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Characterization and functional analysis of grouper (*Epinephelus coioides*) MEK1 and MEK2

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ABSTRACT

MEK dual-specificity protein kinases are a group of mitogen-activated protein kinase kinases, which act as an integration point by transferring extracellular signals to the nucleus. To investigate the function of MEK in teleost fish, we cloned MEK1 and MEK2 cDNA sequences from the orange-spotted grouper (*Epinephelus coioides*). EcMEK1 and EcMEK2 shared 80% amino acid identity with each other. EcMEK1 had 89–99% amino acid identity with teleosts or mammals, whereas EcMEK2 shared 85–97% amino acid identity. The exon structures of the grouper MEK1/2 genes were conserved with zebrafish and human MEK1/2. Tissue distribution analysis showed that *EcMEK1* and *EcMEK2* had a similar expression pattern in grouper tissues and was mainly transcribe in systemic immune organs. Both EcMEK1 and EcMEK2 were distributed throughout the cytoplasm of transfected GS or HEK293T cells. Overexpression of EcMEK1 or EcMEK2 activated Activator protein 1 dependent luciferase. The phosphorylation levels of EcMEK1/2 and EcERK1/2 were significantly increased in head kidney leukocytes by stimulation with PMA treatment. The grouper MEK1/2-ERK1/2 axis was activated in *Cryptocaryon irritans* infection and showed an enhanced phosphorylation after immunization.

1. Introduction

Mitogen-activated protein kinase (MAPK) signaling pathways are responsible for the regulation of cell growth and differentiation and are activated by a variety of extracellular stimulations [1–3]. In mammals, there are at least four distinct groups of MAPK signaling cascades: the extracellular signal-regulated kinase (ERK), the c-Jun N-terminal kinases (JNK), the p38 MAP kinase, and the ERK5 groups [4–7]. The MAPKs are inactive in their base form and activated by the phosphorylation of a characteristic TxY (threonine-x-tyrosine) motif (TEY in ERK1/2, TPY in JNKs, TGY in p38, and TDY in ERK5) [8].

Dual-specificity protein kinases (MEK1 and MEK2), also known as dual-specificity mitogen-activated protein kinase kinases, are specific ERK1/2 activators [9]. Structurally, both MEK1 and MEK2 contain a dual-specificity protein kinase (PK) catalytic domain, which can phosphorylate and activate the specific TEY motif in ERK1/2 [10]. There are two regions between MEK1 and MEK2 that show reduced homology: (1) the N terminus region, which was reported to be an ERK-binding site, and (2) the proline-rich region, which is required for signaling complex formation and ERK activation [11–14].

In mammals, although MEK1 and MEK2 share almost 80% amino acid identity and 90% amino acid similarity, the functions of MEK1 and MEK2 are not the same. Only MEK1 but not MEK2 can form a signaling complex with RAS and c-RAF in NIH 3T3 fibroblasts, suggesting that the RAS signaling pathway preferentially activates MAPK via MEK1 [14]. Moreover, MEK1-deficiency in mice leads to embryonic lethality whereas MEK2 mutant mice are viable and fertile [15,16]. These studies also showed that MEK1 but not MEK2 is responsible for placenta development and suggests that MEK2 is functionally redundant. In addition, the MEK1/2-ERK1/2 signal pathway plays an important role in inflammation response by mediating the production of inflammatory factors such as interleukin (IL)-1β, IL-8, and tumor necrosis factor-α (TNF-α) [17,18].

In fish, although many MEK1/2 sequences have been released in public network databases, the functional analysis of fish MEK1/2 was mainly focused on zebrafish [19–22]. As a model organism, zebrafish is suitable to study the function of MEK1/2 in development. A recent study on *Clarias gariepinus* found that *Aeromonas hydrophila*-infected

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head kidney macrophages induced an increase in the phosphorylation level of MEK1/2-ERK1/2, and suggested that *A. hydrophila* infection mediated the cPKC-MEK1/2-ERK1/2-TNFa axis by the alteration of cytosolic calcium [23]. However, the activation of MEK1/2 was poorly illustrated in other fish infected with pathogens.

