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Research paper

Comparative expression and regulation of duplicated *fibroblast growth factor 1* genes in grass carp (*Ctenopharyngodon idella*)

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ABSTRACT

Fibroblast growth factor 1 (Fgf1) is known as a mitogenic factor involved in the regulation of cell growth, proliferation, and differentiation in vertebrates. Here, we report the isolation and characterization of two *fgf1* genes in grass carp (*Ctenopharyngodon idella*). Grass carp *fgf1a* and *fgf1b* cDNAs are highly divergent, sharing a relatively low amino acid sequence identity of 50%, probably due to fish-specific gene duplication. *fgf1a* and *fgf1b* mRNAs were detected in the zygote and expressed throughout embryogenesis. Both *fgf1a* and *fgf1b* mRNAs were primarily detectable in the notochord at 12 hpf. At 24 hpf, *fgf1a* mRNA was mainly expressed in the gut and somites, while *fgf1b* transcript persisted in the notochord and was detected in the tailbud. At 36 hpf, both *fgf1a* and *fgf1b* transcripts were detected in the brain, somites, and tailbud. In addition, the *fgf1a* mRNA was detected at the base of the yolk sac, whereas the *fgf1b* mRNA was expressed in the pectoral fin. In adult fish, duplicated *fgf1a* and *fgf1b* mRNAs were distributed in most tissues. After 2–6 days of starvation, both *fgf1a* and *fgf1b* mRNAs were upregulated in the muscle and liver. In the brain, *fgf1a* mRNA was upregulated, while *fgf1b* mRNA was significantly downregulated at 6 days. Furthermore, both *fgf1a* and *fgf1b* mRNA levels were significantly decreased in the brain and muscle after administration of 10 or 50 μg of the human growth hormone (hGH),while their mRNA levels were no significant difference in the liver. These results suggest that duplicated *fgf1s* may play important but divergent roles in the grass carp development.

1. Introduction

Fibroblast growth factors (FGFs), including 23 related polypeptides that combine with fibroblast growth factor receptors (FGFRs) (Haugsten et al., 2005; Partanen et al., 1992), are mitogenic factors involved in regulating cell growth, proliferation, and differentiation (Billottet et al., 2008; Ornitz and Itoh, 2001; Philippe et al., 1996). FGFs are composed of 150–200 peptides, and they have 50–70% amino acid identity with respect to their FGF domains (Han et al., 2009). Binding of FGFs to FGFRs is stabilized by heparan sulfate proteoglycans (HSPGs) and results in a dimer receptor–ligand complex that activates the intracellular tyrosine kinase domain by autophosphorylation, which triggers complex signal transduction in mammalian cells, such as the protein kinase C pathway, PI3K pathway, and Ras/ERK pathway (Dorey and Amaya, 2010).

FGF1/FGFR1 signaling is involved in multiple signal regulation, including energy metabolism and growth in mammals (Kim et al., 2002; Lim et al., 2006). FGF1 is expressed in adipose tissues under the control of PPARγ, and it plays an essential physiological role in maintaining adipose tissue plasticity during feeding–fasting cycles (Fernandes-Freitas and Owen, 2015). Additionally, FGF1 plays critical roles in metabolic homeostasis, which restores blood glucose levels and insulin sensitivity in mice (Jonker et al., 2012). During fasting, enhanced expression of FGF21 inhibited growth hormone (GH) signaling and impacted chondrocyte functions (Inagaki et al., 2008). These functions of FGF21 in mice tissue were governed by FGF1 receptor signaling (Adams et al., 2012). However, there is limited information on whether starvation regulates FGF1 and has an effect on growth in teleost fish.

