Molecular identification and functional analysis of Ctrp9 in *Epinephelus coioides*

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Abstract

CTRP9 is a member of the C1q/TNF-related protein (CTRP) superfamily and has been studied in mammals, whereas the comparative studies of CTRP9 in non-mammalian species are still absent. In this study, *ctrp9* was isolated and characterized from the orange-spotted grouper (*Epinephelus coioides*). The full-length cDNA of *ctrp9* was 1378 bp in size with an ORF (open reading frame) of 1020 bp that encodes a 339 amino acid pre-pro hormone. The mRNA expression of *ctrp9* showed a rather high level in the kidney and brain, but a low level in other tissues. Furthermore, the mRNA expression of *ctrp9* decreased significantly in the liver after fasting for 7 days and restored to the normal levels after refeeding. In contrast, the *ctrp9* mRNA level increased in the hypothalamus after fasting. The recombinant gCtrp9 (globular Ctrp9) was prepared using the *Pichia pastoris* expression system and was verified by Western blot as well as mass spectrometry assays. In the primary hepatocytes culture, the recombinant gCtrp9 could inhibit the glucose production after 12-h treatment. After i.p. (intraperitoneal) injection with recombinant gCtrp9, in hypothalamus, mRNA expression levels of *npv* and *orexin* (orexigenic factors) decreased, whereas the expression levels of *crh* and *pomc* (anorexigenic factors) increased. Moreover, i.p. injection with the recombinant gCtrp9 could reduce the serum concentrations of glucose, TG and low-density lipoprotein cholesterol but increase the content of high-density lipoprotein cholesterol. Our studies for the first time unveil the structure of Ctrp9 and its potential role as a regulatory factor of metabolism and food intake in teleost.

Key Words

- ▶ Ctrp9
- ▶ food intake
- ▶ metabolism
- ▶ grouper

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Introduction

Adipose tissue is not only an organ for energy storage but also an active endocrine organ (Scherer 2006). It can release a variety of cytokines, including adiponectin, resistin, leptin, IL (interleukin) 1B, IL6, TNFA (tumor necrosis factor α), MCP1 (monocyte chemoattractant protein-1), adipsin and PAI1 (plasminogen activator inhibitor-1) (Trujillo & Scherer 2006). It is well known that adiponectin is an important adipocytokine, which can improve insulin sensitization and can regulate glucose as well as lipid metabolism of the liver and muscle (Wong *et al.* 2009, Seldin *et al.* 2014). As the crucial cytokine, it has anti-atherogenic and anti-inflammatory properties and also protects against metabolic syndrome (Kadowaki *et al.* 2006, Esfahani *et al.* 2015). The C1q/TNF-related proteins (CTRPs) are a highly conserved family of adiponectin paralogs, containing 15 members (Schaffler & Buechler 2012). Protein structure of CTRPs is similar to adiponectin, including an N-terminal signal peptide, a short variable domain, a collagen domain with variable numbers of Gly-X-Y repeat and a C-terminal globular domain. However, CTRP4 contains two globular domains (Wong *et al.* 2008, Peterson *et al.* 2012, Byerly *et al.* 2014). All the members' globular domains are homologous to those of the immune complement C1q, and their 3D structures show similarity to those of TNFA (Wong *et al.* 2008, Peterson *et al.* 2009). Moreover, CTRP9 can form heterotrimers, heterohexamers and hetero-oligomeric complexes with adiponectin (Peterson *et al.* 2009). Due to the highest homology to adiponectin, CTRP9 has similar functions as adiponectin in mammals.

A lot of studies show that Ctrp9 is a pluripotent factor in mammals. CTRP9 can induce vascular relaxation, protect against myocardial injury after ischemia–reperfusion, alleviate cardiomyocyte apoptosis and fibrosis through adiponectin receptor 1/AMPK and PKA signaling pathway (Zheng *et al.* 2011, Kambara *et al.* 2012, 2015, Su *et al.* 2012, Sun *et al.* 2013). The lipid oxidation was enhanced in the muscle, and TG (triglyceride), insulin and serum glucose were decreased in the *Ctrp9* transgenic mice (Peterson *et al.* 2013). The expression of orexigenic neuropeptides in hypothalamus was upregulated, and the levels of leptin and resistin were increased in mice lacking CTRP9 (Wei *et al.* 2014). Serum concentrations of CTRP9 had a positive correlation with arterial stiffness (Jung *et al.* 2014), and CTRP9 attenuated vascular smooth muscle cell proliferation and neointimal formation by increasing cAMP levels (Uemura *et al.* 2013). A recent study reported that gCTR9 was the biologically active isoform that was

digested in the necessary post-translational modification (Yuan *et al.* 2015).

The studies on CTRP9 are restricted to mammals, and the comparative aspects of CTRP9 in non-mammalian species, especially in lower vertebrates, are still unclear. To date, the function of Ctrp9 has never been reported in teleost. As the first step to investigate the functional roles of Ctrp9 in teleost, we isolate and characterize the gene of *ctrp9* in the orange-spotted grouper (*Epinephelus coioides*). The recombinant protein of gCtrp9 was prepared by using *Pichia pastoris* system, and the roles of gCtrp9 in the regulation of energy metabolism and food intake were evaluated. To the best of our knowledge, this is the first report about CTRP9 in non-mammalian vertebrates.

Materials and methods

Animals

All of the animal experiments were approved by the Animal Care Committee of Sun Yat-Sen University. For the tissue distribution experiments and hepatocytes isolation experiments, approximately two-year-old female orange-spotted groupers (*Epinephelus coioides*) with a body weight of 550–650 g were purchased from the Huangsha aquatic products wholesale market in Guangzhou, China. The fish were acclimated to indoor tanks with recirculating seawater at room temperature under a cyclic light–darkness photoperiod (12h:12h) for at least one week. During the acclimation period, the fish were fed commercial pellets daily until satiety as described previously (Wang *et al.* 2014). All the animal experiments were carried out in accordance with the guidelines and approval from the Sun Yat-Sen University Animal Care and Use Committee.

Molecular cloning of grouper *ctrp9*

Molecular cloning of *ctrp9* was conducted in grouper by nested PCR coupled to 3'/5' RACE according to the procedures described previously (Qin *et al.* 2014). The first-strand cDNA was synthesized using the M-MLV kit (Invitrogen). Using primers (Table 1) designed based on the conserved regions of ORF of fugu rubripe, medaka, three-spined stickleback and spotted green pufferfish *ctrp9*, nested PCR was performed and a partial fragment of grouper *ctrp9* cDNA was isolated. Based on the nucleotide sequence obtained, new primers were synthesized and used in 3'/5' RACE (Table 1). To confirm the composite ORF sequence, RT-PCR was performed using a pair of

Table 1 Primers used in this study.

