High level of GHR nuclear translocation in skeletal muscle of a hyperplasic transgenic zebrafish

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Abstract

It has been reported that nuclear translocation of growth hormone receptor (GHR) may directly activate cell proliferation in mammals and birds. However, this phenomenon has not yet been described in fish. Recently, we have developed a transgenic zebrafish that overexpresses GHR in a muscle-specific manner. Considering that this transgenic model exhibits hyperplasic muscle growth, the present work aims at verifying the relationship between GHR nuclear translocation and muscle cell proliferation. This relationship was evaluated by the phosphorylation state of the proliferative MEK/ERK pathway, expression of nuclear import-related genes, immunostaining of phospho-histone H3 (PH3) as a proliferation marker, and nuclear GHR localization. The results showed a significant decrease in the phosphorylation state of ERK1/2 proteins in transgenics. Moreover, there was an increase in expression of three out of four importin genes analyzed parallel to a large flow of GHR displacement toward and into the nucleus of transgenic muscle cells. Also, transgenics presented a marked increase in PH3 staining, which indicates cell proliferation. These findings, as far as we know, are the first report suggesting a proliferative action of GHR in fish as a consequence of its increased nuclear translocation. Thus, it appears that the nuclear migration of cytokine receptors is a common event among different taxonomic groups. In addition, the results presented here highlight the possibility that these membrane proteins may be involved more directly than previously thought in the control of genes related to cell growth and proliferation.

Key Words

- *Danio rerio*
- growth hormone receptor
- hyperplasia
- nuclear transport

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Introduction

Growth hormone (GH) is a pluripotent hormone produced and secreted by vertebrate pituitary glands. The hormone's actions are mediated via the GH receptor (GHR), which is widely expressed by GH target cells. GHR is the key regulator of post-natal growth and holds important actions over metabolic, reproductive,

gastrointestinal, cardiovascular, hepatobiliary, and renal systems (Lichanska & Waters 2008). Concerning muscular tissue, GH and insulin-like growth factor 1 (IGF1) seem to regulate hypertrophy and hyperplasia, these processes being influenced by myogenic regulatory factors (MRFs) (Sabourin & Rudnicki 2000, Hawke & Garry 2001).

GHR is a class-1 cytokine membrane receptor and it is assumed to trigger intracellular signalization by the JAK2/STAT pathway (Argetsinger & Carter-Su 1996, Moutoussamy *et al.* 1998, Waters *et al.* 2006). However, it has been reported that GHR may signal through additional JAK2-independent mechanisms (Zhu *et al.* 2002, Barclay *et al.* 2010). The MEK/ERK signaling pathway is an example of JAK2-independent signaling, which is implicated in cell proliferation induced by GH (Liang *et al.* 1999). Alternatively, significant amounts of GHR molecules have been found in the cell's interior, including its intriguing nuclear localization (Lobie *et al.* 1991, 1994, Mertani *et al.* 2003). The processes leading to GHR nuclear localization is believed to depend upon the receptor's escape from degradation mechanisms and its subsequent transportation to the nucleus by the classic importin system (Bryant & Stow 2005, Swanson & Kopchick 2007, Conway-Campbell *et al.* 2008). Nuclear localization of GHR seems to be a common characteristic of tissues and cells exhibiting high proliferative levels (Conway-Campbell *et al.* 2007, Martínez-Moreno *et al.* 2011).

Studies addressing the nuclear translocation of GHR have almost exclusively used *in vitro* models. However, *in vivo* models could shed more light on this fascinating mechanism. Recently, we developed a transgenic zebrafish (*Danio rerio*) strain overexpressing approximately 100 times more GHR in skeletal muscular tissue (Figueiredo *et al.* 2012). This *in vivo* model can be an interesting tool to study the relationship between GHR nuclear localization and muscle cell proliferation. Indeed, individuals from this transgenic strain show an increased number of muscle fibers with no weight gain compared to non-transgenics. This hyperplastic condition was related to increase in MRF expression. However, the JAK2/STAT signaling pathway was significantly diminished in transgenics, leading to a decrease in IGF1 transcription levels (Figueiredo *et al.* 2012). Altogether, these observations suggest that GHR-induced hyperplasia is taking place for some JAK2/STAT-independent pathway. Thus, the objective of this study was to determine whether there is a relationship between the observed hyperplasia in our transgenic zebrafish strain and an increase in GHR nuclear translocation in muscle cells overexpressing this membrane receptor.