The MAPK signaling pathway plays a central role in B cell activation and inflammation response [24–28]. Our previous studies showed that the B cell antigen receptor signal pathway and early inflammation were involved in response to *Cryptocaryon irritans* infection [29–34]. Recently, we cloned the ERK1/2 cDNA sequences from grouper and analyzed their expression pattern in response to infection by *C. irritans* [35]. To further this research, we cloned the MEK1 and MEK2 cDNA sequences from the orange-spotted grouper (*Epinephelus coioides*) and analyzed the expression pattern of *EcMEK1* and *EcMEK2* in various tissues. Localization of EcMEK1/2 was determined and a luciferase reporter assay was performed. Grouper head kidney leukocytes (HKLs) treated with phorbol 12-myristate 13-acetate (PMA) showed an increase in the phosphorylation level of EcMEK1/2 and EcERK1/2. The phosphorylation level of MEK1/2 and ERK1/2 could be induced by *C. irritans* infection, suggesting grouper MEK-ERK was activated in the immune response to parasitic infection.

2. Materials and methods

2.1. Fish

Healthy orange-spotted groupers (15.4 ± 1.2 g) were purchased from the Marine Fisheries Development Center of Guangdong Province, Guangdong, China, and maintained at 24 °C in a flow-through water system (300 L) as described previously [36]. Samples of the thymus, gill, head kidney, skin, muscle, liver, spleen, fore intestine, mid intestine, hind intestine, stomach, trunk kidney, heart, and brain tissues were taken from the groupers for tissue distribution analysis. All samples were immediately frozen in liquid nitrogen and stored at −80 °C.

2.2. C. irritans infection and grouper immunization

C. irritans were originally isolated from an infected *Trachinotus ovatus* obtained from a local farm in Daya Bay, Guangdong Province, China, and cultured using *T. ovatus* as a host, as described previously [33] with minor modification. Immune groupers (n = 30) were prepared by exposure to living *C. irritans* theronts every 2 weeks at a dose of 4000 theronts per fish in the dark for 2 h and then transferred into a clean tank every 3 days to avoid secondary infection. The uninfected fish were also transferred. Six weeks after the first immunization, both the uninfected groupers and immune groupers were challenged with living *C. irritans* theronts at a dose of 12,000 theronts per fish. After anesthesia with MS-222, the skin near the dorsal fin of each group was sampled at 3 days post-challenge. Meanwhile, skin samples from control groupers were collected as negative controls.

2.3. RNA extraction and cDNA synthesis

Total RNA from healthy grouper samples was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). cDNA for expression analysis was synthesized from total RNA from all collected samples using a ReverTra Ace-a-Kit (Toyobo, Katata, Ohtsu, Japan). cDNA for obtaining the full-length grouper MEK1 and MEK2 was synthesized from total RNA of healthy grouper spleen using a SMARTer™ RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA).

2.4. Cloning of gene sequences

Expressed sequence tag (EST) sequences of MEK1 and MEK2 were identified from grouper transcriptome data using the BLASTx program [32]. However, both MEK1 and MEK2 were short of their 5′ regions. To obtain the 5′ unknown regions of MEK1 and MEK2, the primers MEK1 5′GSP1/UPM and MEK1 5′GSP2/NUP, MEK2 5′GSP1/UPM and MEK2 5′GSP2/NUP (Table 1) were designed for primary and nested PCR to obtain the full-length sequence of MEK1 and MEK2 from groupers. cDNA was synthesized from healthy grouper spleen total RNA with the SMARTer™ RACE cDNA Amplification Kit (Clontech) following the manufacturer's protocol. The amplification protocol was performed as follows: (98 °C, 10 s; 65 °C, 15 s; 72 °C, 2 min) × 35 cycles, and one cycle of 72 °C for 5 min. The open reading frame (ORF) of MEK1 and MEK2 was amplified using the primers MEK1 ORF F/R and MEK2 ORF F/R (Table 1). The amplification protocol was performed as follows: (98 °C, 10 s; 59 °C, 15 s; 72 °C, 2 min) × 35 cycles, and one cycle of 72 °C for 5 min. The amplification products were purified and ligated into pEASY-Blunt Cloning Vector (TRANS, Beijing, China) for sequencing.

Table 1
Primers used in this study.