Name	Sequences (5'–3')	Purpose
Partial CDS-F1	CCYGGAATACCAGGRGACCC	Partial CDS clone
Partial CDS-F2	CCHGGRAAAATGGGVCCCCA	
Partial CDS-R1	AAGACRGTGATGTGRTAGGTRAAG	
Partial CDS-R2	ACYTGCAGCCASACCTTGTC	3'RACE
3'RACE-F1	GAAGTTGGCCTTAGAGGTGACAGA	
3'RACE-F2	CTCACAGCACAAAGTAACTCCCTA	
3'RACE-F3	GCGGGAGCATATTTCTTCACCTAC	5'RACE
5'RACE-R1	TAGGGAGTTTACTTTGTGCTGTGAG	
5'RACE-R2	ATATTGCCTTTGTGACCTGGAAC	
5'RACE-R3	GGTCCAGGCAGCCCAAGAT	ORF clone
ORF-F	TTTTGCATTGTTGATAGATAGG	
ORF-R	AGACAGGCTGATTTCTTATGAC	
<i>ctrp9</i> -RT-F	CTTAGAGGTGACAGAGGCATTCT	<i>ctrp9</i> real-time PCR
<i>ctrp9</i> -RT-R	CTGTGGATCGTAGTGATTCTGC	
<i>npv</i> -RT-F	ACGCTCCACAGTCAAGATAC	
<i>npv</i> -RT-R	GCGGCTCATAGAGGTAAAGG	<i>orexin</i> real-time PCR
<i>orexin</i> -RT-F	TGTTGCTTTGTCGCTCTGG	
<i>orexin</i> -RT-R	TCTTCAGTCCTCTTGCCCAT	
<i>pomc</i> -RT-F	TCCCAGGCGACAACCAC	<i>pomc</i> real-time PCR
<i>pomc</i> -RT-R	TCGGCTGACTCTTCTTCTAC	
<i>crh</i> -RT-F	TACATCTACCAAGGTCCCA	
<i>crh</i> -RT-R	CAGAGGCAGCAGCACTA	Reference gene
18S-F	CCTGAGAAACGGCTACCACATCC	
18S-R	AGCAATTAGTATACGCTATTGGAG	
AP	GGCCACGCGTCGACTAGTAC(T)16	Universal primers
AAP	GGCCACGCGTCGACTAGTAC	
AUAP	GGCCACGCGTCGACTAGTAC	
rgCtp9-F	CGGAATTCCATCATCATCATCATCTTGTATCTCTAAAAGCGCTCTAAAAGCG	Expression gCtp9
rgCtp9-R	ATAGTTTAGCGCCGCTAAGCCCCAAAGATTA	

Degenerate bases: Y = C + T; R = A + G; H = A + T + C; V = A + G + C; S = C + G.

gene-specific primers designed at the upstream and downstream of protein coding sequence. The following procedure was used for performing all PCRs: 94°C, 3 min; 94°C, 30 s, 50–62°C, 30 s, 72°C, 1–1.5 min, 35 cycles; 72°C, 10 min. All the PCR products were purified by the E.Z.N.A Gel Extraction Kit (OMEGA, bio-tek) and subcloned into pCR2.1 vector (Invitrogen) for DNA sequencing. The ORF finder in NCBI (<http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi>) was used to get the ORF, and the molecular mass and isoelectric point of Ctrp9 precursor protein were analyzed with ExPASy (http://web.expasy.org/compute_pi/). The signal peptide of Ctrp9 was predicted using the SignalP4.1 Server (<http://www.cbs.dtu.dk/services/SignalP/>). The phosphorylation and glycosylation sites of the Ctrp9 pre-pro hormone were analyzed by NetPhos 2.0 Server (<http://www.cbs.dtu.dk/services/NetPhos/>) and NetNGlyc 1.0 Server (<http://www.cbs.dtu.dk/services/NetNGlyc/>), respectively. Sequence alignment at the protein level was performed using ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). The phylogenetic tree was constructed with MEGA6.0 by neighbor-joining method (bootstrap phylogeny test, 2000 replicates).

Tissue distribution and effects of nutritional status on *ctrp9* mRNA expression

Three two-year-old female groupers were anesthetized by MS222 and decapitated for tissue distribution. Various tissue samples were collected quickly and snap-frozen in liquid nitrogen, and then stored in –80°C until RNA extraction.

Juvenile fish with a body weight of 55–65 g and body length of 13–14 cm were used in the fasting and refeeding experiment as described previously (Qin *et al.* 2014). After acclimation for 1 week, all fish were divided into 3 groups ($n=6$ fish/group). The first group of fish was fed for 7 days (fed 6 h before sampling on Day 7) and used as the control group ('Fed'), the second group of fish was maintained under food deprivation for 7 days ('Fasted') and the third group of fish was fasted for 7 days and refed 6 h before sampling on Day 7 ('Refed'). At the end of the experiments, all fish were anesthetized by MS222 and decapitated. The liver, adipose tissue, muscle and hypothalamus samples were collected quickly and snap-frozen in liquid nitrogen and then stored in –80°C until RNA extraction.

RNA extraction, reverse transcription and real-time quantitative PCR

All experiments were performed according to the standard procedures established in our lab (Wang *et al.* 2015a). Briefly, total RNA was extracted from tissues using TRIzol reagent (Invitrogen) according to the manufacturer's instruction. The purity and yield of RNA were assessed by a NanoDrop 2000C spectrophotometer (Thermo Scientific) with OD260: 280 ratios between 1.8 and 2.0. Two micrograms of total RNA was incubated with RNase-free DNase-I (NEB, USA) at 37°C for 10 min to eliminate contaminating genomic DNA and then treated with 5 mM EDTA at 75°C for 10 min to inactivate DNase-I. The first-strand cDNA was synthesized with M-MLV Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. The gene expression levels were determined with real-time quantitative PCR. A total of 10 µL of the PCR reaction volume contained 5 µL of Thunderbird SYBR Green qPCR Mix (TOYOBO, Japan), 0.3 µL of forward and reverse primers (10 µM each), 1 µL of diluted cDNA templates and 3.4 µL of water. Amplification of samples was carried out with the Roche LightCycler 480 Real-time PCR Detection System using the following thermal cycling profiles: 95°C for 1 min, 40 cycles of 95°C for 15 s, 56°C for 15 s and 72°C for 30 s. The 18S gene was used as the internal reference, and the 18S levels remained stable between various treatments throughout the study. The relative gene expression levels were normalized to the 18S levels and were calculated by the comparative Ct method (Schmittgen & Livak 2008).

Production of recombinant protein by *Pichia pastoris*

Recombinant global Ctrp9 (gCtrp9) was obtained by *Pichia pastoris* as described previously (Chen *et al.* 2012). The cDNA fragment coding globular domain of ctrp9 was cloned from pCR2.1-ctrp9 vector by PCR with specific primers. EcoRI and NotI sites were introduced at the upstream and downstream ends of the fragment, respectively. Moreover, a 6× HIS tag was introduced to the site between the EcoRI site and N-terminal sequence of gCtrp9. The primer sequences were shown in Table 1. The sequence was inserted to form an in-frame fusion peptide with the α-factor secretion signal of pPICZαA and the KEX2 cleavage site. The cloned fragment was digested with restriction enzymes (EcoRI and NotI; NEB), purified and subcloned into pPICZαA (Invitrogen) (Supplementary Fig. 1 for details, see section on supplementary data given at the end of this article). The recombinant

plasmid was transformed into *E. coli* DH5α and selected on LB low-salt Zeocin plate (Invitrogen). The positive clones were sequenced by ABI 3730 DNA sequencer (Applied Biosystems).