Material and methods

Transgenic fish

The GHR-transgenic zebrafish strain used here holds a genetic construct assembled by GHR cDNA under

transcriptional control of the myosin light chain 2 (*myl2*) promoter (Figueiredo *et al.* 2012). All sequences used were obtained directly from the zebrafish. Additionally, this strain carries a reporter transgene containing the same promoter driving the expression of the red fluorescent protein (DsRed) from *Discosoma* sp. (Fig. 1). This strategy allowed transgenic identification under ultraviolet light as soon as they hatched. Mating hemizygous transgenic males with non-transgenic females produced transgenic and non-transgenic fish from the same breed in a Mendelian proportion.

RNA extraction and qPCR

Forty-five-day-old fish had their total RNA extracted from skeletal muscle tissue by the TRIzol reagent method (Invitrogen) according to the manufacturer's instructions. The extracted RNA was treated with DNase I Amplification Grade (Invitrogen) and used as a template for cDNA

Figure 1

Schematic representation of transgenes used for production of the GHR-transgenic zebrafish (*Danio rerio*) strain. The myosin light chain 2 (*myl2*) promoter was used to drive expression of both *GHR* (target gene) and *DsRED* (red fluorescence as transgenesis marker). 'pA' represents the termination signal. Transgenic zebrafish express both GHR and red fluorescence only in skeletal muscle tissue. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-15-0185>.

synthesis through the high capacity cDNA reverse transcription kit (Applied Biosystems), following manufacturers' protocols. Expression of four importin genes (*impa1*, *impa3*, *impB1* and *impB2*) was analyzed by quantitative Real Time PCR (qPCR). Seven individuals from each experimental group were used. Gene-specific primers (Table 1) were designed using the software Primer Express 3.0 (Applied Biosystems), based upon sequences available at GenBank (<http://www.ncbi.nlm.nih.gov>). The qPCR reactions were performed in a 7500 Real Time PCR System platform (Applied Biosystems) using the Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen). Each sample was analyzed in triplicate to avoid plate deviations. Serial dilutions were made for all primers to determine the qPCR reaction efficiency. PCR reaction 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 elongation factor 1 alpha (*ef1a*) gene expression, which showed no significant differences among the experimental groups (data not shown). Data were analyzed by relative expression, according to the mathematical model proposed by [Fernandes et al. \(2006\)](#).

Western blot analysis

Muscular tissue was collected from four animals from each experimental group, lysed in a protein homogenization solution (100 mM Tris-HCl, 2 mM EDTA, 5 mM MgCl₂, and 250 ml MilliQ water; pH 7.75), and centrifuged for 20 min at 2000 g at 4 °C. The supernatant was recovered and centrifuged for 45 min at 10 000 g at 4 °C. The supernatant was recovered, and its protein content was determined by the Qubit method (Invitrogen). Samples were analyzed using SDS-PAGE in 7.5% gels using migration buffer (124 mM Tris-base, 1 M glycine, 0.5% SDS, and 500 ml MilliQ water; pH 8.3) in miniVE Electrophoresis and Electrotransfer Unit (Amersham Bioscience, São Paulo, Brazil). Each lane contained 30 µg of protein or 5 µl of MagicMark XP Western Standard (Novex, São Paulo, Brazil). Samples were analyzed under reducing conditions (5% 2-mercaptoethanol). After

electrophoresis, gels were equilibrated in transfer buffer (25 mM Tris-HCl, 192 mM glycine, and 20% methanol (v/v); pH 8.3) for 30 min and electro-transferred (up to 1.0 A, 30 min) in Trans-Blot Turbo Blotting System (BIO-RAD) to a 0.2-µm PVDF membrane (Invitrogen), according to the manufacturer's instructions. Membranes were dried and re-wet with methanol followed by two 20-ml water washes for 5 min. For the protein immunodetection process, we used the Western Breeze Chromogenic western blot Immunodetection system anti-rabbit kit (Novex, Brazil), according to the manufacturer's instructions. The rabbit monoclonal primary antibody used was p44/42 MAPK (Erk1/2) Rabbit mAb (Cell Signaling, São Paulo, Brazil) for total Erk1/2, and Phospho-p44/42 MAPK (Erk1/2) XP Rabbit mAb (Cell Signaling) for phosphorylated Erk1/2. All primary antibodies were used at a 1:1000 dilution.

