Grouper (*Epinephelus coioides*) **CXCR4** is expressed in response to pathogens infection and early stage of development

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Abstract

Chemokine (C-X-C motif) receptor 4 (CXCR4) from orange-spotted grouper (*Epinephelus coioides*) was identified and characterized in this study. gCXCR4 shared common features in protein sequence and predicted structure of CXCR4 family. This suggested that gCXCR4 is a member of G protein-coupled receptors with seven transmembrane domains. The expression patterns revealed that gCXCR4 may play a key role in early development of grouper. Furthermore, overexpression of gCXCR4-GFP for 48 h had significant effects on the GF-1 cell viability. gCXCR4 protein was mainly expressed in the marginal zone of head kidney and on the surface of intestinal villi. gCXCR4 expression can be detected in all the examined tissues and significantly up-regulated in eye and brain, which are the main targets for nervous necrosis virus (NNV) infection and replication. gCXCR4 gene expression can be induced in the spleen and eye by lipopolysaccharide and NNV, respectively. Our data suggested that gCXCR4 may not only play a role in the early immune response to microbial infection but also restrain to the immune system and central nervous system.

Keywords: *Epinephelus coioides*; CXCR4; G-protein-coupled receptors; chemokine
1. Introduction

The orange-spotted grouper, *Epinephelus coioides*, is a commercially important fish that is widely farmed in tropical waters of many countries. Considerable economic losses have been sustained in grouper aquaculture due to the infection of grouper by piscine nodavirus, i.e. nervous necrosis virus (NNV). The virus causes viral nervous necrosis (VNN) on grouper hatchery larvae and juveniles, resulting in a high mortality rate (80-100%) (Kuo et al., 2011; Munday et al., 2002).

Chemokines are a group of small molecular weight (6–14 kDa) cytokines which play important roles not only in against microbial infection by guiding leukocyte migration but also in embryonic growth and development (Kim et al., 1999; Olson et al., 2002). Chemokines can be classified into four different kinds, CXC, CC, C and CX3C (Murphy et al., 2000), according to its cysteine motif in the N-terminal region. Chemokine receptors are a group of G protein-coupled receptors with seven transmembrane domains. Upon stimulation by chemokines, chemokine receptors trigger a series of intracellular signal transductions via interaction with the G proteins. To this day, different chemokine receptors have been found on various cells such as monocytes, T lymphocytes, B lymphocytes, natural killer cells, macrophages, endothelial cells and neuron cells in mammals (Horuk et al., 2009). To date, CXCR4 has been identified in several species including human (Federsppiel et al., 1993), mouse (Heesen et al., 1996) and dog (Tsuchida et al., 2007), but
less is known in fish (Daniels et al., 1999; Chong et al., 2001; Alabyev et al., 2000; Jia and Zhang, 2009), and no functional characterization of CXCR4 in orange-spotted grouper has been reported.

Chemokines and their receptors serve an important role in viral infections and among the chemokine receptors, CXCR4 is also a co-receptor for human immunodeficiency virus (HIV) entry into target cells (Feng et al., 1996; Berson et al., 1996). Nonetheless, CXCR4 is not only involved in the pathogenesis of viral infections but also plays a critical role in organogenesis and embryonic development-related vascularization, lymphopoiesis and myelopoiesis (Tachibana et al., 1998). CXCR4 deficiency produces a lethal phenotype and abnormal development of central nervous system, such as abnormal migration of granule cells and an altered location of Purkinje cells in mice malformed cerebellum (Ma et al., 1998). In fish, CXCR4 has been found in the early stage of zebrafish embryo, it can be detected in the lateral mesoderm and posterior midbrain (Chong et al., 2001), moreover the migration of lateral-line-primordium is impeded in CXCR4 homologue-mutated zebrafish and is completely absent in SDF-1a defective zebrafish (Valentin et al., 2007). In addition, CXCR4 plays a crucial role for tissue polarity (Haas and Gilmour, 2006). CXCR4 homologue deficiency leads to the random migration of cells and the loss of coordinated motility within the posterior lateral line primordium in zebrafish (Haas and Gilmour, 2006).

These observations indicate that CXCR4 is multifunctional and plays crucial roles in
embryonic growth development and hematopoiesis.