The expression plasmid was linearized by Sac I (NEB) and transformed into *Pichia pastoris* X-33 (Invitrogen) by electroporation according to manufacturer's protocol (Invitrogen, 2008) and selected on YPDS (1% yeast extract, 2% peptone, 2% agar, 20% glucose and 0.1 M D-sorbitol) containing Zeocin (250 µg/mL). After incubation of 60 h at 28°C, the clones were picked, and the expression condition was optimized. The procedure of expression was as follows: single clone was grown in 10 mL BMGY medium (1% yeast extract, 2% peptone, 1% glycerol, 1.34% yeast nitrogen base w/o amino acid, 10 mM K₂HPO₄, 100 mM KH₂PO₄ and 0.4 mg/L biotin), 200 r.p.m shaking 18–24 h at 28°C, centrifuged and resuspended in 100 mL BMGY. After shaking 200 rpm for 18–24 h at 28°C, the cells were centrifuged and resuspended in 100 mL BMMY (1% yeast extract, 2% peptone, 0.5% methanol, 1.34% yeast nitrogen base w/o amino acid, 10 mM K₂HPO₄, 100 mM KH₂PO₄ and 0.4 mg/L biotin), and TWEEN-80 (Sigma) was added to a final concentration of 0.4%. After shaking at 200 rpm for 18–24 h at 28°C, the suspension was added with 100% methanol to a final concentration of 0.5%. After shaking at 200 rpm for 18–24 h at 28°C, culture media were centrifuged and the harvested supernatant were loaded onto a 1 mL Ni²⁺-NTA resin column (GE, USA). The recombinant gCtrp9 was eluted with 150 mM imidazole. Purified proteins were dialyzed against PBS buffer. The purified protein was analyzed by SDS-PAGE, Western blot and mass spectrometry. The protein concentrations were quantified using BCA assay (Beyotime, China).

Hepatocyte isolation, culture and treatments

Hepatocytes were prepared by collagenase IV/DNase II digestion method and seeded in 24-well culture plates at a density of 5 × 10⁵ cells/well in 1 mL of L15 medium with 10% FBS (fetal calf serum) at 25°C and saturated humidity with plain air in an incubator as described previously (Wang *et al.* 2015a). After overnight incubation, the medium was gently changed to serum-free fresh L15 medium (no phenol red), and the cell cultures were incubated for 1 h before adding recombinant gCtrp9 (10 µg/mL) for 6- and 12-h treatments. At the end of the incubation, the supernatant was collected for the measurement of glucose concentration using the glucose assay kit (Robio, China), and the cells were lysed by RIPA (Beyotime), and the protein content was measured

by BCA assay for correcting for cell number variations (Wei *et al.* 2011).

I.p. injection of the recombinant gCtp9

For *in vivo* experiments, female fish with an average length of 9–11 cm and weight of 35–45 g were maintained in a 12-h/12-h light/darkness cycle and fed twice daily at least one week before the experiment. The recombinant gCtp9 was injected intraperitoneally at concentrations of 0.5 and 5 µg/g B.W. (body weight). For the control

groups, fish were injected with PBS based on the body weight. Blood samples were collected from the caudal vein of each fish after gCtp9 injection for 3, 6 and 12 h ($n=12$ for each group). These blood samples were transferred to sterile centrifuge tubes and incubated at room temperature at least for 30 min. Then, the samples were centrifuged at 7500g for 10 min, and the serum was isolated. The serum was stored at -80°C for the determination of glucose (GLU), triacylglycerol (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C). The

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1                               ttgttttctctgttgggtgccagtagtacaagtggttat 39
40  gagggcaactatgtactcaatttaacgtcttttagtctagaagttttgcattgttgatagataggatgcacaggtaca 118
119  ATG CTG CGG ATG AGG TTT AGT GTC ATT CTC TTG CTC CTT CTA TTG GCA GTC AGG TGT GTT 178
1   M L R M R F S V I L L L L L L A V R C V 20
179  ACA CAG GAA GTA ACC CCA AAC AAA GAC TGC GTT TGT GGA CAT CCT GGT ATA CCT GGG GAC 238
21  T Q E V T P N K D C V C G H P G I P G D 40
239  CCC GGA CAC AAT GGC ACA CCT GGC AGA GAT GGC CGA GAT GGA CTC AGA GGT GAA AAA GGT 298
41  P G H N G T P G R D G R D G L R G E K G 60
299  GAT CAA GGT CAA GTT GGC CCA GTT GGA GCA GCT GGC AAT GAT GGC CAC AGA GGA GAC AAA 358
61  D Q G Q V G P V G A A G N D G H R G D K 80
359  GGG GAA CCT GGT GCA GTT GGT CCA GCA GGG CTC AAA GGA AAA AGG GGA GAA AAT GGC GAA 418
81  G E P G A V G P A G L K G K R G E N G E 100
419  CGG GGG CCT CCT GGG AAA ATG GGG CCC CAA GGA GTC CAA GGG CCC ATT GGA CTC AAA GGA 478
101  R G P P G K M G P Q G V Q G P I G L K G 120
479  AGT AAG GGA GAT CTT GGG CTG CCT GGA CCC CAA GGA CCT AAA GGG GAT TTG GGG CCT CTT 538
121  S K G D L G L P G P Q G P K G D L G P L 140
539  GGA CCA CAG GGT CCC AAG GGA GAA GTT GGC CTT AGA GGT GAC AGA GGC ATT CAG GGG CCA 598
141  G P Q G P K G E V G L R G D R G I Q G P 160
599  CTG GGA CCC CCT GGC AGG CCA GGG CCC AAG GGG GAA ATT GGT GTT CCA GGT CAC AAA GGC 658
161  L G P P G R P G P K G E I G V P G H K G 180
659  AAT ATT GGT TAT CGT GGG GAG AGA GGG TCT CGA GGA GAG AAA GGT GAC AAA GGT GAG AAG 718
181  N I G Y R G E R G S R G E K G D K G E K 200
719  GGA GAA GAA CTT GTT ATC TCT AAA AGC GCC TTC TCA GTG GGA CTC ACA GCA CAA AGT AAA 778
201  G E E L V I S K S A F S V G L T A Q S K 220
779  CTC CCT ACA CCT AAT GCT CCG ATC CGG TTT GAC AAG ATC ATT TAC AAT GTG CAG AAT CAC 838
221  L P T P N A P I R F D K I I Y N V Q N H 240
839  TAC GAT CCA CAG ACA GGA AGA TTC ACA TGC TCC GCA GCG GGA GCA TAT TTC TTC ACC TAC 898
241  Y D P Q T G R F T C S A A G A Y F F T Y 260
899  CAC ATC ACA GTC TTC CCG AAC GTG AAG GTT GCT CTG GTG AAG AAT GGT GCA AAG ATC 958
261  H I T V F S R N V K V A L V K N G A K I 280
959  ATT CAC ACC ACG GAT AAC TAC CAG AGC AGC GAG GAT CAG GCA GCA GGG GGC GCT GTG CTG 1018
281  I H T T D N Y Q S S E D Q A A G G A V I 300
1019  AAC CTG GAT GTG GGA GAC AAG GTG TGG CTG CAG GTG ACT GGG GGA GAG CTG TAC AAT GGG 1078
301  N L D V G D K V W L Q V T G G E L Y N G 320
1079  CTC TTT GCT GAT GAA GAT GAT GAC ACG ACC TTC TCT GGG TTC TTA ATC TTT GGG GCT TAG 1138
321  L F A D E D D D T T F S G F L I F G A ※ 339
1139  atgaggcaactttctttataattgatgttgaattatttctaaagaatagaatgagtcataagaatcagcctgtctct 1217
1218  aaacttttggttgactacataatgaatgtacagtgacctaagtcatttactacactgtactactaagaacaaattt 1296
1297  gaggtactgtaccttaattaagttatttctatttacttaatacttatactcaaatcataaaaaaaaaaaaaaa 1375
1376  aaa 1378