Primer	Sequence (5′ to 3′)
MEK1 5'GSP1	CCTGGTCTCCCAGGGGGCCGTGG
MEK1 5'GSP2	ACAGATCTTTTCAAAGTCATCG
MEK2 5'GSP1	CAGTGCCGCGGGTCGGAGTACTG
MEK2 5'GSP2	TCTTCTGGGATTCTTCTGGCTTCC
MEK1 ORF F	CAGCCCGTGTGTTTCTACAGCAGCA
MEK1 ORF R	CCCATAATACAAACCCAGTCACCAG
MEK2 ORF F	CATTAGCAGCTTCCTAGCTCA
MEK2 ORF R	TTATGTGTATGCAAGCAAGACC
MEK1 RT F	AGAAGAGAAGGAAGCCAGAGCC
MEK1 RT R	TCCCACCTTCTGCTTCTGTGTCAGG
MEK2 RT F	CTGCTAAAAAAAGACCCGTTCCC
MEK2 RT R	CCCAACCTGAGCTTTCTGGGTGAGA
MEK1 EGFP F	tcgagctcaagcttcgaattcATGCAGAAGAGAAGGAAGCCAG
MEK1 EGFP R1	ggatcccgggcccgcggtaccGTCATTGCTGTGCCATGGGT
MEK1 EGFP R2	ggatcccgggcccgcggtaccTCACATTGCTGTGCCATGGGT
MEK2 EGFP F	tcgagctcaagcttcgaattcATGCCTGCTAAAAAAAGACCCG
MEK2 EGFP R1	ggatcccgggcccgcggtaccGTCTCAGTGCCGCGGGTC
MEK2 EGFP R2	ggatcccgggcccgcggtaccTCACTCAGTGCCGCGGGTC
UPM	Long: CTAATACGACTCACTATAGGGCAAG CAGTGGTATCAACGCAGAGT Short: CTAATACGACTCACTATAGGGC
NUP	AAGCAGTGGTATCAACGCAGAGT
β-actin F	TGCTGTCCCTGTATGCCTCT
β-actin R	CCTTGATGTCACGCACGAT

2.5. Gene structure and phylogenetic analysis

The ORF of EcMEK1 and EcMEK2 were searched using ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/). The theoretical isoelectric point (pI) and molecular weight (Mw) of EcMEK1 and EcMEK2 were predicted using the Compute pI/Mw tool (http://web.expasy.org/compute_pi/). Conserved domains were searched in the CDD tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The gene information of human and zebrafish MEK1/2 was downloaded from NCBI, and the gene sequences of grouper MEK1 and MEK2 were kindly provided by Dr. Zhao Mi (unpublished genome data). Alignment and phylogenic analysis of EcMEK1 and EcMEK2 were performed using the MEGA 5.04 program. All GeneBank accession numbers used in this study are listed in Supplementary Table 1.

2.6. Tissue distribution analysis

The expression level of EcMEK1 and EcMEK2 in various tissues was determined using the SYBR Green Real-time PCR Master Mix (Toyobo) as previously described [29]. Gene-specific primers of EcMEK1 and EcMEK2 (MEK1 RTF/R and MEK2 RTF/R in Table 1) were used in real-time PCR. β-actin primers (β-actin F/β-actin R) were used to amplify the reference gene. The cycling protocol was: 94 °C for 2 min and (94 °C, 15 s; 58 °C, 15 s; 72 °C, 20 s) × 40 cycles. Melting curve analysis and

sequencing were used to detect the specificity of PCR products. The PCR products were verified by sequencing. All samples were analyzed in triplicate. The expression of the target gene was normalized to the β-actin gene and calculated with the $2^{-\Delta\Delta Ct}$ method [37].

2.7. Subcellular localization

Plasmids (pEGFP-EcMEK1-SL and pEGFP-EcMEK2-SL) used for subcellular localization were constructed by amplification with primers MEK1 EGFP F/R1 and MEK2 EGFP F/R1 (Table 1), which contain 20-bp end sequences identical to pEGFP-N1 at the 5′-termini, respectively. pEGFP-N1 were double-digested using EcoRI and KpnI to generate linearized pEGFP-N1. The PCR product was mixed with linearized pEGFP-N1 and ligated using a ClonExpress Ultra One Step Cloning Kit (Vazyme, China) following the manufacturer's protocol. The pEGFP-EcMEK1-SL and pEGFP-EcMEK2-SL plasmids were extracted using an E.Z.N.A. Endo-free Plasmid Mini Kit (Promega, Madison, WI, USA) for subsequent transformation.

