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Effects of Double Transgenesis of Somatotrophic Axis (GH/GHR) on Skeletal Muscle Growth of Zebrafish (*Danio rerio*)

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Abstract

Transgenic fish for growth hormone (GH) has been considered as a potential technological improvement in aquaculture. In this study, a double-transgenic zebrafish was used to evaluate the effect of GH and its receptor (GHR) on muscle growth. Double transgenics reached the same length of GH transgenic, but with significantly less weight, featuring an unbalanced growth. The condition factor of GH/GHR-transgenic fish was lower than the other genotypes. Histological analysis showed a decrease in the percentage of thick muscle fibers in GH/GHR genotype of ~80% in comparison to GH-transgenic line. The analysis of gene expression showed a significant decrease in genes related to muscle growth in GH/GHR genotype. It seems that concomitant overexpression of GH and GHR resulted in a strong decrease of the somatotrophic axis intracellular signaling by diminishing its principal transcription factor signal transducer and activator of transcription 5.1 (STAT5.1).

Introduction

According to FAO,[1] aquaculture is currently one of the fastest growing production sectors in the world, with a series of tools being developed for perfecting production techniques. In this context, modern biotechnology presents great potential for meeting production demands and improving the phenotypical characteristics of aquatic organisms. Transgenesis is an important genetic manipulation tool used for modifying the traits of commercially important species such as fish. For aquaculture purposes, this manipulation has focused mainly on increasing growth rates of lines.[2] After the first transgenic fish was produced in China by Zhu *et al.*,[3] this technology has been successfully applied to dozens of fish species, including some with high commercial interest such as carps, tilapias, salmonids, and catfish.[4]

The growth process in fish begins with growth hormone (GH) synthesis in the hypophysis, followed by its release into the bloodstream where it acts on certain organs through association with specific receptors (GHR) present in the membranes of target cells. Activation of intracellular signaling pathways culminates with the transcription of genes involved in the development of biological responses to GH. In this manner, GH effects are produced and mostly controlled by the somatotrophic axis, with insulin-like growth factor 1 (IGF1) as the main physiological mediator.[5,6]

Although manipulation of the GH gene has shown promising results for increasing fish growth,[7–9] it is known that GH also acts in other processes producing pleiotropic effects on morphology, physiology, metabolism, and behavior.[10] Figueiredo *et al.*[11] developed a transgenic zebrafish (*Danio rerio*) model overexpressing silverside (*Odontesthes argentinensis*) GH and reported a significant increase in transgenic fish growth rates. More recently, Figueiredo *et al.*[12] created a new transgenic line overexpressing GHR in muscle tissue, to increase skeletal muscle intracellular signaling independently of supraphysiological levels of circulating GH and its resulting pleiotropic effects. However, this line did not present the expected muscle mass, seeming that hypertrophy is independent from GHR overexpression alone. In this study, the main objective was to evaluate if double transgenesis with increased circulating GH and a higher number of GH receptors on muscle membranes results in increased muscle growth. For achieving this, analyses of growth, gene expression, and muscle hypertrophy of double-transgenic (GH/GHR) zebrafish were performed.

Material and Methods

Zebrafish genotypes

All analyzed genotypes were obtained by breeding males from the F0104 line[11] with females from the MYO-GHR line.[12] Besides GH, the F0104 line also expresses the green

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fluorescent protein (GFP) under transcriptional control of the β-actin promoter of carp, *Cyprinus carpio*. In contrast, the MYO-GHR line expresses, apart from the GHRa gene, the red fluorescent protein (DsRED) in skeletal muscle tissue (zebrafish myosin light chain promoter). Breeding the two lines generated mendelian proportions of four genotypes (1:1:1:1), identified by epifluorescence microscopy: non-transgenic (NT) = no fluorescence; F0104 (GH) line = green fluorescence; MYO-GHR (GHR) line = red fluorescence; and double transgenic (GH/GHR) = green and red fluorescence (Fig. 1).

Growth analysis

Fish were separated by genotypes and cultivated for 165 days in 15-L aquariums ($N = 10$ per aquarium, in triplicate). Culture conditions were maintained according to zebrafish requirements.[13] Fish were fed twice daily *ad libitum* with commercial feed (47.5% crude protein). For final biometric analysis, fish were anesthetized with tricaine (0.1 mg/mL), weighed, and photographed in lateral decubitus for standard length measurements (L_s), obtained through ImageJ (*Image processing and analysis in Java*, http://rsb.info.nih.gov/ij/). Condition factor (K) was calculated using the equation $K = (W.\ L^{-3}) \times 10^3$, where W is mass (mg) and L the total length (mm).