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Figure 1

The cDNA and deduced amino acids of grouper *ctrp9*. Single underline represented signal peptide; dotted line represented variable domain; double underline represented collagenous domain and box represented domain of globular. The asterisk represented the stop codon. The gray shadow showed predicted glycosylation site, and the trilaterals showed predicted phosphorylation sites.

concentrations of GLU, TG, HDL-C and LDL-C were measured with commercial kits (Jiancheng, China). Then, the fish were killed by decapitation. The hypothalamus was separated quickly and snap-frozen in liquid nitrogen.

Statistical analysis

All data were shown as mean $\pm$ S.E.M., and statistical analysis was performed with SPSS 18.0 software (SPSS). The significant differences were identified by one-way ANOVA using Fisher's least significance difference (LSD) test. A probability of less than 0.05 ($P < 0.05$) was considered as a significant difference.

Results

Molecular cloning and sequence analysis of grouper *ctrp9* cDNA

Using 3'/5' RACE coupled to nested PCR, the full-length cDNA of grouper *ctrp9* was isolated. The *ctrp9* cDNA was 1378bp in size with a 118bp 5'UTR and 240bp 3'UTR. The ORF of 1020bp encoding 339 amino acids precursor with a deduced molecular mass of 35.0964kDa and isoelectric point of 8.76, respectively (Fig. 1). Based on sequence analysis using SignalP program, the first 22 amino acids were predicted to be the signal peptide. Ctrp9 consisted of three domains, including the variable domain (10 amino acids), the collagenous domain (171 amino acids) containing 57 Gly-X-Y elements, and the globular domain (136 amino acids). The globular domain was namely the functional domain (Fig. 1). Seven phosphorylation sites were predicted in the grouper Ctrp9 precursor by NetPhos including four serine residues, two threonine residues and one tyrosine residue. Furthermore, there was one N-glycosylation site (NGT) in the grouper Ctrp9 protein predicted by NetNGlyc (Fig. 1). The amino acid sequence similarities were aligned between grouper Ctrp9 and other species using ClustalW2 program (Fig. 2). As shown in Table 2, the grouper Ctrp9 precursor displayed a high degree of identity with other fish Ctrp9 precursors and a low degree of identity with mammals and birds (less than 60%). The identity of globular domain of Ctrp9 in groupers showed a higher degree of identity with other species. The phylogenetic tree was constructed using MEGA 6.0 based on Ctrp9 amino acid sequences of grouper and other species. The phylogenetic tree formed two distinct clusters (Fig. 3). Grouper Ctrp9 clustered