Culture and transfection of GS and HEK293T cells were performed as previously described [30,38] with some modifications. Briefly, GS cells were cultured in Leibovitz's L-15 medium containing 10% fetal bovine serum at 27 °C. HEK293T cells were cultured in Dulbecco's Minimum Essential Medium containing 10% fetal bovine serum at 37 °C under 5% (v/v) CO_2. At 90% confluence, pEGFP-EcMEK1-SL, pEGFP-EcMEK2-SL, or pEGFP-N1 Endo-free plasmid (1 μg for each plasmid) were transfected into GS or HEK293T cells using Lipofectamine™ 2000 Reagent (Invitrogen). Twenty-four hours after transfection, the cells were fixed with 4% paraformaldehyde (Beyotime) for 15 min and then 1 mg/mL of 4′, 6-diamidino-2-phenylindole (DAPI) was added followed by a further 5 min incubation. The intracellular localization was observed and photographed used an NIH-Elements System (Nikon, Tokyo, Japan).

2.8. Luciferase reporter assay

Plasmids (pEGFP-EcMEK1-LR and pEGFP-EcMEK2-LR) used for the luciferase reporter assay were constructed by amplification with primers MEK1 EGFP F/R2 and MEK2 EGFP F/R2. The luciferase reporter assay was performed as described previously [30]. In brief, the pEGFP-EcMEK1-LR (150 ng) and pEGFP-EcMEK2-LR plasmids (150 ng), and pEGFP-N1 (150 ng) were co-transfected with 40 ng of Activator protein 1 (AP-1) dependent firefly luciferase reporter vector and 10 ng of Renilla luciferase vector. Twenty-four hours after transfection, the cells were collected and lysed for dual-luciferase reporter assay analysis according to the manual. The relative luciferase activity was measured by the ratio between firefly luciferase activity and Renilla luciferase activity. All data were analyzed using SPSS 16.0 (SPSS, Chicago, IL, USA) and expressed as mean ± SE.

2.9. Cells preparation and in vitro stimulation

Grouper HKLs were prepared as described [29] with some modification. After anesthesia with MS-222, the head kidney was isolated and gently sieved through a 70-mm nylon mesh filter. After 5 min incubation, suspensions were filtrated again to remove cell masses and overlaid on Ficoll-Paque PLUS (1.077 g/mL; GE Healthcare) with centrifugation at 2500 rpm for 25 min at 20 °C. The leukocyte layer was collected and washed twice with PBS then cultured in L-15 (Gibco) at 28 °C. HKLs were treated with DMSO, 100 nM PMA (Selleck) or 200 nM PMA for 15 min at 28 °C and washed with PBS. Cells lysates were prepared and western blotting analysis was performed as described below.

2.10. Western blotting

Total proteins of HKLs or skin were extracted using RIPA lysis buffer (Beyotime) by adding an extra 1 mM phenylmethanesulfonyl fluoride (Beyotime) and 1 mM pervanadate (Sigma). The supernatant was mixed with SDS sample buffer and boiled for 10 min. Protein samples were electrophoresed and transferred to polyvinylidene fluoride (PVDF) membranes as described previously [29]. The PVDF membranes were blocked in 5% dried milk (diluted with PBST) for 1 h at room temperature, followed by incubation with rabbit anti-MEK1/2 IgG (1:1000 dilution, CST), rabbit anti-pMEK1/2 IgG (1:1000 dilution, CST), rabbit anti-ERK1/2 IgG (1:1000 dilution, CST), rabbit anti-pERK1/2 IgG (1:1000 dilution, CST) or mouse anti-actin IgG (1:1000 dilution, Proteintech) overnight at 4 °C with gentle shaking. Membranes were washed with PBST three times and incubated with goat anti-rabbit IgG antibodies conjugated to horseradish peroxidase (HRP) or goat anti-mouse IgG antibodies conjugated to HRP for 1 h at room temperature. Membranes were washed in PBST three times and incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo), then exposed and analyzed using the Tanon 5200 chemiluminescence imaging analysis system (Tanon).

