20:85–89

Rowlerson A, Veggetti A (2001) Cellular mechanisms of post-embryonic muscle growth in aquaculture species. In: Johnston IA (ed) Muscle development and growth. Academic Press, London, pp 103–140

Rudnicki MA, Jaenish R (1995) The MyoD family of transcription factors and skeletal muscle myogenesis. Bioessays 17:203–209

Sabourin LA, Rudnicki MA (2000) The molecular regulation of myogenesis. Clin Genet 57:16–25

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Singleton JR, Feldman EL (2001) Insulin-like growth factor-I in muscle metabolism and myotherapies. Neurobiol Dis 8:541–554

Sjögren K, Jansson J-O, Isaksson OGP, Ohlsson C (2002) A model for tissue-specific inducible insulin-like growth factor-I (IGF-I) inactivation to determine the physiological role of liver-derived IGF-I. Endocrine 19(3):249–256

Sotiropoulos A, Ohanna M, Kedzia C, Menon RK, Kopchick JJ, Kelly PA, Pende M (2006) Growth hormone promotes skeletal muscle cell fusion independent of insulin-like growth factor 1 up-regulation. Proc Natl Acad Sci USA 103:7315–7320

Studzinski AL, Almeida DV, Lanes CF, Figueiredo MA, Marins LF (2009) SOCS1 and SOCS3 are the main negative modulators of the somatotrophic axis in liver of homozygous GH-transgenic zebrafish (*Danio rerio*). Gen Comp Endocrinol 161:67–72

Valente LMP, Rocha E, Gomes EFS, Silva MW, Oliveira MH, Monteiro RAF, Fauconneau B (1999) Growth dynamics of white and red muscle fibres in fast- and slow-growing strains of rainbow trout. J Fish Biol 55:675–691

Vanderkuur JA, Wang X, Zhang L, Campbell GS, Allevato G, Billestrup N, Norstedt G, Carter-Su C (1994) Domains of the growth hormone receptor required for association and activation of JAK2 tyrosine kinase. J Biol Chem 269:21709–21717

Vanderkuur JA, Wang X, Zhang L, Allevato G, Billestrup N, Carter-Su C (1995) Growth hormone-dependent phosphorylation of tyrosine 333 and/or 338 of the growth hormone receptor. J Biol Chem 270:21738–21744

Velloso CP (2008) Regulation of muscle mass by growth hormone and IGF-I. Br J Pharmacol 154:557–568

Vielkind JR (1992) Medaka and zebrafish: ideal as transient and stable transgenic systems. In: Hew CL, Fletcher GL (eds) Transgenic fish. World Scientific, Singapore

Walters TD, Griffiths AM (2009) Mechanisms of growth impairment in pediatric Crohn's disease. Nat Rev Gastroenterol Hepatol 6:513–523

Watabe S (1999) Myogenic regulatory factors and muscle differentiation during ontogeny in fish. J Fish Biol 55:1–18

Watabe S (2001) Myogenic regulatory factors. In: Johnston IA (ed) Muscle development and growth. Academic Press, London, pp 19–41

Waters MJ, Hoang HN, Fairlie DP, Pelekanos RA, Brown RJ (2006) New insights into growth hormone action. J Mol Endocrinol 36:1–7

Weatherly AH, Gill HS (1987) The biology of fish growth. Academic Press, London

Westerfield M (1995) The zebrafish book: a guide for the laboratory use of zebrafish *Danio rerio*, 3rd edn. University of Oregon Press, Eugene

Xu Y, He J, Tian HL, Chan CH, Liao J, Yan T, Lam TJ, Gong Z (1999) Fast skeletal muscle-specific expression of a zebrafish myosin light chain 2 gene and characterization of its promoter by direct injection into skeletal muscle. DNA Cell Biol 18:85–95

Zbikowska HM (2003) Fish can be first—advances in fish transgenesis for commercial applications. Transgenic Res 12:379–389

Zhu T, Goh ELK, Graichen R, Ling L, Lobie PE (2001) Signal transduction via the growth hormone receptor. Cell Signal 13:599–616