However, no functional characterization of grouper CXCR4 has been reported. Previously, a partial portion of grouper CXCR4 cDNA was identified by subtractive cDNA hybridization from healthy and NNV-infected groupers (Chen et al., 2010). In the present study, orange-spotted grouper CXCR4 (gCXCR4) was cloned and the expression profile that response to lipopolysaccharide (LPS) and NNV infection was investigated. In addition, we showed that cell proliferation was impeded after gCXCR4 overexpression for 48 h.
2. Materials and methods

2.1. Fish and challenge experiments

Fish weighing approximately 300g (150 days post-hatching) and different ages (1-40 days-old) of orange-spotted grouper (*E. coioides*) were collected from an indoor fish farm in Linyuan and maintained in 10L containers at 27 ± 1 °C. For challenge experiments, 15 300g-in-weight-fish were divided into five groups (n=3 per group) and challenged with 100 μl phosphate buffered saline (PBS) contained approximately 20 μg of purified *Escherichia coli* 0127:B8 LPS (Sigma-Aldrich, St. Louis, MO) per fish via intraperitoneal injection. Fish with 100 μl PBS injection was used as a control group. The fish were sacrificed and the spleens were collected at 0, 6, 24, 48 and 72 h post-injection. In the experiments of virus challenging, juvenile groupers (about 0.8 g in weight, 40–45 days post-hatching) were collected from Linyuan fish farms in southern Taiwan. Twelve juvenile groupers were divided into two groups, NNV infection group and control group. Each six fish were immersed into 500 ml of rearing water which contained 50 ml of a viral solution (10^6 TCID₅₀/0.1 ml) or saline for 2 h. The fish were transferred to a virus-free aquarium, which had been exposure to ultraviolet (UV) light for 24 h, and cultured at 28 °C. Real time PCR was then used to confirm the fish was infected by NNV after 72 h of challenging.

2.2. Total RNA extraction and cDNA synthesis

Eye or whole fish samples (n=3 per group) were used for total RNA extraction by
homogenized using a MagNALysis homogenizer (Roche, Basel, Switzerland) following the manufacture’s recommendations of TRIzol™ (Invitrogen) method. cDNA was synthesized with 2 μg RNA, 0.1 μM oligo dT primer, 12.5 μM dNTP (Bioman Scientific Co. Ltd., Taipei, Taiwan) and 50 units Molony Murine Leukemia Virus (MMLV) reverse transcriptase (Promega, Madison, WI) at 42 °C for 1 h.

RNA and cDNA were quantified using an Ultrospec 3300 Pro spectrophotometer (Amersham Biosciences, Piscataway, NJ, USA); nucleic acids were diluted using sheared salmon sperm DNA (5 ng mL⁻¹) as a carrier.

2.3 RNA gel electrophoresis

To confirm the integrity of RNA samples, the extracted RNA was evaluated by RNA electrophoresis. In brief, 2 μl RNA sample was mixed with 18 μl 1× reaction buffer (1× MOPS, 20 % formaldehyde and 50 % formamide), 2 µl of 10× formaldehyde gel loading buffer (50 % glycerol, 10 mM EDTA, pH8, 0.25% bromphenol blue and 0.25 % xylene cyanol) and was visualized by using ethidium bromide staining.

2.4. Rapid amplification of cDNA ends (RACE)

A 950 bp cDNA fragment obtained from our previous study by subtractive cDNA hybridization from healthy and NNV infected groupers (Chen et al., 2010). The sequence showed 75% similarity to CXCR4 from Psetta maxima by blasting (http://www.ebi.ac.uk/blastall/). Full length cDNA was obtained by 5’/3’ RACE which was
performed by using 5’/3’ RACE Kit. Gene-specific primers (Table 1) for 5’ and 3’ RACE were designed based on partial sequence of gCXCR4. For 5’ RACE, mRNA was transcribed by MMLV reverse transcriptase (Sigma-Aldrich) with primer gCXCR4-5SP1 and gCXCR4-5SP2 and gCXCR4-5SP3 were used for PCR and nested PCR. 3’ RACE was performed by using primers gCXCR4-3SP1 and gCXCR4-3SP2 for PCR and nested PCR, respectively. PCR condition was one cycle of 3 min at 95 °C, followed by 35 cycles each at 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 10 min. The primers gCXCR-F and gCXCR-R were used to amplify the gCXCR4 cDNA fragment.