3. Results

3.1. Characteristics of EcMEK1/2 and phylogenetic analysis

The ORFs of EcMEK1 (GenBank no. MH685929) and EcMEK2 (GenBank no. MH685930) were 1188 and 1200 bp in length, which encoded 395 and 399 amino acids with a theoretical pI of 6.19 and 6.16, and molecular mass of 43.85 and 44.24 kDa, respectively. Both EcMEK1 and EcMEK2 sequences contained a PKc_MEK domain and a proline-rich region (Fig. 1). EcMEK1 and EcMEK2 shared 80% amino acid identity with each other. Homology analysis showed that EcMEK1 had 89%–99% amino acid identity with teleosts or mammals, while EcMEK2 shared 85%–97% amino acid identity. Both EcMEK1 and EcMEK2 shared the highest amino acid identity with the Asian seabass *Lates calcarifer* MEK1 or MEK2 (Table 2). Similar to the human and zebrafish MEK1/2 genes, grouper MEK1 and MEK2 had conserved exon structures (Fig. 2). Both grouper MEK1 and MEK2 genes contained 11 exons. The exons sizes of the grouper MEK1 gene were 83, 211, 147, 80, 50, 125, 208, 65, 59, 46, and114 bp, while those of the grouper MEK2 gene were 89, 211, 147, 82, 48, 125, 214, 65, 62, 46, and 111 bp. The phylogenetic tree indicated the formation of eight clades, which represent Mammalia MEK1, Aves MEK1, Reptilia MEK1, Teleostei MEK1, Mammalia MEK2, Aves MEK2, Reptilia MEK2, and Teleostei MEK2, of which EcMEK1/2 shared the closest homology with the Asian seabass (Fig. 3).

3.2. Tissue distribution of EcMEK1/2 in healthy grouper

EcMEK1 and *EcMEK2* had a similar expression pattern in grouper tissues. Our results showed that *EcMEK1/2* were transcribed in all tissues tested and *EcMEK1* showed the highest transcript levels in head kidney, followed by spleen, gill, stomach, trunk kidney, fore intestine, skin, heart, hind intestine, thymus, mid intestine, brain, muscle, and liver. *EcMEK2* showed the highest transcript levels in spleen, followed by head kidney, gill, brain, thymus, mid intestine, heart, muscle, fore intestine, skin, stomach, trunk kidney, hind intestine, and liver (Fig. 4). Overall, *EcMEK1/2* was mainly expressed in systemic immune organs.

3.3. Subcellular localization and luciferase reporter assay

Plasmids (pEGFP-EcMEK1-SL and pEGFP-EcMEK2-SL) used for subcellular localization were successfully constructed and transfected into grouper GS cells or human HEK293T cell. Green fluorescence was observed. The results showed that both EcMEK1 and EcMEK2 were distributed throughout the cytoplasm of transfected GS (Fig. 5A) or HEK293T cells (Fig. 5B), while fluorescence was distributed throughout the cytoplasm and nucleus in the mock-transfected cells. To study the function of EcMEK1 and EcMEK2 in the activation of AP1, two plasmids