Immunohistochemistry

Seven zebrafish from each experimental group were euthanized with Tricaine methosulfonate (0.5 mg/ml) for collection of muscle tissue. Samples were immediately fixed (6 h) in 4% paraformaldehyde in PBS and preserved in 70% ethanol. Tissue samples were dehydrated (ethanol), diafanized (xylol), and impregnated (Paraplast Xtra; Sigma) using an automated vacuum tissue processor (Leica, São Paulo, Brazil). Transverse histological sections (4 µm) from muscle fibers were obtained with an automated rotatory microtome (Leica). The sections were deparaffinized and rehydrated in xylene and ethanol series. Antigen retrieval was carried out in a microwave oven (boiling for at least 20 min in citrate buffer), cooled slowly, and then transferred to PBS at 4 °C. The slides were then blocked for 1 h in 5% normal goat serum + BSA at 4 °C. Individual sections were washed in PBS and covered with 3 µg/ml of GHR antibody (MAB 263) FITC (Santa Cruz Biotechnology) and 4 µg/ml of PH3 Antibody (Ser 10)-R (Santa Cruz Biotechnology). Antibodies were diluted in PBS. The slides were placed in a humidified chamber and kept in the dark overnight at 4 °C. Next day, slides

Table 1 Gene specific primers used for qPCR analyses.

Gene	Forward primer	Reverse primer	GenBank
<i>ef1a</i>	5'-gggcaagggtccttcaa-3'	5'-cgctcggccttcagtttg-3'	NM_131263
<i>impa1</i>	5'-ccacactcatctgccttctg-3'	5'-gcagggtgtccgcaagca-3'	BC075790
<i>impa3</i>	5'-tccttccatccccataacct-3'	5'-ttcggcacagggttgacaatg-3'	BC055253
<i>impB1</i>	5'-gcacgcagggatcaaga-3'	5'-ctgggccggtatgtctctgt-3'	NM_001037702
<i>impB2</i>	5'-gaccaacacacccaaactct-3'	5'-cacgtatccagtcgaccaat-3'	BC080218

were thoroughly rinsed with PBS and re-covered with 4 µg/ml Anti-Rabbit-IgG-Atto 647N (Sigma) for at least 3 h. Slides were rinsed again with PBS and covered with 1 µg/ml DAPI (Sigma) for nuclei labeling. Finally, slides were mounted with Fluoroshield (Sigma) and 0.7 mm coverslip.

Images were acquired with BX52 epi-fluorescent microscope (Olympus) or TCS SP8 scanning spectral confocal microscope (Leica). For confocal images, settings were fixed at the beginning of both acquisition and analysis steps and were unchanged. Images were acquired sequentially using the following settings: FITC (green) channel (confocal PMT = 500–560 nm), Atto 647 (red) channel (confocal PMT = 650–700 nm), and DAPI (blue) channel (confocal HyD = 410–470 nm).

Colocalization analysis

The presence of GH receptor within the myocyte nuclei was analyzed using CoLocalizer Express Software (CoLocalization Research Software, Tokyo, Japan). The software determined the degree of colocalization of green channel signal (GHR) and blue channel signal (myocyte nucleus) within each selected nucleus, using the following algorithm according to the software manual (<http://www.colocalizer.com/probasics.html>):

$$m_1 = \frac{\sum_i S1_{i,coloc}}{\sum_i S1_i} \quad m_2 = \frac{\sum_i S2_{i,coloc}}{\sum_i S2_i}$$

Colocalization coefficients m_1 and m_2 describe the contribution of each channel to the image ROI. They are not sensitive to the intensities of signals and can be used when the numbers of objects are not equal: where S1 represents signal intensity of pixels in the channel 1 and S2 represents signal intensity of pixels in the channel 2 (Zinchuk *et al.* 2007). The term m_1 relates to the percentage of blue pixels from the selected region (nucleus) that also contains a green channel signal (GHR), thus determining their amount of colocalization. The result m_1 is a value that states what percentage of the two-dimensional area of nucleus also contains GHR. The m_1 values obtained from six unique transgenic fish and from seven unique WT were compared to negative controls. The negative control group represents the noise in our measurement system. These negative controls were obtained from randomly selected nuclei from transgenic and WT fish ($n=10$) and performing the colocalization analysis only using the blue channel images without the green channel overlay. Theoretically, this group should show no degree of colocalization. The small signal seen in the negative

control column is a sum of common fluorescent noise, principally fluorophore cross-talk, which can reveal the limitations of the optical system.

Statistical analysis

Student's *t*-test was used for paired comparisons between transgenic and non-transgenic fish for both gene expression and western blot data analyses. Results were expressed in mean $\pm$ S.E.M. One-way ANOVA was used for colocalization analysis. In all cases, the adopted α was 0.05.