Gene expression analysis

At the end of the growth experiment, five individuals of each group were randomly sampled for gene expression analysis. Genes related to the muscle growth such as *igf1*, myogenic regulatory factors (*myod* and *myf5*), and muscle structural proteins (*mylz2* and *myhc4*) were chosen. To evaluate intracellular signaling, we analyzed the signal transducer and activator of transcription 5.1 (*stat5.1*) gene. Fish were sacrificed with tricaine (0.5 mg/mL) for removal of skeletal muscle, total RNA extraction using the TRIzol reagent (Invitrogen), and cDNA synthesis through High Capacity cDNA Reverse Transcription kits (Applied Biosystems). All procedures were conducted as suggested by manufacturers.

Expression was analyzed through quantitative Real Time polymerase chain reaction (qPCR), with each sample analyzed in triplicate. Table 1 shows primer sequences used for each gene, designed using Primer Express 3.0 software (Applied Biosystems) based on sequences available in GenBank (www.ncbi.nlm.nih.gov). qPCRs were performed using Platinum SYBR Green qPCR SuperMix-UDG kits (Invitrogen). Serial dilutions were done for all primers to determine the reaction's efficiency (data not shown). 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. Expression of target genes was normalized by the constitutive genes elongation factor 1-alpha (*ef1*α) and beta-actin (β*actin*), which did not vary between experimental groups (data not shown).

Histological analysis

Eight fish of each group were sacrificed with tricaine (0.5 mg/mL) for skeletal muscle removal. Muscle tissue was positioned according to methods described by Lillie,[14] fixed in Bouin's solution for 4 h, and preserved in 70% ethanol. Histological samples were prepared using classic methods, cut into 4 μm sections, stained with hematoxylin/eosin, and analyzed following methods described in Weibel and Scherle[15] modified by Romano *et al.*[16] Muscle fibers were classified according to their diameter as follows: thin ($\leq$40 μm) and thick (>40 μm) fibers.

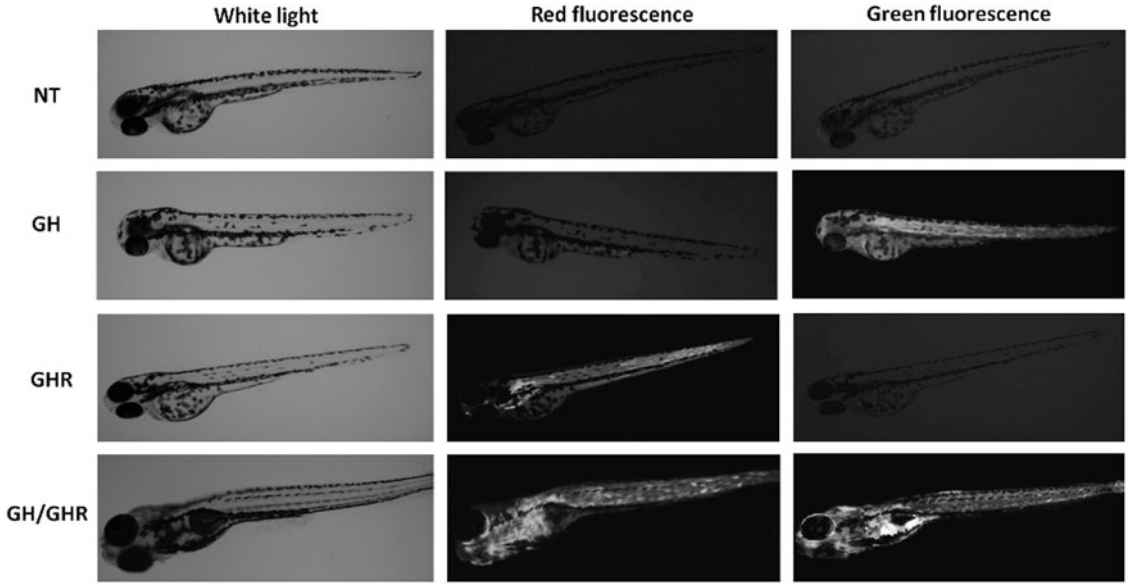

FIG. 1. Side view of zebrafish (*Danio rerio*) larvae from different genotypes under white light, red and green fluorescence. Non-transgenic (NT) fish do not show fluorescent expression, growth hormone (GH) line shows the expression of green fluorescent protein (GFP; excitation = 485 nm, emission = 520 nm), GH receptor (GHR) line shows the expression of red fluorescent protein (DsRed; excitation = 557 nm, emission = 579 nm), and GH/GHR (double) transgenic shows the expression of both red and green fluorescent protein.