Mammals and *Xenopus* have single copies of the *fgf1* gene; however, teleosts may have duplicate *fgf1* genes, which is believed to be due to an additional genome-wide duplication event (Taylor et al., 2003). Two *fgf1* genes have been identified in the zebrafish genome, with *fgf1a* on chromosome 14 and *fgf1b* on chromosome 21. During embryogenesis, *fgf1a* expression in zebrafish is the strongest in somites at 28 hpf, and *fgf1a* is required for normal differentiation of erythrocytes during primitive hematopoiesis (Songhet et al., 2007). In common carp, *in situ* hybridization was used to show that *fgf1a* was expressed specifically in developing

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scale tissues, and it was also observed in the tissues of the tail and dorsal or ventral fins (Ma et al., 2014). These findings reveal the vital roles of *fgf1a* in earlier fish embryo development. However, to the best of our knowledge, there is no information on functions and expression patterns of duplicate *fgf1* genes in teleosts.

The grass carp, *Ctenopharyngodon idellus*, is an important species for freshwater aquaculture in China, with the highest output in fish farming worldwide; its production accounts for 15.6% of global freshwater aquaculture (FAO, 2011). In 2013, total production of grass carp was reportedly around 5 million tons (FBMA, 2013). Fish growth is an integrated process that depends, for the most part, on nutrient availability as well as GH (Cao et al., 2014; MacDonald and Webber, 1995). Fasted fish reduce energy storage and display a decrease in the growth rate (Cao et al., 2014). In this study, duplicated *fgf1* cDNAs were cloned from the grass carp, and their expression patterns were examined in adult tissues and different embryo stages during embryogenesis. In addition, effects of fasting and human growth hormone (hGH) treatments on the mRNA expressions of *fgf1a* and *fgf1b* were investigated in juvenile grass carp.

2. Materials and methods

2.1. Experimental fish

All experimental materials, including embryos and adult grass carp, were obtained from the Qingpu Fish Breeding Experiment Station, Shanghai, China. Embryos were obtained using artificial insemination. Fertilized eggs (~200) were plated in petri dishes (10 cm in diameter). Embryo development occurred at room temperature (~22 °C). Every 4 h after fertilization (0–40 hpf), the embryos were stored by immersion in RNA Store (Tiangen, Shanghai, China) and maintained at 4 °C overnight and then at −80 °C until use. Water in the petri dishes was replaced every 2–3 h with well-aerated water to maintain normal dissolved oxygen (DO) levels during embryogenesis. Embryos at different developmental stages were collected and fixed as reported previously for *in situ* hybridization (Jiang et al., 2012). Tissues from adult grass carp, namely, brain, muscle, liver, eyes, heart, gill, spleen, kidney, intestine, and skin, were rapidly dissected, frozen in liquid nitrogen, and stored at −80 °C until use. All experiments were conducted according to the guidelines approved by the Shanghai Ocean University Committee on the Use and Care of Animals.

2.2. Fasting and hGH treatments

For the fasting treatment, 36 juvenile grass carp (~20 g each) were cultured in two 150-L indoor tanks within a continuous flow system. After 1 week of acclimation, a total of 6 fish (3 per tank) were collected on day 0, 2, 4, and 6 during fasting treatment and on day 3 and 6 during re-feeding treatment. Six fish in a feeding control tank were collected simultaneously with the fasted or re-fed group at every sampling time. hGH treatment was performed according to a published method (Yuan et al., 2011). Nine juveniles were cultured in three 150-L indoor tanks. After 3 days without feeding, 3 fish from each tank were anesthetized using MS222 and administered an intraperitoneal injection of phosphate-buffered saline (PBS, control) or 10 or 50 μg of recombinant hGH (Shanghai United Cell Biotechnology Company, China) per gram body weight at a volume of 100 μL. Each experiment was performed in triplicate. After 12 h, a total of 9 fish were sampled for each experimental treatment. Brain, liver, and muscle were immediately excised, frozen in liquid nitrogen, and stored at − 80 °C until use.