2.5 Bioinformatic analyses of gCXCR4

The transmembrane domains, extracellular domains and cytoplasmic domains of CXCR4 were identified by the TMHMM Server 2.0 program (http://www.cbs.dtu.dk/services/TMHMM/). The protein sequences of different CXCR4 species were obtained from GenBank and were aligned using Vector NTI 10 software. A phylogenetic tree of CXCR4 was constructed by the neighbor-joining method using MEGA4.0. The reliability of the tree was established by bootstrap analysis, based on 1,000 bootstrap replications.

2.6 RT-PCR

The tissue distribution of gCXCR4 gene expressions was investigated by RT-PCR. Total RNA was extracted from different tissues of grouper such as eye, fin, gill, muscle, head...
kidney, heart, spleen, intestine and brain. PCR condition was one cycle of 3 min at 95 °C, followed by 35 cycles each at 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 10 min. To detect the expression of gCXCR4 in different developmental stages of grouper larvae, total RNA were prepared from pooled larvae that contained 20 fish fry in each group at 1, 2, 4, 6, 8, 10, 14, 18 and 20-days post hatch (dph), and 3 fish larvae were pooled at 24, 26, 28, 30, 32, 34, 38 and 40 dph.

2.6. Real Time-quantitative PCR

Real-time quantitative PCR was performed by StepOne™ real-time PCR system (Applied Biosystems, Foster City, CA, USA). 1μl of cDNA (from 100 ng RNA) was mixed with 12.5 μl 2×SYBR® Green Master Mix (Applied Biosystems) and 1 μl of each 10 μM specific primer (Table 1). The thermal profile for real-time PCR was 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s and a final stage at 95 °C for 15 s, 60 °C for 1 min, 95°C for 15 s. The results of real-time PCR were analyzed with StepOne Software v2.1.

2.7 Statistical analysis

The CT for gCXCR4 and β-actin were determined for each sample. β-actin was used as internal control. △CT (Differences between gCXCR4 and β-actin) was calculated to normalize the differences in the efficiency of reverse transcription reactions. The △CT for each sample was subtracted from the △CT of the calibrator, and the difference was
designated as the $\Delta \Delta C_T$ value. The relative expression level of gCXCR4 could be calculated by $2^{-\Delta \Delta C_T}$. All real-time PCR data were subjected to analysis of t-test and are presented as the mean ± S.E. of the relative mRNA expression. $P$-values of < 0.05 were considered significantly different.

2.8 Plasmid construction

To prepare anti-gCXCR4 antiserum, extracellular domain I and III of gCXCR4 were constructed into pET29b expression vector (Novagen, USA) by PCR using primers. Primers gCSCR4-EXI-F (BamHI) and gCSCR4-EXI-R (SalI) were used for extracellular domain I and gCXCR4-EXIII-F (SalI) and gCXCR4-EXIII-R (XhoI) were used for amplifying extracellular domain III (181-215 a.a.). This recombinant plasmid was named pET29b-gCXCR4-EXI-EXIII which can express a fusion protein of gCXCR4 extracellular domains I and III along with a 6×His tag.

The gCXCR4 overexpression vector, pcDNA3.1-gCXCR4-GFP, was constructed by PCR amplifying gCXCR4 using primers gCXCR-F and gCXCR4-GFP-R (Table 1) and the PCR products were then cloned into the pcDNA3.1-CT-GFP-TOPO expression vector (Invitrogen). The inserted DNA fragments of each clone were confirmed by sequencing (Mission Biotech Co., Ltd., Taipei, Taiwan).

2.9 Recombinant protein and anti-gCXCR4 antiserum preparation

The gCXCR4-EXI-EXIII-His recombinant protein was expressed by transforming
pET-29b-gCXCR4-EXI-EXIII into BL21(DE3) cells (Novagen) and induced by adding isopropyl-beta-D-thiogalactopyranoside (IPTG; MDBIO, Frederick, MD) to a final concentration of 0.1 mM. Protein purification was performed using a HisTrap HP 1 ml column (Amersham Biosciences, Piscataway, NJ). Antisera against gCXCR4 was obtained by immunizing (injection of 1 mg/ml protein which was mixed with Freund's complete adjuvant [Sigma-Aldrich] on days 1, 14 and 28) a New Zealand White rabbit (Taiwan Livestock Research Institute, Tainan, Taiwan) with recombinant gCXCR4-extracellular domain I-extracellular domain III fusion protein. The antiserum collected at day 0 (before treatment) was used as control. The blood samples were incubated at 37°C for 1 h and left overnight at 4°C. The supernatant (containing rabbit anti-gCXCR4 antiserum) was collected after centrifuging at 900 ×g for 10 min at 4 °C. The rabbit anti-gCXCR4 antiserum was stored at –20 °C.