Table 1. Gene-Specific Primers for qPCR Analyses

Gene	*Sequence*	*GenBank*	*Efficiency (%)*
igf1	F: 5′-CAGGCAAATCTCCACGATCTC-3′ R: 5′-TTTGGTGTCCTGGAATATCTGT-3′	NM131825	86
myf5	F: 5′-TCCAATGGGCCTGCAAA-3′ R: 5′-CGGCGGTCCACCGTACT-3′	AF270789	105
myod	F: 5′-GGAGCGAATTTCCACAGAGACT-3′ R: 5′-GTGCCCCTCCGGTACTGA-3′	BC114261	104
mylz2	F: 5′-TGGAGGCCATGATCAAGGAA-3′ R: 5′-TGGTGAGGAAAACGGTGAAGT-3′	BC045520	90
myhc4	F: 5′-GCGCGCTGACATTTCTGA-3′ R: 5′-CAGCGTCACGGCTTTTGG-3′	AY921650	85
stat5.1	F: 5′-AAATTGGCGGCATCACTATAGC-3′ R: 5′-CCTTTCCCCTGCTTTGTTAGG-3′	NM194387	90.7
ef1α	F: 5′-GGGCAAGGGCTCCTTCAA-3′ R: 5′-CGCTCGGCCTTCAGTTTG-3′	NM131263	101
ßactin	F: 5′-CTGTCCACCTTCCAGCAGAT-3′ R: 5′-GATGGACCTGCCTCGTCGTA-3′	AF180887	100

qPCR, quantitative real-time polymerase chain reaction.

Statistical analyses

Growth data were submitted to one-way ANOVA followed by Tukey's *post hoc* test. Relative gene expression was calculated through $2^{-\Delta\Delta CT}$.[17] Muscle fiber variance was analyzed through an r × c table with a 5% significance level. When significant difference was encountered, the Marascuillo multiple comparison test was applied (National Institute of Statistics, www.itl.nist.gov/div898/handbook).

Results

Growth

The evaluated genotypes presented final weight (mg ± SE) of NT = 254 ± 15, GH = 734 ± 37, GHR = 309 ± 39, and GH/GHR = 476 ± 35. Statistical analysis showed that the GH and GH/GHR groups weighed significantly more than the NT and GHR groups (Fig. 2A). No differences were observed when comparing NT versus GHR, but the GH genotype presented a significant weight increase in relation to GH/GHR. In terms of standard length (mm ± SE), all transgenic fish (GH = 33 ± 0.5; GHR = 26 ± 0.9; GH/GHR = 31 ± 0.7) were significantly larger than the NT (23 ± 0.4) ones. However, the GHR was significantly smaller than the GH and GH/GHR groups (Fig. 2B). No significant difference in condition factor (±SE) was observed between NT (2 ± 0.04), GH (2.1 ± 0.04), and GHR (1.8 ± 0.09) fish. In contrast, the GH/GHR group (1.6 ± 0.08) presented a lower condition factor when compared to the NT and GH groups (Fig. 2C). The survival of the animals at the end of the growth experiment was 100%.

Gene expression

The GH/GHR genotype presented, in general, a significant decrease in skeletal muscle expression of *stat5.1*, *igf1*, *myod*, *myf5*, and *myhc4* when compared to the other groups (Fig. 3A, B, C, D, E). Expression of the *mylz2* was significantly decreased in all transgenics in relation to the NT group (Fig. 3F).