2.3. Molecular cloning of grass carp fgf1a and fgf1b cDNAs

Total RNA was isolated from grass carp embryos at 40 hpf by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and subsequently treated with DNase (Promega, Madison, WI, USA) to remove contaminant genomic DNA. First-strand cDNA was reverse-transcribed from the total RNA by using reverse transcriptase M-MLV (TaKaRa, Japan), according to the manufacturer's instructions. PCR was performed to amplify partial cDNA fragments of *fgf1a* and *fgf1b*. The primer pairs were fgf1a-PS-F/-R and fgf1b-PS-F/-R (Table 1), designed using conserved regions of known sequences of zebrafish (GenBank Accession No. NP_957054.1 for *fgf1a* and NP_001098748.1 for *fgf1b*) and common carp (BAQ36072.1 for *fgf1a* and BAQ36073.1 for *fgf1b*). PCR fragments (185 bp for *fgf1a* and 306 bp for *fgf1b*) were cloned, sequenced, and used to design nested gene-specific primers for 3′ RACE analysis (fgf1a-3RACE-O and fgf1a-3RACE-I; fgf1b-3RACE-O and fgf1b-3RACE-I) and 5′ RACE analysis (fgf1a-5RACE-O and fgf1a-5RACE-I; fgf1b-5RACE-O and fgf1b-5RACE-I) (Table 1). The 5′ and 3′ ends of *fgf1a* and *fgf1b* mRNAs were amplified using the SMART RACE cDNA amplification kit (Clontech, CA, USA), according to the manufacturer's protocol. PCR products were gel-purified, ligated into the T/A cloning vector pMD-19T (TaKaRa, Dalian, China), and transformed into *Escherichia coli* DH5α competent cells. Positive clones were examined using PCR and direct sequencing.

2.4. Sequence and phylogenetic analyses

Nucleotide sequences of *fgf1a* and *fgf1b* were analyzed using BioEdit 7.0.0.1 (Jeon et al., 2014). Sequences of Fgf1a and Fgf1b proteins from different species were compared using the National Center for Biotechnology Information BLASTP search program. Alignment of putative amino acid sequences of the Fgf1a and Fgf1b proteins was performed with the Clustal X 1.83 program (Thompson et al., 1997). Phylogenetic analysis was performed using coding sequences with the neighbor-joining method in MEGA 5.05 (Tamura et al., 2011). Gap sites in the alignment were used for the phylogenetic reconstruction, and reliability of the estimated tree was evaluated using the bootstrap method with 1000 pseudo-replications.

Table 1
Primer sequences used in this study.

Primer name	Primer sequence (5′–3′)
fgf1a-PS-F	AATGGAGGATTTCACCTTCAGA
fgf1a-PS-R	TCCATCTTCTCCAGGAAATAAC
fgf1b-PS-F	CAACCTAACGGGACTGTGGA
fgf1b-PS-R	GCCCTTGTGTGTTTTGGAGC
fgf1a-5RACE-O	TCTGTTCCTTCAATGACCACCA
fgf1a-5RACE-I	ATGCGCAGTATGCTGTAGATGTTC
fgf1a-3RACE-O	TGGTGGTCATTGAAGGAACAGA
fgf1a-3RACE-I	CGTCAGCATTAGTAACGGATGATAGT
fgf1b-5RACE-O	CCACATTTATCCATTGCCAGG
fgf1b-5RACE-I	ACCTTCAAAAGAGTGTAAACGTCG
fgf1b-3RACE-O	ACCTGGCAATGGATAAATGTGG
fgf1b-3RACE-I	CACATCCCGTTCACAGAGGTATC
fgf1a-qRT-F	TTTCAAACAGCTCAGAATAGAT
fgf1a-qRT-R	GGTAAAGTCCTGCCTCTGTTCC
fgf1b-qRT-F	TTCACAGGGGTGTTCACGC
fgf1b-qRT-R	ACAGTCCCGTTGGGTTGG
β-actin-qRT-F	TGCCATGTATGTGGCCATCC
β-actin-qRT-R	TCTTTCGGCTGTGGTGGTGA
fgf1a-P-F	GCAGTGAAATTCAACCCGCC
fgf1a-P-R	TCACACCATGTCATAGAAGCGTTTT
fgf1b-P-F	TTCACACTGCTACCGACGAC
fgf1b-P-R	AAAAAAGTTCTTTACATTCACCTG