2.10 Immunofluorescence staining

The head kidney and intestines were obtained from healthy groupers and treated with 30% sucrose at 4 °C overnight. The different tissue blocks were covered with an optimal cutting temperature compound (Tissue-Tek®; Sakura Finetek, Tokyo, Japan), and the samples were slowly placed into liquid nitrogen. The frozen tissue block was transferred into a cryotome cryostat and 5 μm-thick sections were cut. Each slide was fixed with 3% paraformaldehyde (Kanto Chemical, Tokyo, Japan) and incubated at room temperature for 30 min. The
samples were then washed with 1x PBST (0.1% Tween 20, 1x PBS) and blocked with 5% skim milk. Rabbit anti-gCXCR4 antisera (1:200 dilution) were added and subsequent Alexa Fluor® 594 goat anti-rabbit IgG (1:200 dilution) (H+L) (Invitrogen) secondary antibody was added. The nuclei were stained with Hoechst 33342 (Invitrogen) at room temperature for 20 min, then washed extensively with 1xPBS and mounted on a coverslip with mounting medium.

2.11 Cell proliferation assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT) assay was performed using Cell Proliferation Kit I (Roche) to analyze the effects of gCXCR4-GFP overexpression. 5×10^4 *Epinephelus coioides* fin cells (GF-1, BCRC 960094) were seeded in 24-well plate and grown in a humidified incubator operating at 28°C in an antibiotic-free L15 medium (Life Technologies, Carlsbad, CA, USA) supplemented with 5% v/v heat-inactivated fetal bovine serum (FBS) (Chen et al., 2008). After 24hr, when cells attached completely, pcDNA3.1-CT-GFP-CXCR4 or pcDNA3.1-CT-GFP vector was transfected using Lipofectamine 2000 (Invitrogen). Then 20μl of MTT labeling reagent was added to each well and cultured for 4 hours in incubator. To dissolve formazan crystals, 200μl solubilization solution was added to each well. Covered with tinfoil and agitate cells on orbital shaker for 10 min. Read absorbance at 570 nm.
3. Results

3.1. Characterization of full-length gCXCR4

Orange-spotted grouper CXCR4 (gCXCR4) contained an open reading frame (ORF) of 1,104 nucleotides encoding a 367 a.a. protein with a predicted molecular weight of 40.37 kDa (Fig. 1). The structure of the protein was predicted to have seven-transmembrane domains, four extracellular domains and four cytoplasmic domains. The DRY motif was found at the second intracellular loop. A conserved cysteine residue on each of the four extracellular domains located at positions Cys$^{33}$, Cys$^{118}$, Cys$^{198}$ and Cys$^{292}$. The C-terminal is a region rich in serine and threonine residues.

The results of different species CXCR4 alignment (Fig. 2) showed that gCXCR4 not only similar to fish (zebrafish [58%], carp [54%], rainbow trout [53%] and turbot [52%]) but also to mammalian (human [51%] and mouse [50%]) and the conserved regions appeared in seven putative transmembrane domains (Fig. 2). The sequence of extracellular domains showed very diverse in different species. gCXCR4 has 22.1%, 8.5%, 28.8%, 32.2%, 33.6% and 29.3% similarity to human, mice, common carp, zebrafish, rainbow trout and turbot, respectively (Table 2).

3.2. Phylogenetic analysis of gCXCR4

There are six groups of CXCRs on the phylogenetic tree. Interestingly, within the group of CXCR4, mammalian and fish origins of CXCR4s was clear separated except for gCXCR4
and *Petromyzon marinus* CXCR4 (Fig. 3). This indicated that gCXCR4 might be a common ancestor to other CXCR4 proteins.

### 3.3. *in vitro* and *in vivo* expression of gCXCR4

To clarify the role of gCXCR4 in grouper, the expression of gCXCR4 in different growth stages of grouper was measured by real-time PCR. *gCXCR4* can be detected in all examined fish samples (from 1dph to 40 dph). The expression levels of gCXCR4 was up regulated and fluctuated in the period 1-4 dph and 6-8 dph, the *gCXCR4* expressions are < 50. Two higher expression peaks (>100, *p* < 0.05) were observed at 18 dph and 38 dph (Fig. 4).

