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Forkhead box O1 in grass carp *Ctenopharyngodon idella*: Molecular characterization, gene structure, tissue distribution and mRNA expression in insulin-inhibited adipocyte lipolysis

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ABSTRACT

Factor forkhead box O1 (FoxO1) is a transcription factor and plays an important role in insulin-mediated lipid metabolism. In the present study, two distinct FoxO1 cDNAs, designated FoxO1a and FoxO1b, were firstly isolated and characterized from grass carp *Ctenopharyngodon idella*, encoding peptides of 654 and 631 amino acids, respectively. Phylogenetic and synteny analyses suggested that FoxO1a and FoxO1b were derived from paralogous genes that could be originated from teleost-specific genome duplication (TSGD) event. Analysis of the exon–intron structures clarified that grass carp FoxO1a and FoxO1b comprise 3 coding exons and contain a extra intron compared with human and mouse FoxO1. Both FoxO1a and FoxO1b mRNAs were expressed in a wide range of tissues, but the abundance of each FoxO1 mRNA showed the tissue-dependent expression patterns. Time-course analysis of FoxO1 expressions indicated that the level of FoxO1a mRNA reached almost maximal level at day 2, while that of FoxO1b mRNA reached almost maximal level at day 4 during grass carp primary preadipocyte differentiation. In insulin-inhibited adipocyte lipolysis, only FoxO1a showed a significant decrease in adipocyte, indicating that two FoxO1 isoforms may serve somewhat different roles in the regulation of lipolysis by insulin. These results suggested that grass carp FoxO1a and FoxO1b may play different roles in tissues, and their expression levels were differently modulated by insulin in adipocyte.

1. Introduction

Lipids are the predominant source of energy for fish and fatty acids are very important substrates used to maintain metabolic homoeostasis (Weil et al., 2013). In aquaculture, metabolic disorders resulted from excessive fat accumulation has been reported in many fish species (Tacon, 1996); however, the mechanisms involved in fat metabolism in fish have not been revealed clearly. Adipose tissue is characterized as a form of connective tissue that plays a major role in nutrient homeostasis and functions as an endocrine organ at the center of energy homeostasis (Rosen and Spiegelman, 2014). The excessive fat deposited in the adipose tissue is closely related to metabolic disease (Eckel et al., 2005). Because of its central role in energy homeostasis, interest in "solving" the mechanisms involved in adipocyte lipid metabolism has been given more and more research concerns in fish in recent years.

Many transcription factors are involved in the lipid metabolism in adipose tissue. FoxO1, a member of the evolutionarily conserved FoxO subfamily of forkhead transcription factors, functions in adipose cells to couple insulin signalling to adipogenesis, which involves switching preadipocytes from proliferation to terminal differentiation (Birkenkamp and Coffer, 2003; Nakae et al., 2003). FoxO1 is expressed in tissues involved in energy metabolism such as liver, muscle, and adipose tissue (Farmer, 2003). FoxO1 proteins promote triacylglycerol (TAG) catabolism in adipose tissue by stimulating the expression of adipose TAG lipase (ATGL) (Chakrabarti and Kandror, 2009), which mediates the first step in lipolysis, and thereafter other lipases, including hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MGL), promote the removal of additional fatty acids from the glycerol backbone of TAG (Coleman and Mashek, 2011; Zechner et al., 2009). FoxO1 protein interacts with response elements in the ATGL gene promoter and stimulates ATGL gene expression in adipose tissue (Chakrabarti and Kandror, 2009). However, information is very scarce about the function of FoxO1 gene in fish.