Results

Western blot analysis was carried out to evaluate the phosphorylation state of the MEK/ERK intracellular signaling pathway. Phosphorylated ERK1/2 was significantly reduced in muscle of GHR-transgenics (0.044 ± 0.009), when compared to non-transgenic controls (0.193 ± 0.086) (Fig. 2). Expression analyses of nuclear import-related genes revealed that *impa1*, *impa3*, and *impB1* increased twofold in transgenics, while *impB2* was not altered (Fig. 3). Analyses of confocal images indicated the GHR presence in membranes of muscle cells in both experimental groups, with a significant increase in the nuclear region of transgenics (Fig. 4F) when compared with non-transgenics (Fig. 4B). In addition, the proliferation marker PH3 was significantly increased in transgenics (Fig. 4G), when compared to non-transgenics (Fig. 4C). Colocalization GHR/DAPI analysis confirmed

Figure 2

Western blot analyses of ERK1/2 proteins compare GHR-transgenic (T) and non-transgenic (NT) zebrafish. Graph shows pERK1/2 data normalized by total ERK1/2. Asterisk represents statistically significant differences ($P < 0.05$; $n = 4$).

Figure 3

Relative expression of nuclear import related genes compare GHR-transgenic (T) and non-transgenic (NT) zebrafish. Asterisks represent statistically significant differences ($P < 0.05$). In all cases $n = 7$.

that 78.6% of the two-dimensional area of transgenic nuclei showed evidence of the receptor, while only 49.1% of the nuclear area of non-transgenic fish showed the same characteristic (Fig. 5). Finally, a more detailed picture of the GHR migration toward the nuclear region is shown in Fig. 6, where it is possible to observe the receptor surrounding the nuclear membrane.

Discussion

The majority of what is known regarding the intracellular signaling of the somatotrophic axis has been elucidated through cellular models. *In vitro* studies provide important evidences and are indispensable in illuminating specific physiological mechanisms. Unfortunately, much information obtained from these studies cannot be extrapolated to the organismal level due, in part, to the great complexity of interactions that occur at the intermediate systemic levels. The use of *in vivo* models provides additional information, since the operation of specific mechanisms can be evaluated under the influence of intra- and extracellular signals in association with environmental parameters. In this study, we employed a transgenic zebrafish model overexpressing GHR specifically in skeletal muscle cells. This transgenic strain has been previously characterized as hyperplastic regarding muscle growth (Figueiredo *et al.* 2012). Here, the relationship between muscle cell proliferation and GHR overexpression was tested through analyses of phosphorylation state of the proliferative MEK/ERK pathway, expression of nuclear import-related genes, immunostaining of the proliferative marker PH3, and nuclear localization of GH receptor.

The MEK/ERK pathway represents an alternative pathway for GH signaling as well as for IGF1 (Herrington

Figure 4

Confocal images from skeletal muscle sections of non-transgenic (A, B, C, and D) and transgenic (E, F, G, and H) zebrafish. All sections were triple labeled using DAPI for nuclei staining (blue), GHR antibody (green) and phospho-

histone H3 (PH3) antibody (red). (D and H) are overlaid images from non-transgenic and transgenic respectively. Scale bars = 25 μ m. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-15-0185>.

Figure 5

Percentage of GHR/DAPI (nuclei) colocalization in skeletal muscle cells of GHR-transgenic (T) and non-transgenic (NT) zebrafish. Control group (C) represents system noise (see 'Material and methods' section). Different letters represent statistical differences determined by one way ANOVA. Transgenics: $n=7$, non-transgenics: $n=6$, and controls: $n=10$.

& Carter-Su 2001), and it is related to proliferative and cell differentiation processes (Coolican *et al.* 1997, Clemmons 2009). MEK/ERK signaling pathway activation occurs after the hormone binding to its receptor, leading to tyrosine phosphorylation mediated by serine/threonine kinases such as Shc, Raf, and MAPK (Chiou *et al.* 2007). Among these factors, MAPK is crucial for regulation of cell functions in response to mitotic stimuli (Seeger & Krebs 1995, Robinson & Cobb 1997). There are three MAPK classes and in the present work, MEK and ERK were investigated. We performed a western blot analysis to measure the phospho-ERK1/2 concentrations in the muscle tissue. A very lower quantity of phosphorylated ERK1/2 in transgenics was observed when compared to non-transgenics, indicating a substantial reduction of this signaling cascade of approximately 77%. This observation

suggests that hyperplasia previously found in GHR-transgenic strain was not due to the activation of MEK/ERK signaling pathway. Thus, the main question that must be answered is how GHR overexpression might be increasing MRF transcription and consequently inducing hyperplasia?