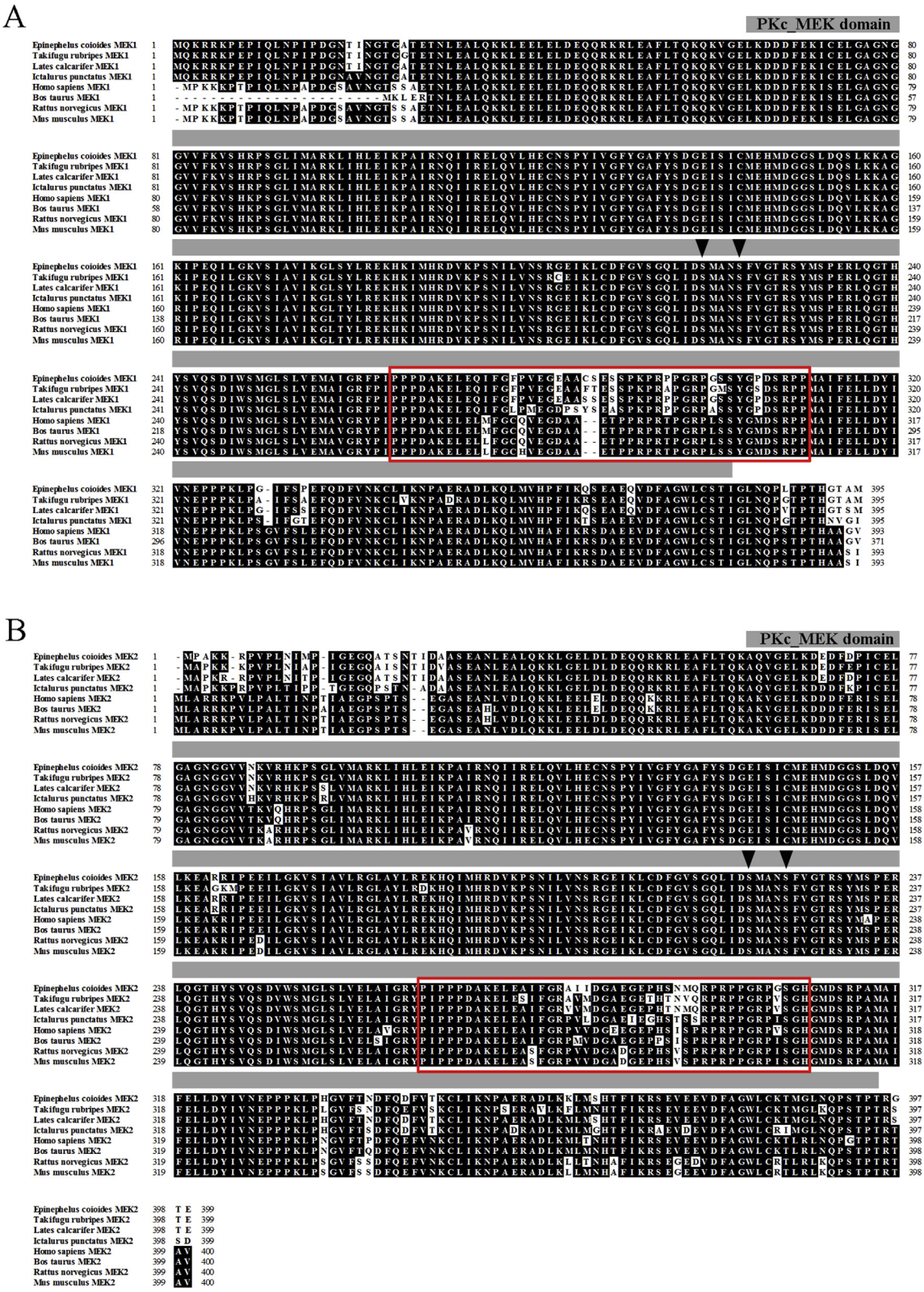

Fig. 1. Alignment of MEK1 (A) and MEK2 (B). Gaps, show as dashes (——), had been introduced to maximize the alignment to the sequences. Residues identical in all the sequences were shaded by black. The Pkc_MEK domain was indicated by black letters on a grey background. The conserved serine residues were indicated by triangle. The proline-rich region was indicated by red box. All Gene Bank accession numbers used in this study are listed in Supplementary Table 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Amino acid identities (%) between EcMEK1/2 with other vertebrates.

	EcMEK1	EcMEK2
Lates calcarifer	99	97
Takifugu rubripes	97	93
Ictalurus punctatus	95	91
Rattus norvegicus	89	85
Mus musculus	89	86
Bos Taurus	91	87
Homo sapiens	89	86

pEGFP-EcMEK1-LR and pEGFP-EcMEK2-LR were constructed and dual-luciferase reporter assays were performed. Compared with mock-transfected cells, both EcMEK1 and EcMEK2 activated AP1, and EcMEK2 showed an enhanced activity (Fig. 6).

3.4. PMA treatment increases the phosphorylation level of grouper MEK1/2 and ERK1/2

HKLs were isolated and treated with PMA for 15 min and cell lysates were probed with either anti-MEK1/2, anti-pMEK1/2, anti-ERK1/2, anti-pERK1/2 or anti-actin antibodies. The phosphorylation level of EcMEK1/2 and EcERK1/2 were increased in HKLs at a concentration of 100 nM or 200 nM PMA compared to the vehicle (Fig. 7). This suggested that the grouper MEK1/2-ERK1/2 axis could be triggered by PMA stimulation.

3.5. Activation of MEK-ERK post-C. irritans infection

To study whether grouper MEK-ERK was activated in *C. irritans* infection, immune groupers were prepared by exposure to a low dose of living *C. irritans* (4000 theronts per fish) for three times and then challenged with 12,000 theronts per fish. Skin was collected from the immune groupers, infected groupers (challenged with 12,000 theronts per fish for the first time), and negative control groupers, and the total protein level and phosphorylation level of MEK1/2 and ERK1/2 were detected. The phosphorylation level of MEK1/2 and ERK1/2 were significantly upregulated in the infected skin and immune skin, while the immune skin showed an enhanced signal (Fig. 8). The total protein level of ERK1/2 was upregulated in the infected skin and immune skin, while MEK1/2 was only upregulated in the immune skin but not in the infected skin. Taken together, the results indicated that infection of *C. irritans* activated MEK1/2 and ERK1/2 in grouper skin.