Larimichthys crocea	---MRFTFTVLLLLLARCVAQEETPNKGCVCYGPYIPGDPGHNGAPGRDGRDLRGEK 57
Dicentrarchus labrax	---MRSTVTLTLLLLTARCAVEETPNKGCVCYGPYIPGDPGHNGTPGRDGRDLRGEK 57
Epinephelus coioides	MLRMFSYVLLLLLLAVRCVTQEVTPNKGCVGHPYIPGDPGHNGTPGRDGRDLRGEK 60
Oreochromis niloticus	MLKTRFIALLLLLLAVRCVAQEETPNKGCVCYGPYIPGDPGHNGTPGRDGRDLRGEK 60
Takifugu rubripes	-----MGIFLSLLMVSAAQEIPNKGVCYGPYIPGDPGHNGTPGRDGRDLRGEK 63
Cynoglossus semilaevis	MMQIMFVALLLLLLLWCAAQDEQKRCVCYGPYIPGDPGHNGTPGRDGRDLRGEK 60
Oryzias latipes	MFQLKLSVLLLLLFAVRCVAEETTPNKGVCYGPYIPGDPGHNGTPGRDGRDLRGEK 60
Danio rerio	MLCPRLICTLFLVLLVNAEASDPSKPCYGHPIPGDPGHNGTPGRDGRDLRGEK 60
Gallus gallus	-----MKSWILLIAIVSTEEAQKQDICTQAYPIPGDPGHNGTPGRDGRDLRGEK 63
Alligator sinensis	-----MNRWILLIAIVVTEETRKQDSCQGHPIPGDPGHNGTPGRDGRDLRGEK 63
Homo sapiens	-----MRIWILLIAIEICTGNINSQDTCRQGHPIPGDPGHNGTPGRDGRDLRGEK 63
Mus musculus	-----MRIWILLIYMGACTRSVFSQDTCRQGHPIPGDPGHNGTPGRDGRDLRGEK 63
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Larimichthys crocea	DQ--GDICPTGRAGMDHGRGEKGETAGVACGLKGRKGRDGERPPKMGPCQVQGPGL 115
Dicentrarchus labrax	DQ--GEIPGVQGTNDHGRKGEAGTVAGLKGKRGDGERPPKMGPCQVQGPGL 115
Epinephelus coioides	DQ--GQVGPVGAAGNDHGRDGEAGTVAGLKGKRGDGERPPKMGPCQVQGPGL 118
Oreochromis niloticus	DR--GELGPTGLAGNHGRDGEAGTVAGLKGKRGDGERPPKMGPCQVQGPGL 118
Takifugu rubripes	DQ--GEFPGIPGPGNLQKQDGEAGTVAGLKGKRGDGERPPKMGPCQVQGPGL 111
Cynoglossus semilaevis	DT--GEAGASGPGAHGKQDGEAGTVAGLKGKRGDGERPPKMGPCQVQGPGL 118
Oryzias latipes	DK--CGTGPAGVPTDGRHGRGEAGTVAGLKGKRGDGERPPKMGPCQVQGPGL 118
Danio rerio	DR--CTCDGVKPGP--LKQDGRDGEAGTVAGLKGKRGDGERPPKMGPCQVQGPGL 115
Gallus gallus	DTVLGEAGLPGIPGKDGAIHGRGEAGTVAGLKGKRGDGERPPKMGPCQVQGPGL 113
Alligator sinensis	DT--GEVGLPGFPGKDGANGKGAQIDGRVEEKGKRGDGERPPKMGPCQVQGPGL 111
Homo sapiens	DA--GEPGRPSGKDGTSERGERGADKVEAKIKDQSGRSPGKMGPCQVQGPGL 111
Mus musculus	DA--GEPHGPGRKDGIRGEGERGADKVEAKIKDQSGRSPGKMGPCQVQGPGL 111
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Larimichthys crocea	KGNKGLGLPGQSGKDLGPPGPEPKGEIQLGRDGIQGLPIGRPGKGEIGVPH 175
Dicentrarchus labrax	KGNKGLGLPGQSGKDLGPPGPEPKGEIQLGRDGIQGLPIGRPGKGEIGVPH 175
Epinephelus coioides	KGSKGLGLPGQSGKDLGPPGPEPKGEIQLGRDGIQGLPIGRPGKGEIGVPH 178
Oreochromis niloticus	KGNKGLGLPGQSGKDLGPPGPEPKGEIQLGRDGIQGLPIGRPGKGEIGVPH 178
Takifugu rubripes	KNGEGEILPGLQPKDELGPPGPEPKGEIQLGRDGIQGLPIGRPGKGEIGVPH 171
Cynoglossus semilaevis	KGNKGLGLPGQSGKDLGPPGPEPKGEIQLGRDGIQGLPIGRPGKGEIGVPH 178
Oryzias latipes	KGNKGLGLPGQSGKDLGPPGPEPKGEIQLGRDGIQGLPIGRPGKGEIGVPH 178
Danio rerio	KQKQGLGLPGQSGKDLGPPGPEPKGEIQLGRDGIQGLPIGRPGKGEIGVPH 175
Gallus gallus	KQKQGLGLQSGKDLGPPGPEPKGEIQLGRDGIQGLPIGRPGKGEIGVPH 173
Alligator sinensis	KQKQGLGLQSGKDLGPPGPEPKGEIQLGRDGIQGLPIGRPGKGEIGVPH 171
Homo sapiens	KGLRGETGPGQSGKDLGPPGPEPKGEIQLGRDGIQGLPIGRPGKGEIGVPH 171
Mus musculus	QGLRGETGPGQSGKDLGPPGPEPKGEIQLGRDGIQGLPIGRPGKGEIGVPH 171
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Larimichthys crocea	KGNIGYRGDRSGRGEKDRGEKDAFVLSKSAFVSLTAQSLPAANPIRFDKI 235
Dicentrarchus labrax	KGNIGYRGDRSGRGEKDRGEKDAFVLSKSAFVSLTAQSLPAANPIRFDKI 235
Epinephelus coioides	KGNIGYRGDRSGRGEKDRGEKDAFVLSKSAFVSLTAQSLPAANPIRFDKI 238
Oreochromis niloticus	KGNIGYRGDRSGRGEKDRGEKDAFVLSKSAFVSLTAQSLPAANPIRFDKI 238
Takifugu rubripes	KGNIGYRGDRSGRGEKDRGEKDAFVLSKSAFVSLTAQSLPAANPIRFDKI 231
Cynoglossus semilaevis	KGNIGYRGDRSGRGEKDRGEKDAFVLSKSAFVSLTAQSLPAANPIRFDKI 238
Oryzias latipes	KGNIGYRGDRSGRGEKDRGEKDAFVLSKSAFVSLTAQSLPAANPIRFDKI 238
Danio rerio	KGNIGYRGDRSGRGEKDRGEKDAFVLSKSAFVSLTAQSLPAANPIRFDKI 235
Gallus gallus	KGNIGYRGDRSGRGEKDRGEKDAFVLSKSAFVSLTAQSLPAANPIRFDKI 233
Alligator sinensis	KGNIGYRGDRSGRGEKDRGEKDAFVLSKSAFVSLTAQSLPAANPIRFDKI 231
Homo sapiens	KGNIGYRGDRSGRGEKDRGEKDAFVLSKSAFVSLTAQSLPAANPIRFDKI 231
Mus musculus	KGNIGYRGDRSGRGEKDRGEKDAFVLSKSAFVSLTAQSLPAANPIRFDKI 231
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Larimichthys crocea	NHYSPTQGRFTCSAAGAYFTYTHITVFSRNKVALKNGAKVILHTDNYQSSDAQGA 295
Dicentrarchus labrax	NHYSPTQGRFTCSAAGAYFTYTHITVFSRNKVALKNGAKVILHTDNYQSSDAQGA 295
Epinephelus coioides	NHYSPTQGRFTCSAAGAYFTYTHITVFSRNKVALKNGAKVILHTDNYQSSDAQGA 298
Oreochromis niloticus	NHYSPTQGRFTCSAAGAYFTYTHITVFSRNKVALKNGAKVILHTDNYQSSDAQGA 298
Takifugu rubripes	NHYSPTQGRFTCSAAGAYFTYTHITVFSRNKVALKNGAKVILHTDNYQSSDAQGA 291
Cynoglossus semilaevis	NHYSPTQGRFTCSAAGAYFTYTHITVFSRNKVALKNGAKVILHTDNYQSSDAQGA 298
Oryzias latipes	NHYSPTQGRFTCSAAGAYFTYTHITVFSRNKVALKNGAKVILHTDNYQSSDAQGA 298
Danio rerio	NHYSPTQGRFTCSAAGAYFTYTHITVFSRNKVALKNGAKVILHTDNYQSSDAQGA 295
Gallus gallus	NHYSPTQGRFTCSAAGAYFTYTHITVFSRNKVALKNGAKVILHTDNYQSSDAQGA 293
Alligator sinensis	NHYSPTQGRFTCSAAGAYFTYTHITVFSRNKVALKNGAKVILHTDNYQSSDAQGA 291
Homo sapiens	NHYSPTQGRFTCSAAGAYFTYTHITVFSRNKVALKNGAKVILHTDNYQSSDAQGA 291
Mus musculus	NHYSPTQGRFTCSAAGAYFTYTHITVFSRNKVALKNGAKVILHTDNYQSSDAQGA 291
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Larimichthys crocea	VHLHLDGDKVWLQVGGELNGLFADEDDDTTFSGLIFD----- 336 86.90%
Dicentrarchus labrax	VHLHLDGDKVWLQVGGELNGLFADEDDDTTFSGLIFGA----- 336 88.10%
Epinephelus coioides	VHLHLDGDKVWLQVGGELNGLFADEDDDTTFSGLIFGA----- 339 100.0%
Oreochromis niloticus	VHLHLDGDKVWLQVGGELNGLFADEDDDTTFSGLIFGA----- 339 83.19%
Takifugu rubripes	VHLHLDGDKVWLQVGGELNGLFADEDDDTTFSGLIFGP----- 332 78.61%
Cynoglossus semilaevis	VHLHLDGDKVWLQVGGELNGLFADEDDDTTFSGLIFAD----- 339 78.76%
Oryzias latipes	VHLHLDGDKVWLQVGGELNGLFADEDDDTTFSGLIFGA----- 339 78.17%
Danio rerio	VHLHLDGDKVWLQVGGELNGLFADEDDDTTFSGLIFGA----- 336 65.77%
Gallus gallus	VHLHLDGDKVWLQVGGELNGLFADEDDDTTFSGLIFGA----- 342 55.16%
Alligator sinensis	VHLHLDGDKVWLQVGGELNGLFADEDDDTTFSGLIFGA----- 339 56.93%
Homo sapiens	VHLHLDGDKVWLQVGGELNGLFADEDDDTTFSGLIFGA----- 333 59.16%
Mus musculus	VHLHLDGDKVWLQVGGELNGLFADEDDDTTFSGLIFGA----- 333 57.36%
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Figure 2