To evaluate the effect of gCXCR4 overexpression on cell proliferation, GF-1 cells were transfected by pcDNA3.1-gCXCR4-GFP or pcDNA3.1-GFP overexpression vector. The results showed that overexpression of gCXCR4-GFP for 12 h, 24 h, and 36 h had no significant effects on cell viability (Fig. 5) but significantly repressed after 48 h (*p* < 0.05) (Fig. 5) (n=5 per group).

### 3.4. Expression patterns of gCXCR4 on head kidney and intestine

gCXCR4 was mainly expressed in the head kidney (Fig. 6A) and on the surface of intestinal villi of intestine (Fig. 6B).

### 3.5 The expressions of gCXCR4 in different organs of grouper

gCXCR4 was highly expressed in eye, gill, brain and important immune organs such as
spleen and head kidney (Fig. 7). Higher levels of expression were detected in eye, gill, spleen, brain and head kidney tissues. Lower levels of expression were detected in fin, muscle and heart tissues. Barely any gCXCR4 transcript was detected in the intestine (Fig. 7B).

3.6 The expressions of gCXCR4 after LPS or NNV challenge

The expression level of gCXCR4 in spleen was significantly increased after 6 h post-injection of LPS ($p < 0.05$) and decreased at 24 h and 48 h post-injection of LPS ($p < 0.05$). At 72 h post-injection, gCXCR4 had returned to the base level as the control (Fig. 8A).

Forty-eight h after NNV infection, the juvenile groupers exhibited abnormal behaviors such as loss of equilibrium and spiral swimming pattern and NNV can be detected at 72 h. gCXCR4 expression was also up-regulated at the time point which was 72 h post-NNV infection in eyes ($p < 0.05$) (Fig. 8B).
4. Discussion

The chemokine system has an important role in the host immune response against microbial pathogens and provides a link between innate and adaptive immunity (Murphy et al., 2000). The similar structure of gCXCR4 to other species (Alabyev et al., 2000; Tsuchida et al., 2007; Jia and Zhang, 2009) contains seven transmembrane regions, four extracellular regions and four intracellular regions, and a conserved DRY motif (Fig. 2). The predicted function of the gCXCR4 DRY motif was supported by the results of amino acid sequence alignments of gCXCR4 and CXCR4 of other species, which function had been demonstrated as important to G protein coupling (Doranz et al., 1999). The transmembrane regions as well as the cysteine residue positions in the extracellular regions appear to be highly conserved in CXCR4 evolution (Federspiel et al., 1993; Heesen et al., 1996; Alabyev et al., 2000; Tsuchida et al., 2007; Jia and Zhang, 2009). The posttranslational modification, i.e. the tyrosine residues of the N terminus are sulfated in Golgi, of human CXCR4 plays a crucial role on the infective ability of HIV (Farzan et al., 2002). However, these tyrosine residues were not conserved in gCXCR4 (Figs.1 and 2), suggesting that the posttranslational modification of gCXCR4 N-terminus is different. In addition, many serine and threonine residues were identified in the C-terminus of gCXCR4 and might have the modifications, i.e. phosphorylated as a prerequisite of signal transfer (Berson et al., 1996), like other protein in CXCR4 family.
CXCR4 is expressed mainly in immune organs and central nervous system: thymus and spleen of mouse (Heesen et al., 1996), chicken bursa (Liang et al., 2001), primate (Macaca mulatta) brain (Federspriel et al., 1993) and cattle locus coeruleus, cerebellum and pons (Rimland et al., 1991). In grouper, gCXCR4 was highly expressed in NNV major target organs, such as eyes and brain, and major lymphoid organs, such as gill, spleen and head kidney. This also been reported in other fish species (Daniels et al., 1999; Jia and Zhang, 2009) in which CXCR4 expressed in central nervous system and immune system. Interestingly, the expression of gCXCR4 in eye other than the immune related organs or central nervous system has never been reported which raised the other possible function of gCXCR4. Accordingly the grouper major lymphoid organs such as spleen, head kidney, gill and mucosa-associated tissues appeared to be regions of gCXCR4 overproduction (Press and Evensen., 1999). Furthermore, CXC chemokine system originates from the central nervous system and may participate in central nervous system development (Huising et al., 2003).