Muscle histology

Analysis of muscle fibers revealed significantly different profiles between study groups (Fig. 4). Highest percentage of thick fibers was observed in the GH (36%), followed by NT (14%), GH/GHR (7%), and GHR (2%) groups. The fibers

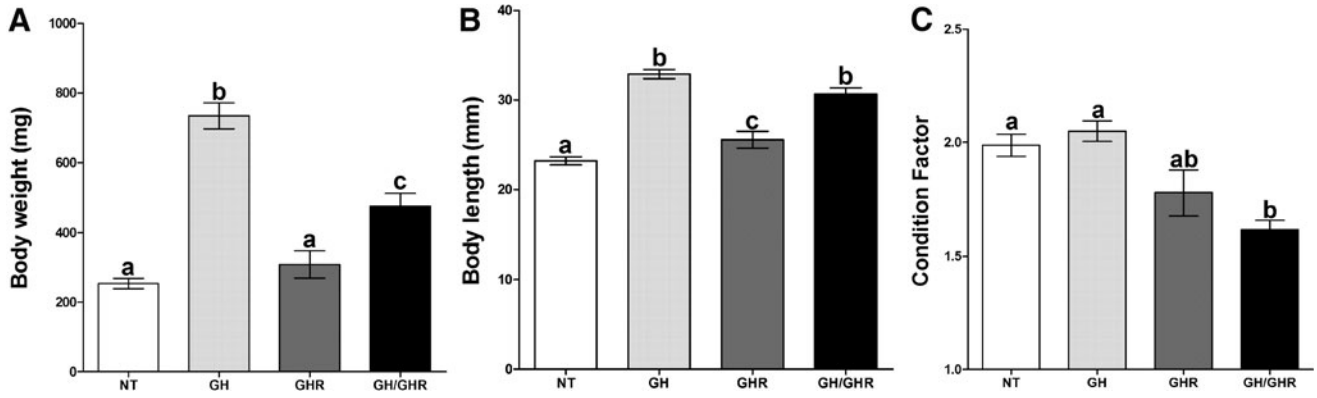

FIG. 2. Biometry of zebrafish (*D. rerio*). GH = GH transgenics; GHR = GHR transgenics; GH/GHR = double transgenics. Zebrafish (165 days old) were measured on the following parameters: body weight **(A)**, body length **(B)**, and condition factor **(C)**. *Different letters* indicate significant differences ($p < 0.05$).

