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Duplicated connective tissue growth factor genes in hypoxia-sensitive blunt snout bream *Megalobrama amblycephala* and their in vivo expression

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ABSTRACT

Connective tissue growth factor (CTGF) is a peptide involved in tissue growth and development, and can be regulated by hypoxia stress. This study aimed to isolate and characterize duplicate *Ctgf* genes in blunt snout bream *Megalobrama amblycephala*, and determine their expression patterns and response to hypoxia. The blunt snout bream Ctgfa and Ctgfb were found to be highly divergent, sharing a relatively low sequence identity of 57%. During embryogenesis, *Ctgfa* mRNA expression levels were low, gradually decreased from zygotes to 12 h post-fertilization (hpf), markedly increased from 16 hpf, and then stabilized from 32 to 40 hpf. *Ctgfb* expression levels were constant but low from zygotes to 20 hpf, then gradually increased from 24 to 40 hpf. *Ctgfa* mRNA was expressed in the adaxial cells of the somites, floor plate, and tailbud at 24 hpf, and in the notochord and ethmoid plate at 36 hpf, whereas *Ctgfb* mRNA was weakly expressed in the adaxial cells and floor plate at 24 hpf, and in the notochord at 36 hpf. In adult fish, *Ctgfa* mRNA was strongly expressed in the kidney, brain, intestine, muscles, and skin, while *Ctgfb* mRNA was detected in all examined tissues. During hypoxic treatment, the mRNA levels of both *Ctgfa* and *-b* were significantly upregulated in the gill and liver, whereas *Ctgfa* mRNAs in the brain and kidney and *Ctgfb* mRNAs in the kidney significantly decreased. These results provide new insights into the functional conservation and divergence of *Ctgf* genes and reveal their responses to hypoxia.

1. Introduction

Connective tissue growth factor (CTGF), also known as CCN2, is a potent fibroblast mitogen and angiogenic factor involved in the regulation of tissue growth and development and was first isolated from human umbilical vascular endothelial cells (Bradham et al., 1991). Studies in mammals have shown that CTGF has an important role in multiple biological processes, such as angiogenesis (Kuiper et al., 2008; Hall-Glenn et al., 2012), chondrogenesis (Ivkovic et al., 2003), skeletogenesis (Arnott et al., 2011), and fibrosis (Liu et al., 2011). Recent studies have indicated that hypoxia can significantly both upregulate and suppress the expression of the *CTGF* gene, depending on the cell type (Lee et al., 2009; Kroening et al., 2010; Tran et al., 2013).

Mammals and *Xenopus* have single copies of the *CTGF* gene (Mercurio et al., 2004); however, teleost fish may have duplicate *Ctgf* genes, believed to be due to an additional genome-wide duplication event (Taylor et al., 2003; Jaillon et al., 2004; Kasahara et al., 2007). Two *Ctgf* genes are found in the zebrafish genome (Chiou et al., 2006; Fernando et al., 2010). Zebrafish *Ctgfa* expression is first detected in the somites, floor plate, and notochord during embryogenesis, while zebrafish *Ctgfb* signals are observed in the neural plate, the posterior region of embryo at the midline, and the posterior notochord (Erwin, 2008; Fernando et al., 2010). A loss-of-function study of zebrafish showed that using antisense morpholino against *Ctgfa* injection led to developmental delays and distortion of the notochord (Chiou et al., 2006). Despite those studies in zebrafish, the existence of duplicated *Ctgf* genes and their temporal and spatial expression are yet unknown in other teleost fish. Moreover, the hypoxia responses for *Ctgf* genes in both hypoxia-sensitive and -tolerant fish species are worthy of study.

Blunt snout bream *Megalobrama amblycephala* is a herbivorous fish, and is widely favored as a delicacy in China (Li et al., 2006). Since 1960, it has been accepted as a principal species in the Chinese freshwater fish polyculture systems (Zou et al., 2004), whose total production amounted to 677,887 tons in 2011 (Guo et al., 2013). The blunt snout bream is a hypoxia-sensitive species and a short period (<2 h) of hypoxia (below 1.0 mg O_2 L^{-1}) at room temperature can be lethal (Shen et al., 2010). In this study, duplicated *Ctgf* cDNAs were cloned and identified from the blunt snout bream. Their expression patterns were determined in adult tissues and different embryo stages, and their response to acute hypoxia was investigated.