The development of adiposity is regulated by many factors. Insulin is an important anabolic hormone that can promote cell functions and signals in mammals, including glycogen synthesis, gene transcription and protein synthesis (Cheatham and Kahn, 1995). Insulin stimulates adipocyte lipogenesis and inhibits adipocyte lipolysis (Choi et al., 2010). In fish, insulin has almost no effect on lipolysis in sea bream (*Sparus aurata*) adipocytes (Albalat et al., 2005b), while insulin decreases the lipolysis in large yellow croaker (*Pseudosciaena crocea*) adipocytes (Wang et al.,

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2012). Information regarding the insulin-mediated control of lipolysis in fish is still limited. Recent study indicated that FoxO1 controls insulin-dependent ATGL expression and lipolysis in 3T3-L1 adipocytes (Chakrabarti and Kandror, 2009). Despite of insulin's important role in lipolysis, the ability of insulin to regulate FoxO1 remains poorly characterized in fish.

Grass carp (*Ctenopharyngodon idella*), a herbivorous freshwater fish, is an important farmed fish in China for its delicious meat and high market value (Wang et al., 2015). It is considered as a good model for the study of lipid metabolism because grass carp store excess fat in liver and visceral adipose tissue. Additionally, the draft genome of grass carp (Wang et al., 2015) released recently is a useful tool for identifying genomic structure of genes involved lipid metabolism. In this study, two different FoxO1 genes were cloned and their tissue-specific expressions and mRNA levels in preadipocytes during differentiation were determined in grass carp. Furthermore, the effects of insulin on adipocyte lipolysis and FoxO1 mRNA expression were evaluated *in vitro* in this fish species. The present study will extend our understanding on the physiological function of FoxO1 gene in adipocyte of fish.

2. Materials and methods

2.1. Fish culture and sampling

Experimental grass carp were obtained from the local fish farm. Animals were acclimated to laboratory conditions for at least 4 weeks prior to experiments. Fish were assigned randomly to one of six treatment groups in 150-L circular tanks (approximately16–20 fish per tank) with a flow-through water supply at 28 °C under a 12L:12D photoperiod. They were fed a commercial pellet diet (crude protein: 35%; crude lipid: 7%) three times a day and provided with continuous aeration to maintain the dissolved oxygen level near saturation. For cDNA cloning and analysis of tissue distribution, grass carp were euthanized by immersion in MS-222 after 24 h post feeding (Sigma, St. Louis, MO, USA). Tissues including spleen, brain, kidney, abdominal fat, heart, white muscle, red muscle, and liver were rapidly dissected, frozen in liquid nitrogen and stored at −80 °C until used. All procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals and approved by the Northwest A&F University Institutional Animal Care and Use Committee.

2.2. Identification and cloning of FoxO1

The complete CDS of human FoxO1 were used as queries for a BLASTN search to acquire sequences of grass carp FoxO1 transcripts from our transcriptomic database (Tian et al., 2015). All the ESTs were assembled *in silico* into a consensus sequence containing the complete open reading frame (ORF) using the SeqMan program of DNAstar software (Burland, 2000). To amplify and confirm the full length of FoxO1 cDNAs, gene specific primers were designed (Table 1). European eel (*Anguilla Anguilla*) and Small spotted catshark (*Scyliorhinus canicula*) FoxO1 transcripts were identified by tblastn in the transcriptomes (http://www.zfgenomics.com/sub/eel; http://skatebase.org/downloads). Other FoxO1 sequences were obtained from NCBI and Ensembl release 83 (http://www.ensembl.org). Suppl. Table 1 summarized sequence information and accession numbers.

For gene cloning, total RNA was isolated from grass carp liver using TRIzol Reagent (TaKaRa, Otsu, Shiga, Japan) according to the instructions of the manufacturer. The purity and the concentration of total RNA were measured by a spectrophotometer at 260 and 280 nm. The integrity was tested by electrophoresis in formaldehyde agarose gels. One microgram of total RNA and 0.1 μg of random primers were used to synthesize first-strand cDNA by reverse transcription using the M-MLV reverse transcriptase in a 10 μL reaction volume (Promega, Madison, WI, USA). The resulting product was used as template for PCR amplification. PCR amplification was made with 2 μL of cDNA, 0.2 μM primers and 1.25 U of PrimeStar HS DNA polymerase (Takara, Otsu, Shiga, Japan) in a total volume of 50 μL. The primers for each amplication were given in Table 1. PCR was performed for 35 cycles at temperatures of 98 °C for 10 s, 55 °C for 5 s, and 72 °C for 2 min. Products of the PCR were electrophoresed on 1.5% agarose gel staining with Goldview. Bands of expected size were purified with the PCR Purification Kit (Tiangen, Beijing, China). The purified fragments were then cloned into a PMD18-T vector following the manufacturer's instructions (TaKaRa, Otsu, Shiga, Japan) and transformed into *E. coli* DH5α. Recombinant bacteria were identified by blue/white screening and confirmed by PCR. Plasmids containing inserts of an expected size were purified and the sequence analysis was carried out by Sangon (Shanghai, China).