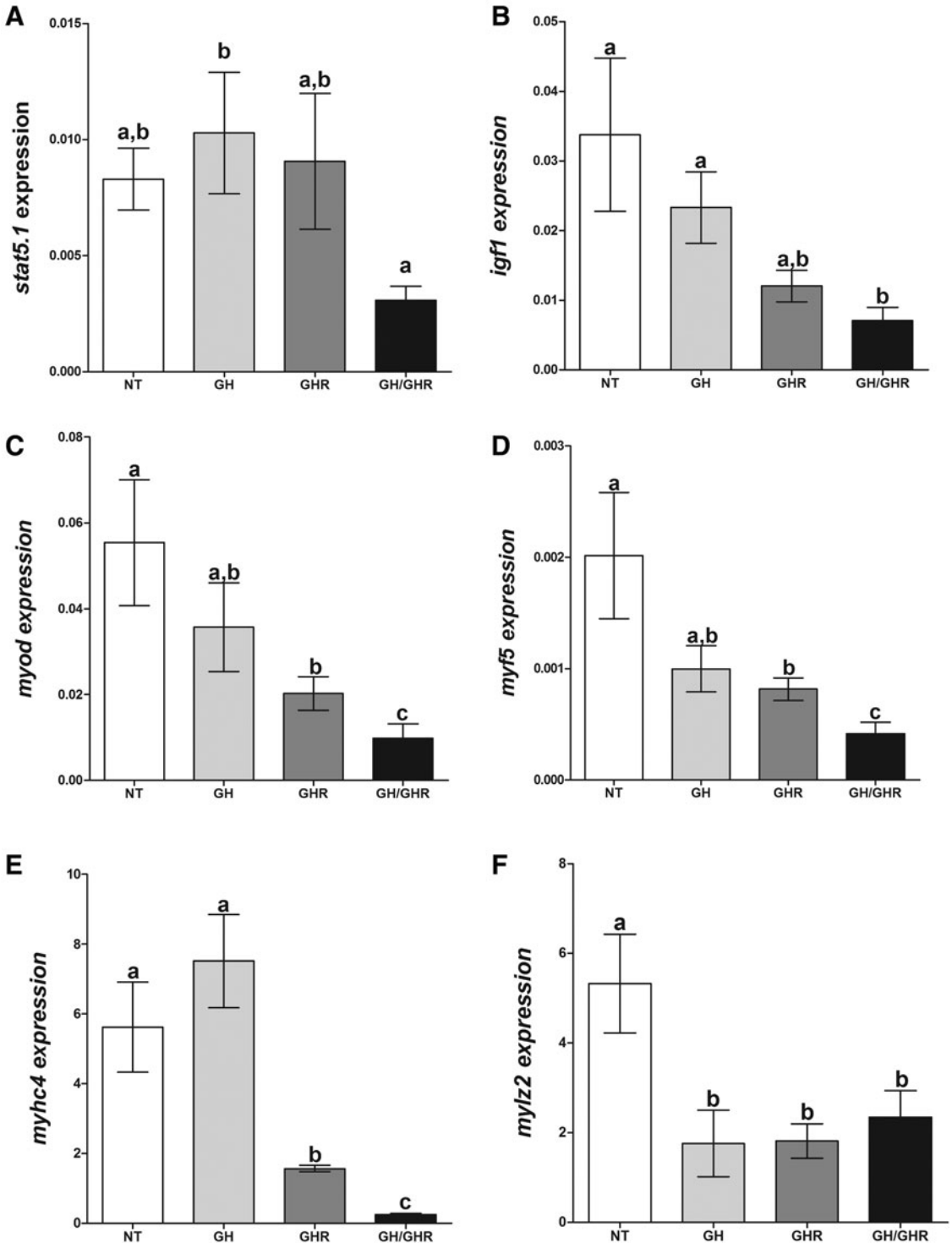

FIG. 3. Expression of growth-related genes in skeletal muscle of 165-day-old zebrafish (*D. rerio*). Analyzed genes: **(A)** *stat5.1*, **(B)** *igf1*, **(C)** *myod*, **(D)** *myf5*, **(E)** *myhc4*, **(F)** *mylz2*. The gene expression was normalized by expression of the constitutive genes: elongation factor 1 alpha (*ef1α*) and beta-actin (*βactin*). Data are expressed as mean ± SEM of $2^{-\Delta\Delta CT}$ ($n = 5$). *Different letters* represent significant differences ($p < 0.05$). The groups represented by ''a'' and ''b'' are significantly different between them, but the *group* represented by ''a,b'' was not significantly different either from ''a'' or ''b.''

showed different sizes, separated by loose connective tissue, the endomysium, and organized into fascicles by a thicker connective tissue septum, forming a structure similar to the perimysium. The same cell types (myoblasts and satellite cells) were found in all genotypes.

Discussion

In this study, we used two transgenic lines, one over-expressing GH and the other GHR, for production of a double-transgenic zebrafish (GH/GHR). Both lines express

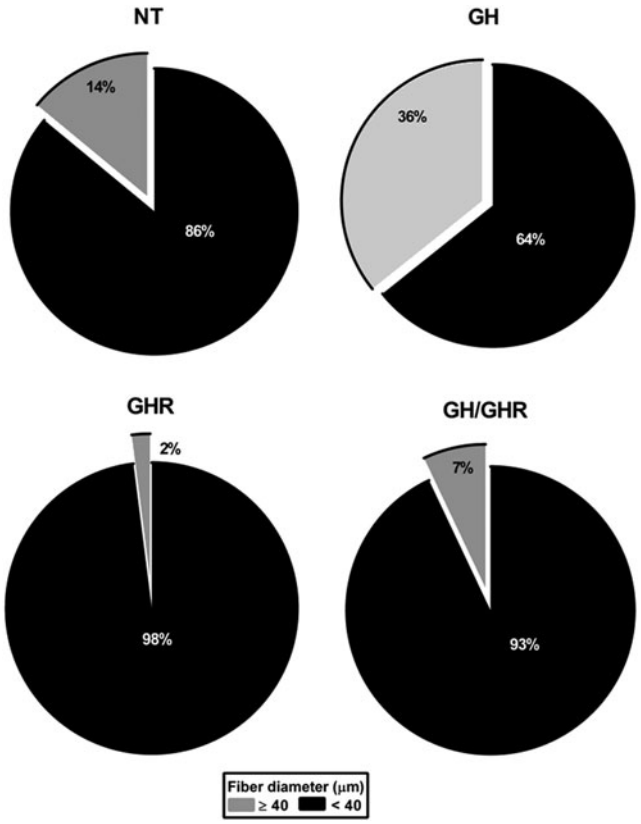

FIG. 4. Proportion of muscle fibers of 165-day-old zebrafish (*D. rerio*) from different genotypes: NT, GH, GHR, GH/GHR. Muscle fibers were classified as thin ($\geq 40\ \mu$m) or thick ($< 40\ \mu$m). The differences were significantly different at $p < 0.05$.

fluorescent proteins for noninvasive genotype identification. The main objective was to evaluate if a breed between the two above-cited lines could increase muscle growth. Results from analyses of growth, muscle structure, and gene expression demonstrated that, differently from our initial hypothesis, muscle growth was not enhanced in the GH/GHR double transgenics when compared to GH genotype.