It has been shown that the complex assembled by the binding of GH to its receptor, localized at the cell surface, is internalized and redistributed to different subcellular compartments (Roupas & Herington 1989). The mechanism by which GH/GHR complex is translocated into the nucleus still has not been well established, and it seems that this type of translocation is not exclusive to GHR. Other receptors closely related to GHR are also translocated into cell's nucleus (Bryant & Stow 2005, Krolewski 2005, Carpenter & Liao 2009, Wang & Hung 2009), and these translocations have been related to cell proliferation (Reilly & Maher 2001, Wang *et al.* 2010). The nuclear translocation process, in mammals, requires importin- β as described for FGFR1, EGFR, and ErbB2 receptors (Reilly & Maher 2001, Giri *et al.* 2005, Lo *et al.* 2006). Moreover, GHR transport into nuclei of murine hematopoietic cells (Ba/F3) seems to be mediated by the classic heterodimer importin α/β mechanism (Conway-Campbell *et al.* 2008). In the present study, we observed a significant twofold increase in expression in three out of four importin genes analyzed, suggesting molecular transport into the nucleus. GHR has been observed in proliferative cells' nuclei from chick testis (Martínez-Moreno *et al.* 2011), as well as in BaF/3 cells stably expressing GHR (Conway-Campbell *et al.* 2007). Thus, it is likely that the increased expression of importin

Figure 6

Representative epifluorescent micrograph showing GHR migration and concentration within the nuclei of transgenic zebrafish muscle fibers. (A) Original 32-bit DAPI channel co-label confirms the highest signal levels indeed originate from the cell nuclei. (B) The original 32-bit FITC channel RGB image was converted to 8-bit grayscale, and then the ICA lookup table

from NIH Image was applied to visually enhance the differences in signal levels. Yellow corresponds to the highest concentration of GHR, orange is intermediate concentrations, and dark brown are the lowest detectable levels of GHR. (C) Superimposed images. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-15-0185>.

genes observed here might be related to nuclear transport of the overexpressed receptor in muscle cells of our transgenic strain.

In order to verify whether GHR overexpression resulted in an increased transport of this receptor to the nucleus, an immunohistochemical analysis was performed. A quantitative analysis the GHR labeling was performed comparing transgenic and non-transgenic groups. We observed that nuclear transport of the GHR also occurs in non-transgenic fish, but at a significantly lower intensity. These results indicate that overexpression of GHR resulted in an increase in the nuclear transport of this receptor. Beyond that, the proliferative state of transgenic muscle cells was confirmed by the increased staining of phosphorylated histones in this experimental group. Together, these results permit us to hypothesize that nuclear translocation of the receptor could be leading to an increased cell proliferation in muscle of GHR-transgenic zebrafish. Figure 6 shows more details about nuclear transport of the GHR in a muscle fiber from a GHR-transgenic fish. One can clearly observe the presence of the receptor in the cytoplasm and an increasing intensity gradient approaching the nucleus, where the highest concentration of the receptor is detected.

Nuclear internalization of cytokine receptors has been associated with cellular proliferation and, in most cases, to cancer (Aleksic *et al.* 2010, Wang *et al.* 2010, Wang & Hung 2012, Wu *et al.* 2012). According to Conway-Campbell *et al.* (2008), nuclear GHR is involved in transcriptional regulation, exhibiting many proprieties normally associated with transcription factors. However, GHR interaction with transcriptional coactivator molecules is likely to be necessary to exert any transcriptional activity, as this receptor does not exhibit DNA binding domains. Among these molecules, the coactivator activator (CoAA) supplies a putative mechanism to nuclear GHR transcriptional activity in mammals and there are data demonstrating that CoAA increases the transcriptional activity of many transcription factors (Auboeuf *et al.* 2004). Identification of a fish CoAA could be helpful to determine the GHR's mechanism of action in the cell's nucleus of this vertebrate group.

This study provides compelling evidence that nuclear translocation of the GHR may be associated with increased cell proliferation in fish, as already observed for birds and mammals. The occurrence of GHR nuclear translocation in fish and its possible action as an ancillary transcription factor is an indication of the high degree of conservativeness of this mechanism from an evolutionary point of view. Taking this into consideration, it is possible that the

role of this receptor is not restricted only to the triggering of intracellular signaling cascades from its interaction with the GH. More than that, cytokine receptors seem to be more directly involved in the regulation of genes related to cellular growth and proliferation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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