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Expression profile of IGF paralog genes in liver and muscle of a GH-transgenic zebrafish

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ABSTRACT

The objective of this study was to investigate the relationship between IGFs produced in the liver and skeletal muscle with muscle hypertrophy previously observed in a line of GH-transgenic zebrafish. In this sense, we evaluated the expression of genes related to the IGF system in liver and muscle of transgenics, as well as the main intracellular signaling pathways used by GH/IGF axis. Our results showed an increase in expression of *igf1a*, *igf2a*, and *igf2b* genes in the liver. Moreover, there was a decrease in the expression of *igf1ra* and an increase in muscle *igf2r* of transgenics, indicating a negative response of muscle tissue with respect to excess circulating IGFs. Muscle IGFs expression analyses revealed a significant increase only for *igf2b*, accompanied by a parallel induction of *igfbp5a* gene. The presence of IGFBP5a may potentiate the IGF2 action in muscle cells differentiation. Regarding JAK/STAT-related genes, we observed an alteration in the expression profile of both *stat3* and *stat5a* in transgenic fish liver. No changes were observed in the muscle, suggesting that both tissues respond differently to GH-transgenesis. Western blotting analyses indicated an imbalance between the phosphorylation levels of the proliferative (MEK/ERK) and hypertrophic (PI3K/Akt) pathways, in favor of the latter. In summary, the results of this study suggest that the hypertrophy caused by GH-transgenesis in zebrafish may be due to circulating IGFs produced by the liver, with an important participation of muscle IGF2b. This group of IGFs appears to be favoring the hypertrophic intracellular pathway in muscle tissue of transgenic zebrafish.

1. Introduction

The insulin-like growth factor (IGF) system works in a coordinated form in vertebrates promoting the animal growth, development, metabolism, and longevity, as well as cellular processes leading proliferation, survival, cellular migration and differentiation (Wood et al., 2005). This system includes three ligands (IGF1, IGF2 and Insulin) evolutionarily conserved, their cell surface receptors (IGF1R, M6P/IGF2R and IR) and six high affinity binding proteins (IGFBP-1–6) (Le Roith, 2003). IGF1 is the main mediator in the postnatal actions of the growth hormone (GH) (Kaplan and Cohen, 2007). Most circulating IGF1 is produced in the liver, although it has been demonstrated that this hormone can be synthesized in other organs where it exerts autocrine and paracrine effects (Kaplan and Cohen, 2007; Eppler et al., 2007). Differently, IGF2 shows little dependence on GH and its main function is related to the prenatal development of mammals (Coan et al., 2008; Murphy et al., 2008).

IGF2 has also been detected in several tissues of fish, but its function is still unclear in this group of vertebrates. (Zou et al., 2009) have identified and characterized four IGF genes (*igf1a*, *igf1b*, *igf2a* and *igf2b*) in zebrafish (*Danio rerio*), each encoding a polypeptide with specific characteristics from a structural and functional point of view. These authors also determined that *igf1a* is orthologous to human *igf1*, whereas *igf2a* and *igf2b* are orthologous to the human *igf2*. It has been recognized that *igf1a* is involved in muscle growth, protein synthesis and myoblast proliferation in fish (Castillo et al., 2004). However, the situation is not so clear about the role of *igf2a* and *igf2b*. The knockdown of these genes in zebrafish demonstrated that the two isoforms have different functions, but both related to the early stages of embryonic development (White et al., 2009). The retention of four IGF genes in zebrafish genome suggests that each one can be involved in different and indispensable functions.

In addition to somatic growth and development, IGFs also play other important functions. Fish IGF1b, initially identified as IGF3, has been related to gonadal and reproductive development (Wang et al., 2008; Zou et al., 2009). Moreover, IGF2b may also be involved in myocardial regeneration process (Huang et al., 2013). In the same sense, IGFs seem to be involved with

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regeneration and hypertrophy of skeletal muscle (Duan et al., 2010). Several studies have demonstrated that both IGF1 and IGF2 can activate different signaling pathways related to skeletal muscle growth and myoblast proliferation such as JAK/STAT (Himpe and Kooijman, 2009), PI3K/Akt (Glass, 2005, 2003; Rommel et al., 2001), and MEK/ERK (Clemmons, 2009; Codina et al., 2008).

Most studies on the effect and function of the IGF system in fish are performed with cultures of myogenic cells (Codina et al., 2008; Garikipati and Rodgers, 2012; Rius-Francino et al., 2011). In addition, some GH-transgenic fish have been produced and contributed to increased knowledge about the regulation of the somatotrophic axis (Ahmed et al., 2011; Rahman et al., 1998). In order to study the effects of GH/IGF axis on body growth, our research group produced a transgenic zebrafish line (named F0104) overexpressing GH ubiquitously (Figueiredo et al., 2007a). Figueiredo et al. (2007b) showed increased growth of F0104 transgenic zebrafish, while Kuradomi et al. (2011) demonstrated that GH-transgenesis induced muscle hypertrophy in an independent way of IGF1 locally produced. In this regard, the objective of this study was to investigate the relationship between the liver and muscle IGFs with the GH-induced muscle hypertrophy previously observed in our line of transgenic zebrafish.