SDF1/CXCR4 signaling plays a critical role in embryonic development and is essential for development of cardiovascular, central nervous system, bone marrow colonization and hematopoiesis in mice (Ma et al., 1998; Tachibana et al., 1998). In fish, CXCR4 has been found in the early stage of zebrafish embryo and related to tissue polarity (Chong et al., 2001; Haas and Gilmour, 2006). The gene expression of gCXCR4 was highly expressed in
the period day14-day20 and day34-day40 larva that is coincided with dorsal spine formation and pigmentation (Katsutoshi and Hiroshi, 2009). The results implied that chemokine system exist in early developmental stage and play a key role in grouper development.

Immunohistofluorescence staining suggested that the protein gCXCR4 is expressed in lymphoid organs (Fig. 6A) and mainly on the surface of intestinal villi. This may be due to eyes, gills and surface of intestinal villi are continuously exposed to an environment which may have potentially pathogenic microbes. LPS is an endotoxin constituent of the outer membrane of Gram-negative bacteria which can induce immune responses and inflammation (Raetz et al., 2008). In fish, it has been demonstrated that LPS can stimulate the proliferation of neutrophils, monocytes, B lymphocytes and macrophages in a response against LPS-induced inflammation (Swain et al., 2008). The results shown that LPS can up-regulate the expression of gCXCR4 in the spleen and this also been showed in head kidney and spleen of turbot after challenging with Vibrio harveyi (Jia et al., 2009).

gCXCR4 mRNA was up-regulated at 3 days post-infection and was significantly increased in the eyes (Fig. 8B), suggesting that gCXCR4 is not only involved in the response to bacteria invasion, but also have response to NNV infection. Although the eye has been known to express chemokine receptors, such as CXCR1 and CXCR2 in mammals (Goczalik et al., 2008), our detection of abundant gCXCR4 in the organs and significantly up-regulated in NNV-infected fish was unexpected. Interestingly, grouper eye is one of the
main organs for NNV replication (Munday et al., 2002) and the immune response of NNV infection involving macrophage-like cells and lymphocytes migrate to the eyes (Grotmol et al., 1997; Nilsen et al., 2001; Munday et al., 2002). Taking those results together, we hypothesized that the gCXCR4 expression is related to NNV infection and which may cause by the immune related cells migration. However, too much gCXCR4 in cell could result in significant growth obstruction which might due to the other functions of CXCR4 (Bleul et al., 1996; Ganju et al., 1998).

In summary, our data indicated that the expression of grouper CXCR4 is regulated by LPS or NNV challenge, and is expressed during embryogenesis, speculating its importance in both immune and early developmental stage. The characterization of gCXCR4 between a teleost fish and mammals has provided valuable information for future functional analysis of the gene.
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References


Legends of figures

Fig. 1. Nucleotide sequence and deduced amino acid sequence of the open reading frame of *Epinephelus coioides* cDNA. The bold letters represent the start codon (ATG) and the stop codon (TAG). The regions of seven-transmembrane domains are at amino acids 50–72, 85–104, 119–141, 161–180, 216–238, 259–281 and 301–323. The Genbank accession number of gCXCR4 is HQ185191.

Fig. 2. Protein alignment and analysis of gCXCR4 with homologues from other species. The other species used for comparison were *Homo sapiens* (GenBank accession number CAA12166), *Mus musculus* (GenBank accession number AAH31665), *Cyprinus carpio*, *Oncorhynchus mykiss* (GenBank accession number CAA04493) and *Psetta maxima* (GenBank accession number ABP48751). The black letters denote the consensus sequence of CXCR4 of the different species. The bars represent transmembrane region of gCXCR4 determined using the TMHMMM program in the ExPASy Proteomics Server database. The DRY motif is boxed. The asterisk represents conserved cysteine residue on each of the four extracellular domains that located at positions 33aa, 118aa, 198aa and 292aa.