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2. Materials and methods

2.1. Animals

The blunt snout bream *M. amblycephala* were obtained from the Bream Genetics and Breeding Center of Shanghai Ocean University, Shanghai, China. Fertilized eggs were generated by artificial insemination of the blunt snout bream. Fertilized eggs (200–300) were plated in each Petri dish (10 cm in diameter). Embryo development was carried out at room temperature, ~25 °C. Adult fish were sacrificed by immersion in MS-222 (tricaine methanesulfonate, Sigma, St. Louis, MO). Tissues, including brain, gill, eyes, muscle, skin, heart, liver, spleen, kidney, intestine, ovary, and testis, were rapidly dissected, frozen in liquid nitrogen and stored at −80 °C until use. All experiments were conducted following the guidelines approved by the Shanghai Ocean University Committee on the Use and Care of Animals.

2.2. Hypoxia treatments

Ten juvenile fish (~32 g) were transferred into each of three 50 L plastic tanks. After one week of acclimation, two groups were exposed to severe hypoxic conditions (1.0 ± 0.1 mg O_2 L^{-1}) for 4 h and a control group was kept under normoxic dissolved oxygen conditions (7.3 ± 0.5 mg O_2 L^{-1}). The hypoxic conditions were maintained by manipulating the N_2 levels, which were controlled by a valve that varied the rate of N_2 bubbling according to the desired level of hypoxia (Shen et al., 2010). The O_2 levels were monitored with an oxygen electrode (YSI Pro ODO, USA). After the exposure period, five fish from each hypoxic treatment and the control group were sampled. The dissolved oxygen levels of the two hypoxic treatments were then adjusted back to normal levels within 1 h by bubbling air into the water. The remaining five fish from each recovery treatment and the control group were sampled 24 h later. Liver, brain, kidney, and gill were immediately excised, frozen in liquid nitrogen and stored at −80 °C until use. The hypoxia experiments were conducted at room temperature.

2.3. Molecular cloning of the blunt snout bream Ctgf cDNAs

Total RNA was isolated from the blunt snout bream embryos at 40 h post-fertilization (hpf) using a 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 total RNA using a TaKaRa RNA PCR Kit (AMV) Ver. 3.0 (TaKaRa, Tokyo, Japan) with oligo-dT primers according to the manufacturer's instructions. Based on conserved regions of known sequences of *Ctgfa* in *Danio rerio*, *Cyprinus carpio*, and *Salmo salar* (GenBank accession numbers NP_001015041.2, EF524110.1, and NM_001139999.1, respectively), PCR was performed to amplify partial cDNA fragments of the blunt snout bream *Ctgfa* using the primers Ctgfa-RT-F1 and Ctgfa-RT-R1 (Table 1). A 745-bp PCR fragment of the blunt snout bream *Ctgfa* was cloned, sequenced, and used to design nested gene-specific primers for 3′ RACE analysis (Ctgfa-3RACE-O, Ctgfa-3RACE-I) and 5′ RACE analysis (Ctgfa-5RACE-O, Ctgfa-5RACE-I) (Table 1). Based on the *D. rerio Ctgfb* sequence (GenBank accession number NM_001102573.1), PCR was performed to amplify partial cDNA fragments of the blunt snout bream *Ctgfb* using the primers Ctgfb-RT-F1 and Ctgfb-RT-R1 (Table 1). A 505-bp partial PCR fragment of the blunt snout bream *Ctgfb* was cloned, sequenced, and used to design nested gene-specific primers for 3′ RACE analysis (Ctgfb-3RACE-O, Ctgfb-3RACE-I) and 5′ RACE analysis (Ctgfb-5RACE-O, Ctgfb-5RACE-I) (Table 1). The 5′ and 3′ ends of the *Ctgfa* and *-b* mRNA were amplified using the SMART RACE cDNA amplification kit (Clontech, Mountain View, CA, USA) following the manufacturer's protocol. The 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 by PCR and direct sequencing.

Table 1
Primer sequences used in this study.