2. Materials and methods

2.1. GH-transgenic zebrafish

Transgenic (T) and non-transgenic (NT) control fish were obtained from crosses between non-transgenic females and hemizygous GH-transgenic males from F0104 line, following a previously described protocol (Figueiredo et al., 2007a). Briefly, the F0104 line was produced by the co-injection of two transgenes comprised by the carp (*Cyprinus carpio*) β-actin promoter driving the expression of the marine silverside (*Odontesthes argentinensis*) GH coding sequence (Marins et al., 2002) or the green fluorescent protein (GFP) reporter gene. The effect of β-actin promoter can be observed through GFP fluorescence (Fig. 1). Taking into consideration that the same promoter controls GH expression, it is assumed that this hormone is being produced with the same location and intensity of GFP. For this study, T and NT zebrafish siblings were reared in a closed circulation water system composed of 15 L aquariums at 28 °C, 14 h light/10 h dark photoperiod, fed with high-protein (47.5%) twice a day, *ad libitum*. Water quality was monitored once a week, and temperature, pH, nitrogen compounds and photoperiod were maintained according to zebrafish requirements (Westerfield, 1995). When the fish reach seven months of age, twelve males from each group ($T = 545.5 \pm 27.8$ mg and NT = 353.2 ± 10.3 mg) were euthanized (Tricaine methosulphonate, 0.5 mg mL^{-1}) for molecular analyzes. All experiments were performed as suggested by the Ethics Committee for Animal Use at the Federal University of Rio Grande (FURG, Brazil).

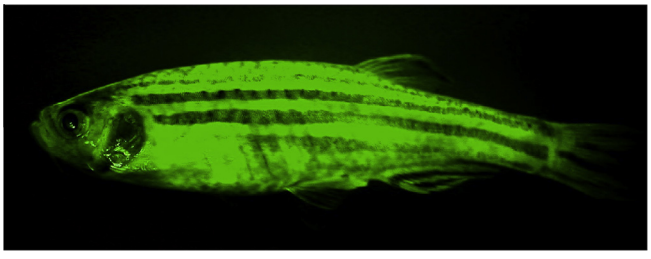

Fig. 1. Transgenic zebrafish (*Danio rerio*) expressing both GH (growth hormone) or GFP (green fluorescent protein) under control of carp (*Cyprinus carpio*) β-actin promoter.

2.2. RNA extraction and cDNA synthesis

Total RNA was isolated from skeletal muscle and liver of six individuals from each group (T and NT) using TRIzol Reagent (Invitrogen, Brazil), following the manufacturer's recommendations. The extracted RNA was treated with DNAse I Amplification Grade (Invitrogen, Brazil). The total amount of RNA was determined with a Qubit Fluorometer and a Quant-iT RNA BR Assay Kit (Invitrogen, Brazil). RNA integrity was assessed through electrophoresis on 1% agarose gels. For cDNA synthesis, 1 μg of total RNA was reverse-transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Brazil), following manufacturers' protocols.

2.3. Gene expression

Gene expression was analyzed by real-time RT-PCR (7500 Real Time System, Applied Biosystems), using SYBR Green PCR Master Mix™ (Invitrogen), according to manufacturer's protocol. Each sample ($n = 6$) was analyzed in duplicate. Specific primers for each gene (Table 1) were designed using the Primer-BLAST tool from GenBank (http://www.ncbi.nlm.nih.gov). Five-point standard curves of a five-fold dilution series from pooled cDNA were used for PCR efficiency calculation. Gene expression was carried out for 50 °C/2 min, 95 °C/2 min, followed by 40 cycles at 95 °C/15 s and 60 °C/30 s. In this study, four housekeeping genes (elongation factor 1 alpha, *ef1α*; beta-2-microglobulin, *b2m*; beta-actin, *actb1*; and ribosomal protein L13 alpha, *rp113a*) were tested using geNorm VBA applet for Microsoft Excel (Vandesompele et al., 2002). Consequently, we calculated a normalization factor based on the expression levels of the best-performing housekeeping genes. *ef1α* and *rp113a* were selected as reference genes.

2.4. Western blotting analyses

For Western blotting analyses, protein samples were obtained from skeletal muscle of fish coming from each experimental group. Tissue samples were lysed in a protein homogenization solution (100 mM Tris-HCl, 2 mM EDTA, 5 mM $MgCl_2$ 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 again for 45 min at 10,000*g* at 4 °C. The supernatant was recovered once more, and its protein content was determined by the Qubit method (Invitrogen, Brazil). Samples were analyzed using SDS-PAGE in 7.5% gels using migration buffer (124 mM Trisma-base, 1 M glycine, 0.5% SDS, and 500 mL MilliQ water; pH 8.3) in miniVE Electrophoresis and Electrotransfer Unit (Amersham Bioscience, Brazil). Each lane contained 30 μg of protein or 5 μL of MagicMark XP Western Standard (Novex, 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, 20% methanol, pH 8.3) for 30 min and electro-transferred (up to 1.0 Å, 30 min) in Trans-Blot Turbo Blotting System (BIO-RAD, Brazil) to a 0.2 μm PVDF membrane (Invitrogen, Brazil), according of the manufacturer's instructions. Membranes were dried, and re-wet with methanol followed by two water washes (20 ml) 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, Brazil) for total Erk1/2 (used as normalizer), Phospho-p44/42 MAPK (Erk1/2) XP Rabbit mAb (Cell Signaling, Brazil) for phosphorylated Erk1/2,