Table 1
Specific Primers used for CDS cloning of FoXO1 and qPCR of ATGL, FoXO1a and FoXO1b genes from grass carp.

Primers	Sequences (5′-3′)	Size (bp)
Primers for complete CDS		
FoXO1a-F	GTATTGCTGGTACCATGGCTG	1987
FoXO1a-R	GAGAGTTTTTACCCAGACACCC	
FoXO1b-F	AAGGACAGTCAGTTGGCTTG	2067
FoXO1b-R	CGACAGCATTCAGTCTGCTT	
Primers for qPCR		
ATGL-F	TCGTGCAAGCGTGTATATG	120
ATGL-R	GCTCGTACTGAGGCAAATTA	
FoXO1a-F	GCATCTCATAGCCATGCCCT	190
FoXO1a-R	CACCTCCAAGATGACCGGAG	
FoXO1b-F	CTCAACCTCATCTCGCCCAA	196
FoXO1b-R	TCGGTATGGCGATTGGACTG	
β-actin-F	TCCACCTTCCAGCAGATGTGGATT	115
β-actin-R	AGTTTGAGTCGGCGTGAAGTGGTA	

2.3. Sequence characterization

The predicted protein sequence was analyzed by ORF Finder (Hancock and Bishop, 2004). The presence of conserved domains was analyzed by using the CDART (Geer et al., 2002) and InterProScan (Quevillon et al., 2005) programs.

2.4. Gene organization and synteny analysis

The exon-intron boundary of grass carp FoxO1 was characterized by aligning the cloned cDNA sequence with the genomic sequence (http://www.ncgr.ac.cn/grasscarp/) and the gene structure was compared with those of human, mouse, xenopus, chicken, medeka and zebrafish.

To investigate whether FoxO1 paralogs exist in teleosts, we used synteny analysis to determine orthologous or paralogous relationships of sequences using Genomicus. Short-range gene linkage comparisons included human, chicken, lizard, xenopus, the coelacanth and 7 ray-finned fish (tetraodon, stickleback, tilapia, medaka, zebrafish, cavefish and gar). The genomic data used in this analysis were derived from Ensembl release 83 (http://www.ensembl.org)

2.5. Adipocyte isolation and insulin exposure

The grass carp pre-adipocytes were cultured as described by Liu et al. (2015) with minor modifications. Briefly, the adipose tissue (~180 g) was isolated by sterile dissection from the abdominal cavity of 4–5 fishes. The tissue was washed three times with phosphate-buffered saline (PBS, pH 7.4) and minced in 0.1% Type I collagenase (Sigma, USA) with 2% BSA (Sigma) at room temperature for 30 min. The cell suspension was filtered through a 200-μm nylon filter and centrifuged at 590*g* for 10 min. The sedimented cell pellet was incubated in erythrocyte lysing buffer for 10 min at room temperature, washed twice and resuspended in growth medium (GM), composed of Dulbecco's modified Eagle's medium (DMEM), 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin. The resuspended cells were seeded in gelatin pre-coated plates at a density of approximately 10 g tissue/25 cm^2. To reach 80–90% confluency, the cells were incubated at 28 °C with 5% CO_2 for one week. The differentiation was induced in adipogenic medium (AM) containing GM