4. Discussion

In this study, we obtained MEK1 and MEK2 cDNA sequences from orange-spotted grouper. EcMEK1 and EcMEK2 share a very high amino acid identity with other vertebrate MEK1/2 molecules and both sequences had the highest amino acid identity with Asian seabass. EcMEK1 and EcMEK2 share 80% amino acid identity with each other, which is also found in mammals [10]. The exon structure of grouper MEK1 and MEK2 is conserved between zebrafish and humans. In addition, as in mammals, there are two reduced homology regions between EcMEK1 and EcMEK2: an N terminus region and a proline-rich region, which is required for the ERK-binding site and signaling complex formation, respectively [11–14]. This suggests that fish MEK1/2 may have a similar function to that in mammals.

Although some studies involving MEK1/2 have been done in fish [22,23], the expression pattern of MEK1/2 in fish tissue is rarely reported. To further understand the tissue distribution of EcMEK1 and EcMEK2, two pairs of gene-specific primers were designed from the 5′ region of EcMEK1 and EcMEK2 ORF sequences, and the transcript level of *EcMEK1* and *EcMEK2* was detected in various tissues. Our results showed that EcMEK1 and EcMEK2 had a similar expression pattern in grouper tissues, and transcripts were mainly in the systemic immune organs: head kidney and spleen, suggesting that EcMEK1 and EcMEK2 may play an important role in hematopoietic organs.

MEK-ERK is a classic MAPK axis which transfers extracellular signals and regulates downstream AP1 activation [39]. AP1 is a transcription factor that regulates various inflammatory cytokines such as TNF-α and IL-1β [40]. To study the function of EcMEK1 and EcMEK2 in the activation of AP1, EcMEK1 and EcMEK2 were overexpressed in GS cells and the luciferase activity of an AP1 dependent firefly luciferase reporter was detected. Both EcMEK1 and EcMEK2 have an increased luciferase activity compared with mock-transfected cells, and EcMEK2 showed an enhanced activity. Because the functions of MEK1 and MEK2 are not the same in mammals [15,16], the discrepancy of EcMEK1 and EcMEK2 in the activation of AP1 seemed reasonable.

As an evolutionarily conserved signaling module, MEK1/2 were also identified in teleosts. Studies on zebrafish MEK1/2 revealed a potential role in notochord development by treatment with a MEK1/2 inhibitor U0126 [22]. *C. gariepinus* head kidney macrophages infected with *A. hydrophila* showed an increased level of phospho-MEK1/2 [23]. However, there is little information on fish MEK1/2 in response to a stimulator. PMA, also known as12-O-tetradecanoylphorbol-13-acetate, is a potent tumor promoter and widely used to activate protein kinase C and the ERK pathway [41,42]. To study whether PMA can activate grouper MEK1/2 or ERK1/2 *in vitro*, we isolated grouper HKLs and stimulated them with 100 nM or 200 nM PMA. Compared to the control, both 100 nM and 200 nM PMA treatment were able to induce phosphorylation in EcMEK1/2 as well as EcERK1/2. This indicated that grouper have an integrity MEK-ERK axis that could be triggered by PMA stimulation. This result can be used to find MEK-ERK inhibitors in further studies.

It has been demonstrated that the production of inflammatory factors IL-1β and IL-8 is regulated by the ERK pathway [17]. Our previous reports showed that grouper IL-1β, IL-8, and ERK1/2 were significantly upregulated in skin infected by *C. irritans* [33,35,43]. In addition, macrophages may be involved in the inflammation response to *C. irritans* infection [32,34]. In this study, we provided further evidence that grouper pMEK1/2 and pERK1/2 levels significantly increased in day 3 of *C. irritans* infection. Furthermore, the immune groupers showed a