The condition factor (K), which represents the degree of well-being and robustness of an animal,[18] showed significant decrease (21%) in the double transgenic when compared to the GH group. This decrease was due to the fact that the GH/GHR group did not present proportional growth, that is, attained the same length as GH fish, but with significantly lower weights. The result of this morphological alteration in double transgenics was a more slender fish than the other analyzed genotypes. In addition, muscle structure alterations were also observed in the double-transgenic group, with an $\sim$80% decrease in thick fibers when compared to the line overexpressing GH. A possible explanation for these observations could be that the excess of circulating GH caused an increase in longitudinal growth, but overexpression of GHR in muscle tissue blocked intracellular signaling of this hormone, with a consequent decrease of hypertrophy.

Regarding gene expression analyses, a significant decrease of *igf1* ($\sim$70%) in GHR and GH/GHR genotypes was observed. IGFs are present in a wide variety of tissues at relatively high concentrations, displaying hypertrophic and hyperplasic properties and being important regulators of myogenesis.[5,19] This decline in *igf1* expression could be result of a generalized decrease in GH intracellular signaling. Phosphorylation is induced when GH links to GHR, with consequent activation of Janus kinases (JAKs), a family of enzymes commonly associated with the intracellular portion of the receptor.[20] Activated JAKs phosphorylate specific tyrosine-rich regions of the receptors, which are anchorage sites for molecules related to different signaling pathways, and culminate with the activation of specific genes for GH-mediated biological actions. Transcriptional activation of *igf1* follows the classic route driven by proteins known as STATs, which when phosphorylated form dimers for nuclear translocation.[21] In fact, *stat5.1* expression analysis showed a decrease of $\sim$75% in GH/GHR-transgenic zebrafish when compared to the GH group. This result could be interpreted as a consequence of the increased intracellular signalization due to concomitant GH and GHR overexpression. In fact, muscle cells of double transgenics would be producing less STATs as a way to avoid excessive signaling due to the presence of large amounts of membrane GHR and circulating GH. Considering that the STATs are the main transcription factors associated with IGF1, the lower production could explain the observed decline in *igf1* expression.

Apart from the JAK/STAT pathway, GH activates two other intracellular signaling pathways. The PI3K/Akt pathway is related to IGF1-induced muscle mass increase, through activation of genes associated with protein synthesis increase, hypertrophy, apoptosis inhibition, and decrease in muscle protein degradation.[22–24] The MEK/ERK pathway is related to cell proliferation and differentiation,[24,25] through phosphorylation of tyrosines mediated by serine/threonine kinases.[26] PI3K/Akt and MEK/ERK pathways can activate genes that code not only for myogenic regulatory factors but also for muscle proteins.[27,28] In this study, expression of *myod* and *myf5* was, respectively, 73% and 58% lower in the double transgenics when compared to the GH group. Regarding muscle proteins, the gene that codifies for the MYHC4 presented the largest decrease (97%) when comparing the GH to the GH/GHR group. When analyzed as a whole, gene expression results support the hypothesis that GH/GHR overexpression causes a significant intracellular signaling decrease in the main somatotrophic axis pathways.

To our knowledge, this is the first work to use a double-transgenic zebrafish overexpressing GH in a constitutive way, and its receptor in a muscle-specific manner. The results obtained with this model allowed an *in vivo* analysis of the main responses related to the effects of GH/GHR axis on muscle growth. In the case of the double-transgenic (GH/GHR) fish used in the present study, a scenario of elevated somatotrophic axis signaling in muscle tissue was expected. However, concomitant overexpression of GH and GHR probably leads to intracellular signaling levels not supported by the cells, which respond by decreasing the production of molecules associated with the major intracellular signaling pathway of the somatotrophic axis.

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Disclosure Statement

No competing financial interests exist.

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