Amino acid sequence alignment of *Epinephelus coioides* Ctrp9 with other species. *Homo sapiens* (NP_848635.2), *Mus musculus* (NP_898998.2), *Gallus gallus* (XP_001234806.2), *Alligator sinensis* (XP_006017705.1), *Danio rerio* (XP_005162770.1), *Oreochromis niloticus* (XP_005471911.1), *Takifugu rubripes* (XP_003968015.2), *Larimichthys crocea* (XP_010736742.1), *Dicentrarchus labrax* (embI|CBN81948.1), *Cynoglossus semilaevis* (XP_008306029.1) and *Oryzias latipes* (XP_004081095.1).

Table 2 Amino acids identities in the full and globular domains of *Epinephelus coioides* Ctrp9 compared to other species.

	Full (%)	Globular domain (%)
<i>Dicentrarchus labrax</i>	88.10	90.44
<i>Larimichthys crocea</i>	86.90	90.44
<i>Oreochromis niloticus</i>	83.19	91.18
<i>Cynoglossus semilaevis</i>	78.76	83.09
<i>Takifugu rubripes</i>	78.61	86.03
<i>Oryzias latipes</i>	78.17	83.09
<i>Danio rerio</i>	65.77	67.65
<i>Homo sapiens</i>	59.16	69.85
<i>Mus musculus</i>	57.36	67.65
<i>Alligator sinensis</i>	56.93	66.18
<i>Gallus gallus</i>	55.16	61.76

with their counterparts of other teleosts with high bootstrap values.

Tissue distribution of *ctrp9* gene expression

To examine the expression pattern of *ctrp9* in different tissues of grouper, real-time quantitative PCR was performed using cDNA samples prepared from selected tissues. As shown in Fig. 4, gene expression of *ctrp9* could be detected at high levels in cerebellum and kidney and to a lesser extent in telencephalon, mesocerebrum and medulla oblongata. The expression was at low levels compared with telencephalon in peripheral tissues except kidney.

Effects of nutritional status on the mRNA expression of *ctrp9*

To assess the effects of nutritional status on *ctrp9* mRNA levels, the fasting and refeeding experiment was performed. In hypothalamus, the mRNA levels of *ctrp9* have an increased trend in fasted group compared to that in fed group (Fig. 5A). However, the hepatic expression of *ctrp9* mRNA decreased significantly after fasting of 7 days and was restored back to the normal levels after refeeding (Fig. 5B). Similarly, the expression of *ctrp9* mRNA in muscle has a declined trend in fasted group and rose back to the normal levels after refeeding (Fig. 5D). However, refeeding did not raise the expression of *ctrp9* mRNA in adipose tissue to the normal levels (Fig. 5C).

Recombinant protein expression and purification of the globular Ctrp9

The pPICZaA-*gctrp9* with 6× HIS expression vector was constructed and transformed into *Pichia pastoris* X-33 using electroporation according to the *Pichia pastoris* expression kit. The positive strain was selected, and the expressed protein was verified by SDS-PAGE and Western blotting with HIS primary antibody. As expected, the molecular weight of recombinant gCtrp9 was 15.8 kDa (Fig. 6A and B). The positive strain was cultured for large scale protein expression, and the protein was purified with Ni²⁺-NTA resin. The molecular mass and

Figure 3

Phylogenetic tree based on amino acid alignment for Ctrp9 in different species. *Homo sapiens* (NP_848635.2), *Bos Taurus* (XP_005888803.1), *Mus musculus* (NP_898998.2), *Gallus gallus* (XP_001234806.2), *Columba livia* (XP_005501564.1), *Alligator sinensis* (XP_006017705.1), *Danio rerio* (XP_005162770.1), *Oreochromis niloticus* (XP_005471911.1), *Takifugu rubripes* (XP_003968015.2), *Xiphophorus maculatus* (XP_005797954.1), *Astyanax mexicanus* (XP_007249048.1), *Oryzias latipes* (XP_004081095.1), *Larimichthys crocea* (XP_010736742.1), *Dicentrarchus labrax* (embICBN81948.1), *Cynoglossus semilaevis* (XP_008306029.1).

Figure 4

The mRNA expressions of *ctrp9* in various tissues of groupers. The mRNA levels were quantified by real-time PCR and normalized against 18S transcripts. The values represent the fold of telencephalon. All data were represented as the mean $\pm$ S.E.M. ($n=3$).

purity of recombinant gCTrp9 were verified by SDS-PAGE and stained by Coomassie Brilliant Blue R250, which exhibited a single protein band, and the purity was over 95% (Fig. 6C). To further confirm the authenticity of recombinant gCTrp9, mass spectrometry assays of gCTrp9 were performed to obtain sequence information. Sequences of Ctrp9 were introduced into MS BLAS program, which demonstrated that these sequences corresponded to gCTrp9 (see Supplementary Fig. 2).

Effects of gCTRP9 on glucose production in hepatocytes of grouper

As shown in Fig. 7, when the primary hepatocytes of groupers were treated with recombinant gCTrp9 of 10 μ g/mL, the glucose concentration in the culture medium showed a slight decrease after 6-h treatment. Furthermore, after 12-h treatment, the glucose production level decreased significantly (Fig. 7).

Effects of gCTrp9 on orexigenic/anorexigenic factors and metabolism via i.p. injection

During *in vivo* experiments, the recombinant gCTrp9 was injected intraperitoneally into groupers. The result showed that both low (0.5 μ g/g B.W.) and high doses (5 μ g/g B.W.) of gCTrp9 could significantly suppress the mRNA expression of *npv* and *orexin* 3 h after injection. Six hours after injection, high dose of gCTrp9 significantly reduced the mRNA expression of *npv* and *orexin*. Only *npv* mRNA expression was suppressed by a low dose of gCTrp9 (Fig. 8A and B). For anorexigenic factors, *crh* mRNA expression increased significantly after being treated with a high dose of gCTrp9 for 6 h and 12 h but was stimulated by the treatment with low dose for 6 h only. *Pomc* mRNA expression was remarkably promoted by both low and high doses of gCTrp9 12 h after injection (Fig. 8C and D). The serum concentrations of GLU, TG, HDL-C and LDL-C were different among different treatment groups. Twelve hours after injection, the concentrations of serum LDL-C was decreased in both low- and high-dose groups, whereas the concentrations of GLU was only decreased in the low-dose group (Fig. 8E and H). The concentration of TG was decreased in high-dose group but maintained at a low level in low-dose group throughout the 12-h treatment (Fig. 8F). On the contrary, the concentration of HDL-C was increased in the low-dose groups with 3-h and 12-h treatment (Fig. 8G).