Primer name	Primer sequence (5′-3′)	Assay technique
Ctgfa-RT-F1	CACAAAGGTCTTTACTGTGACTACGGCTC	Fragment PCR
Ctgfa-RT-R1	ATGCGCAGGTCTTGATGAACATCAT	
Ctgfb-RT-F1	GGATGCCAGAGCATCAGGCTGTATAAGCC	Fragment PCR
Ctgfb-RT-R1	CTAATAACACAAAGTTCTCAGTGCTT	
Ctgfa-3RACE-O	TGGCTGCACCACTACCAAGTCTTAC	Ctgfa 3′RACE
Ctgfa-3RACE-I	CAAGTCTTACCGCCCCAAGTTCTGC	
Ctgfa-5RACE-O	ACGTGGCGTACGGATGCACTTCTTC	Ctgfa 5′RACE
Ctgfa-5RACE-I	GCACTTCTTCCCTTTCCTGATTTTC	
Ctgfb-3RACE-O	CCAGTCAGCAATGGCTGGTAGGTCA	Ctgfb 3′RACE
Ctgfb-3RACE-I	TGGCTGGTAGGTCAATCGCATATAG	
Ctgfb-5RACE-O	TGTGTATGTTACACTGGACAGCTCCTCA	Ctgfb 5′RACE
Ctgfb-5RACE-I	CTGGACAGCTCCTCACATGTCATTATCAT	
β-Actin RT-F	CCGCTGCCTCTTCTTCCTC	RT-PCR
β-Actin RT-R	CTACCTCCCTTTGCCAGTTTCCGC	
Ctgfa-in situ-F	ATGGTCCGCCCTTGTGAGTCACACCT	WISH
Ctgfa-in situ-R	CTTCCACCTTCTCTCAGCTTGATGA	
Ctgfb-in situ-F	GTTCAGACCACTGAATGGAGTGAATG	WISH
Ctgfb-in situ-R	CTAATAACACAAAGTTCTCAGTGCT	
Ctgfa-qRT-F	TCTGGACGGTGCCGTAGGTT	qRT-PCR
Ctgfa-qRT-R	TGCCAAAGCTGATCCCACAA	
Ctgfb-qRT-F	CAGCTGCAAACATCAGTGTG	qRT-PCR
Ctgfb-qRT-R	GTCGTGGGTAGGGACAGTCT	
β-Actin-qRT-F	CGTGCTGTTTTCCCTTCCATT	qRT-PCR
β-Actin-qRT-R	CAATACCGTGCTCAAAGGATACTT	

2.4. Sequences and phylogenetic analyses

The putative peptide sequences of *Ctgf* cDNAs were analyzed using BioEdit 7.0.0.1 (Jeon et al., 2014). Protein domains were analyzed with Search Pfam software (http://pfam.sanger.ac.uk/search/sequence). Signal peptides were analyzed by SignalP software (http://www.cbs.dtu.dk/services/SignalP/). Molecular weight was calculated using Compute pI/Mw software (http://cn.expasy.org/tools/pi_tool.html). The sequences of Ctgf proteins from different species were compared using the National Center for Biotechnology Information BLASTP search program. Alignment of the putative amino acid sequences of the Ctgf proteins was performed with the Clustal X 1.81 program (Thompson et al., 1997). Positions with gaps were eliminated, and a neighbor-joining phylogenetic tree of CTGF putative proteins was constructed using MEGA 5.05 (Tamura et al., 2011) with *p*-distance and 1000 bootstrap replicates.

2.5. RT-PCR

Total RNA was isolated from 20 embryos at the same developmental stage from fertilized eggs to 40 hpf of the blunt snout bream using a TRIzol reagent (Invitrogen). After DNase treatment, 1 μg RNA was reverse transcribed to single-stranded cDNA using Reverse Transcriptase M-MLV (TaKaRa) and oligo-dT primers. PCR primers Ctgfa-RT-F1 and Ctgfa-RT-R1 (Table 1) were used to amplify the blunt snout bream *Ctgfa*. PCR primers Ctgfb-RT-F1 and Ctgfb-RT-R1 (Table 1) were used to amplify the blunt snout bream *Ctgfb*. RT-PCR primers were designed in highly divergent regions to ensure that no cross-amplification would occur. The PCR product sizes for *Ctgfa* and *-b* were 745 and 505 bp, respectively. Both products spanned 1–2 putative introns. The primers β-actin-RT-F and β-actin-RT-R (Table 1) were used to amplify *β-actin*, which was used as a control. The PCR products were analyzed by electrophoresis and stained with ethidium bromide.

2.6. Whole-mount in situ hybridization

A 789-bp PCR fragment of the blunt snout bream *Ctgfa* amplified by primers Ctgfa-in situ-F and Ctgfa-in situ-R (Table 1) and a 718-bp PCR