Fig. 3. Phylogenetic analysis of CXCR4 protein family members. The amino acids of the different CXCR4 species obtained from the NCBI GenBank were aligned using ClustalW. The Neighbor-Joining tree was created by MEGA4.0 software with a bootstrap value of 1,000. Accession numbers of chemokine receptors amino acid sequences obtained from
GenBank were: *Homo sapiens* CXCR1 NP_000625; *Mus musculus* CXCR1 NP_839972; *Cyprinus carpio* CXCR1 BAA31458; *Takifugu rubripes* CXCR1 NP_001072110; *Homo sapiens* CXCR2 NP_001161770; *Mus musculus* CXCR2 NP_034039; *Bos taurus* CXCR2 ABC59060; *Homo sapiens* CXCR2 NP_001072110; *Mus musculus* CXCR2 NP_034039; *Bos taurus* CXCR2 ABC59060; *Homo sapiens* CXCR3 EAX05283; *Danio rerio* CXCR3a NP_001082899; *Cyprinus carpio* CXCR3 BAA31458; *Petromyzon marinus* CXCR4 AAO21209; *Epinephelus coioides* CXCR4 HQ185191; *Homo sapiens* CXCR4 CAA12166; *Mus musculus* CXCR4 AAH98322; *Bos taurus* CXCR4 NP_776726; *Sus scrofa* CXCR4 AAZ32767; *Cyprinus carpio* CXCR4 BAA32797; *Oncorhynchus mykiss* CXCR4 CAA04493; *Salmo salar* CXCR4 BT060355; *Danio rerio* CXCR4 AAF1756; *Ictalurus punctatus* CXCR4 ACS45337; *Acipenser ruthenus* CXCR4 CAB60252; *Xenopus laevis* CXCR4 AAI10722; *Oryzias latipes* CXCR4 ABC41565; *Psetta maxima* CXCR4 ABP48751; *Homo sapiens* CXCR5 AAI10353; *Mus musculus* CXCR5 AAH64059; *Ctenopharyngodon idella* CXCR5 ACZ06880; *Mus musculus* CXCR6 NP_109637; *Homo sapiens* CXCR6 NP_006555; *Bos taurus* CXCR6 NP_001014859; *Homo sapiens* CXCR7 NP_064707; *Mus musculus* CXCR7 NP_031748 and *Xenopus laevis* CXCR7 NP_001082236.

**Fig. 4.** Gene expression profile of gCXCR4 was examined in different development stages of *Epinephelus coioides*. The total RNA was isolated from different stages and gene expression of gCXCR4 was determined by real-time PCR.
Fig. 5. Effects of overexpression of gCXCR4 on GF-1 cell proliferation. The cells proliferation was quantified by measuring MTT absorbance at 570 nm. Vertical bars indicate the mean ± S.E (N=3). **p < 0.01. The Blank was the spontaneous proliferation of GF-1 cells without treating any plasmid; the GFP was the group transfected with the same backbone of the plasmid to the gCXCR4-GFP group without inserting the gCXCR4.

Fig. 6. Expression of gCXCR4 in (A) head kidney and (B) intestine of healthy grouper using immunohistofluorescence staining. (a) and (d): Nucleus was detected using Hoechst 33342 (blue). (b) Control experiments were carried out with control rabbit antiserum as the primary antiserum, and visualized with Alexa Fluor® 594 goat anti-rabbit IgG (H+L) (red). (e) The expression of gCXCR4 was detected using rabbit anti-gCXCR4 antiserum and visualized with Alexa Fluor® 594 goat anti-rabbit IgG (H+L). (c) Merged image from figures (a) and (b). (f) Merged image from figures (d) and (e). Bars = 1 mm (A) and 50 μm (B).

Fig. 7. Gene expression of gCXCR4 in different tissues. (A) RT-PCR and (B) real-time PCR analysis of gCXCR4 gene expression in different tissues including eye, fin, gill, muscle, head kidney, heart, spleen, intestine and brain of healthy adult grouper. β-actin amplification was used as an internal control. Vertical bars indicate the mean ± S.E (N=3). *p < 0.05.

Fig. 8. Expression level of gCXCR4 mRNA in grouper after challenge with LPS (A)
and NNV (B). (A) Relative expression level of gCXCR4 mRNA in spleen of grouper after challenge with LPS or PBS. (B) Analysis of expression of gCXCR4 gene in control or NNV infected juvenile grouper or eye of juvenile grouper groups by real-time RT-PCR. gCXCR4 mRNA levels (relative to β-actin mRNA) between different time were compared by the t-test. Vertical bars indicate the mean ± S.E (N=3). *p < 0.05.
Figure 1
Figure 2
Figure 3
Figure 4

Relative expression of gCXCR4 mRNA

Days post hatch
Figure 5
Figure 6
Figure 7
Figure 8
### Table 1. Primers used in this study

<table>
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<tr>
<td>gCXCR4-5SP1</td>
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Table 2. Protein sequences similarity* of CXCR4 from different species

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*the number was showed in percentage