Discussion

As CTRP9 was first reported in 2009 (Wong *et al.* 2009), the functions of CTRP9 were well investigated in mammals. As the paralog of adiponectin, the studies of CTRP9 were focused on fat metabolism, immunization and diabetes mellitus (Su *et al.* 2012, Peterson *et al.* 2013, Jung *et al.*

Figure 5

Effects of fasting and refeeding on the mRNA expressions of *ctrp9*. The mRNA expression levels of *ctrp9* in the (A) hypothalamus, (B) liver, (C) adipose tissue and (D) muscle of groupers were quantified by real-time PCR and normalized against 18S transcripts. The results were represented as the fold of fed. All data are shown as mean $\pm$ S.E.M. ($n=5-6$). Significant differences ($P<0.05$) were indicated by *.

Figure 6

gCtrp9 expression strain was selected and gCtrp9 protein was expressed. Positive strain was selected and the expressed protein was verified by SDS-PAGE and Western blotting with HIS primary antibody. (A) SDS-PAGE, (B) WB with HIS primary antibody and (C) gCtrp9 protein purified with Ni²⁺-NTA resin was verified by SDS-PAGE.

2014). To clarify the functional roles of Ctrp9 in teleost, firstly, we isolated and characterized the *ctrp9* gene from the orange-spotted grouper. The recombinant gCtrp9 protein was obtained by using *Pichia pastoris* system, and the role of Ctrp9 was evaluated *in vivo* and *in vitro*.

The size of human CTRP9 gene was 12.6kb. It contains four exons and was located on chromosome 13q12.12 (Wong *et al.* 2009). There were two isoforms of CTRP9 (CTRP9 A and CTRP9 B) in human and primates. Although the identity of two isoforms of CTRP9 was 98% in humans, they were encoded by different genes and had

Figure 7

Effect of gCtrp9 on glucose production of grouper primary hepatocytes. The cells were seeded in 24-well plates at 5×10^5 per well in 1 mL L15 with 10% FBS. After 4h, cells were incubated overnight in L15 without phenol red and FBS. The next day, experiments were performed by the addition of vehicle (PBS) or recombinant grouper gCtrp9 protein (10 μg/mL) for the indicated lengths of time. The medium was taken from each sample for the measurement of glucose concentration. Glucose concentration was corrected for protein content in each sample. All data are shown as mean $\pm$ S.E.M. ($n=5-6$). Significant differences at the $P<0.01$ level are indicated by **.

different functions (Peterson *et al.* 2009). One isoform of *ctrp9* gene was isolated and characterized in our study, and the protein structure of Ctrp9 was similar to that of mammals, containing four domains: an N-terminal signal peptide, a short variable domain, a collagen domain and a C-terminal globular domain. 57 Gly-X-Y repeat elements were identified in grouper Ctrp9 protein and a predicted N-glycosylation site was located on the collagen domain. Moreover, a number of phosphorylation sites were predicted in the grouper Ctrp9 protein. All of these were the vital sites in the multiple post-translational modifications. The modifications are indispensable processes in which prolines were hydroxylated and lysines were hydroxylated and glycosylated in the collagen domain (Wong *et al.* 2009). The Gly-X-Y repeat elements were the characteristic structures that formed a triple helical structure (Khoshnoodi *et al.* 2006). It was demonstrated that CTRP9 and adiponectin associated via their globular domain, and this process did not require their N-terminal cysteines or their collagen domains (Wong *et al.* 2009). Cysteines were distributed in the N-terminal domain, which was in line with the results of mammals (Wong *et al.* 2009). In the present study, the cysteines were found to be distributed in collagen and globular domains of grouper Ctrp9. Sequence alignment at the protein level reveals that the grouper Ctrp9 precursor displayed a high degree of identity with those of other fish. For example, the identity of Ctrp9 precursor between groupers with perches, large yellow croaker and tilapia are 88.1, 86.9 and 83.1%, respectively. Phylogenetic analysis based on amino acid sequences has confirmed that grouper Ctrp9 could be grouped in the clade of teleosts. Based on those analyses of amino acid sequences, the sequence we obtained is the prospective *ctrp9* of grouper.

Figure 8

Effects of i.p. injection of recombinant grouper gCtp9 on the mRNA expression of *npy* (A), *orexin* (B), *crh* (C), *pomc* (D) in the hypothalamus and the concentration of glucose (E), TG (F), HDL-C (G), LDL-C (H) in serum of grouper. Fish were injected intraperitoneally with vehicle (PBS) or recombinant gCtp9 (0.5 μg/g B.W., 5 μg/g B.W.) for the indicated duration of time (3, 6 or 12 h after i.p. injection). Fish were killed and the hypothalamus was collected for RNA extraction and RT-PCR. The mRNA expression was determined by real-time PCR. The 18S rRNA acts as an internal control. The GLU, TG, HDL-C and LDL-C concentrations were measured with commercial kits according to manufacturer's protocol. The data are presented as mean $\pm$ S.E.M. ($n=8-10$). The statistical differences were estimated using one-way ANOVA followed by Duncan (D)'s multiple range test (* $P<0.05$, ** $P<0.01$ and *** $P<0.001$).

Adiponectin was mainly expressed in adipose tissue of mammals (Arita *et al.* 1999). However, the highest mRNA expression of *adiponectin* was found in red muscle tissue of rainbow trout (Sanchez-Gurmaches *et al.* 2012). There were two isoforms of *adiponectin* in zebrafish. *Adiponectin A* was mainly expressed in kidney, whereas *adiponectin B* was primarily expressed in the liver and brain regions and slightly in adipose tissue (Nishio *et al.* 2008). Similarly, *Ctp9* mRNA was detected in adipose tissue, and the expression level of *Ctp9* mRNA in female mice was higher than that of male mice (Wong *et al.* 2009). However, recent research indicated that *Ctp9* mRNA was predominantly expressed in heart, at the level of 2.5-fold higher than that of adipose tissue (Yuan *et al.* 2015). Our study showed that *ctp9* mRNA was highly expressed in the cerebellum and kidney. As it is well documented that the major functions of fish kidney are osmotic stress regulation and excretion, we speculated that Ctp9 may play roles in regulating osmotic stress and excretion in groupers.

As the eukaryotic expression system, *P. pastoris* has multiple advantages over bacterial systems such as eukaryotic post-translational modifications (Thor *et al.* 2005, Terpe 2006). Li tried to use *P. pastoris* expression system to express the recombinant protein of human fCTRP2 (full-length CTRP2), but this recombinant hCTRP2 was found to be unstable and easily degraded in the strains of both X-33 and GS-115. This degradation phenomenon in *P. pastoris* was causally associated with the members of Yapsin family (Li *et al.* 2011). Several studies reported that

the members of Yapsin family can recognize not only the neighboring dibasic amino acid residues but also two basic amino acid residues separated by a given distance and degenerate them (Ledgerwood *et al.* 1996, Terpe 2006). In our study, several degradation sites that can be recognized by Yapsin protease were located on the collagen domain of grouper mature Ctp9 protein. The latest study showed that gCTRP9 was the main activated form (Yuan *et al.* 2015). For these reasons, *P. pastoris* expression system was used to produce the recombinant protein of gCtp9. We succeed to obtain a recombinant protein with the molecular weight of 15.8 kDa. The results of Western blotting and mass spectrometry confirmed that the protein we obtained is the globular domain of grouper Ctp9.

To evaluate whether Ctp9 is involved in the control of energy balance and food intake in grouper, the fasting and refeeding experiment was performed. In the fasting and refeeding experiment, expression levels of *ctp9* mRNA were detected in liver, muscle, hypothalamus and adipose tissue. Hypothalamus is an important area in the regulation of feeding. After fasting for 7 days, the *ctp9* mRNA expression was upregulated in the hypothalamus, implying that Ctp9 could involve in food intake in groupers. This result is consistent with that in mice, in that case, CTRP9 was found to be involved in food intake (Wei *et al.* 2014). As an anorexigenic factor, CTRP9 could regulate food intake and expression of orexigenic neuropeptides in hypothalamus (Wei *et al.* 2014). In the present study, *ctp9* mRNA expression was downregulated in the liver, muscle

and adipose tissue after fasting. As an important endocrine factor which protected from metabolic dysfunction (Peterson *et al.* 2013), CTRP9 acted as a regulator when the energy was unbalanced. In mammals, the liver can regulate the content of plasma glucose by storing and secreting actions (Moore *et al.* 2012). In the present study, the glucose production was reduced when hepatocytes were treated with gCtrp9 for 12h. This result was in agreement with *in vivo* studies of mammals in which glucose level was suppressed by CTRP9. Serum glucose levels were significantly lowered in *Ctrp9*-overexpressing mice (Wong *et al.* 2009, Li *et al.* 2015). *Ctrp9* transgenic mice had markedly reduced plasma glucose levels compared to that in littermate controls (Peterson *et al.* 2013). Moreover, adiponectin and CTRP3 reduced glucose production from hepatocytes by inhibiting gluconeogenesis (Pajvani & Scherer 2003, Peterson *et al.* 2010). These results, taken together, suggested that CTRP9 was involved in the regulation of energy balance.

To investigate the roles of gCtrp9 on the metabolism and regulation of mRNA expression of orexigenic/anorexigenic factors in groupers, i.p. injection experiment was performed. Our results showed that the mRNA expression of orexigenic factors (*npv* and *orexin*) were decreased, but those of anorexigenic genes (*pomc* and *crh*) were increased. Previous study showed that the expression of *Npy* and *Agrp* were promoted in hypothalamus in *Ctrp9*-KO mice, but CTRP9 showed no effect on the gene expression of *Pomc* in mice (Wei *et al.* 2014). Similarly, intracerebroventricular injection of adiponectin, a conserved paralog of CTRP9, increased the expression of *Crh* (Qi *et al.* 2004). A study showed that *Ctrp9* transgenic mice are lean and resist HFD-induced obesity by decreasing food intake (Peterson *et al.* 2013). *Ctrp9*-KO mice are fat, which is due to increased food intake (Wei *et al.* 2014). Moreover, results from both mouse models implicated that CTRP9 was involved in food intake modulation. In the present study, mRNA expression of orexigenic factors was decreased, whereas the anorexigenic genes expression were increased when *Ctrp9* was introduced into groupers by i.p. injection. These results indicated that *Ctrp9* could regulate the expression of genes related food intake. The further study need to be performed to investigate whether *Ctrp9* directly regulate feeding in groupers. The results of serum measurement showed that the concentrations of glucose, TG and LDL-C were decreased after i.p. injection of gCtrp9. These results were similar to the study on *Ctrp9*-Tg mice in which the serum glucose and TG content were downregulated in comparison with control groups (Peterson *et al.* 2013). Adenovirus- or lentiviral-mediated overexpression of *Ctrp9* in mice also significantly lowered serum glucose levels

(Wong *et al.* 2009, Li *et al.* 2015). This result was consistent with that we got from the *in vitro* experiment. A previous study in mice demonstrated that CTRP9 could suppress HFD-induced TG accumulation (Jung *et al.* 2015). A study in 3T3-L1 adipocytes showed that TG content was decreased in gAd-treated adipocytes compared to that in untreated cells (Lazra *et al.* 2015). Furthermore, serum TG levels were significantly decreased in gAd-Tg mice (Yamauchi *et al.* 2003). These results suggested that CTRP9 or adiponectin could regulate the serum content of TG. HDL participated in the initiation of reverse cholesterol transport, which was the key antiatherogenic action (Lee-Rueckert *et al.* 2016). Studies have showed that the concentration of CTRP9 was positively associated with HDL-C in the serum of mammal (Jung *et al.* 2014, Wang *et al.* 2015b). In addition, a similar result reported that adiponectin was positively associated with HDL-C in human serum (Katsiki *et al.* 2011, Lubkowska *et al.* 2015). Interestingly, in our study, gCtrp9 was found to upregulate the content of HDL-C in the serum of groupers. Based on these results, we speculated that *Ctrp9* might play a role in cholesterol metabolic process by regulating the content of HDL-C. Our result showed that the LDL-C content was decreased in gCtrp9 injection groups. Similar to our findings, Wang's study suggested an inverse correlation between serum CTRP9 and LDL-C (Wang *et al.* 2015b). A study in human showed that adiponectin was negatively associated with LDL-C (Bansal *et al.* 2006). These results suggested that CTRP9 or adiponectin could regulate the serum content of LDL-C. Results of our *in vivo* study showed that effects of gCtrp9 on Glu, TG and LDL-C contents have no coincident trend at 3 or 6 h after injection. However, at the time point of 12h after injection, the contents of Glu, TG and LDL-C were decreased significantly. The possible reason could be that the effect of gCtrp9 on the change of contents of Glu, TG and LDL-C in serum is not stable after 3-h or 6-h injection. The effects become stabilized when gCtrp9 is acting on the fish 12h after injection. Overall, gCtrp9 could play a role in metabolism and involved in regulating the mRNA expression of orexigenic/anorexigenic factors in groupers.

Conclusions

In the present study, the full-length cDNA of grouper *ctrp9* was isolated and characterized. The mRNA expression of *ctrp9* could be detected at high levels in cerebellum and kidney of groupers. Using the recombinant protein of gCtrp9 produced from *Pichia pastoris*, we firstly found that Ctrp9 may take part in the regulation of energy metabolism

and food intake in teleost. Our further research will focus on exploring the underlying mechanism related to the Ctrp9 in the regulation of energy metabolism and food intake.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-16-0171>.

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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