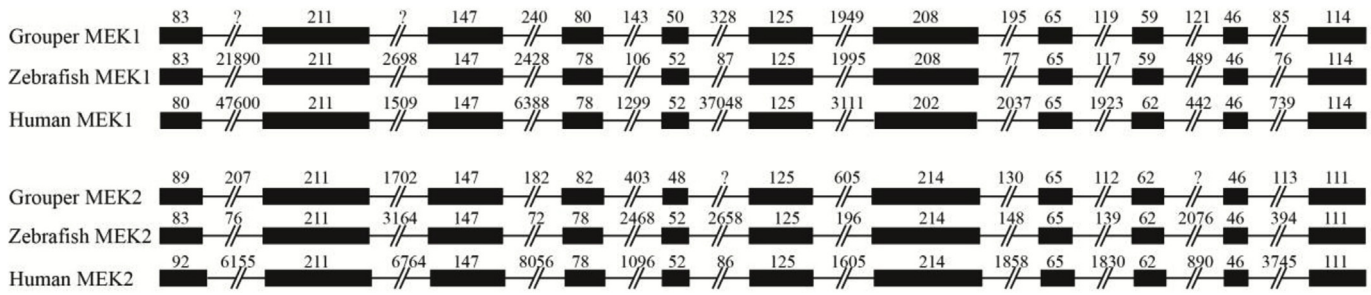

Fig. 2. Genomic structure of MEK in grouper, zebrafish and human. The boxes and lines between each box indicated exons and introns, respectively, and the numbers indicated the sizes (bp) of each exon and intron. ? represents unknown intron sizes.

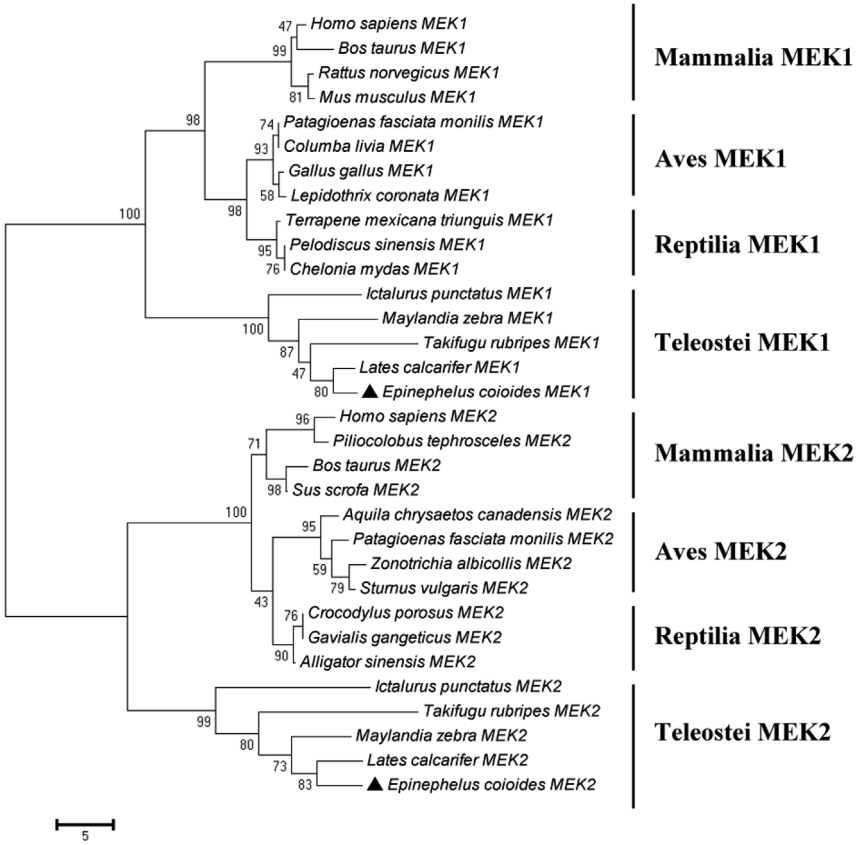

Fig. 3. A phylogenetic tree illustrating the relationship across vertebrate MEK molecules using the neighbor-joining method within the MEGA program. Node values represent percent bootstrap confidence derived from 10,000 replicates. The scale bar is 5.

stronger pMEK1/2 signal than the first infection groupers, which indicated that an immunological memory was generated in immune groupers. The correlation between *C. irritans*-induced IL-1β, IL-8 production, and MEK-ERK activation suggested a MEK-ERK-IL-1β/IL-8 axis may be involved in host anti-*C. irritans* inflammatory response.

To conclude, we cloned the MEK1 and MEK2 cDNA sequences from orange-spotted grouper and found that both *EcMEK1* and *EcMEK2* were mainly transcribed in hematopoietic organs. Overexpression of EcMEK1 and EcMEK2 could activate AP1. PMA treatment increased the phosphorylation levels of EcMEK1/2 and EcERK1/2. Moreover, the EcMEK1/2-EcERK1/2 axis was activated in *C. irritans* infection and showed an enhanced phosphorylation post-immunization. Our future work will explore the upstream molecular events of the MEK-ERK signal pathway and these subsequent studies will help to further understand MAPK pathway activation in fish during parasitic infection.

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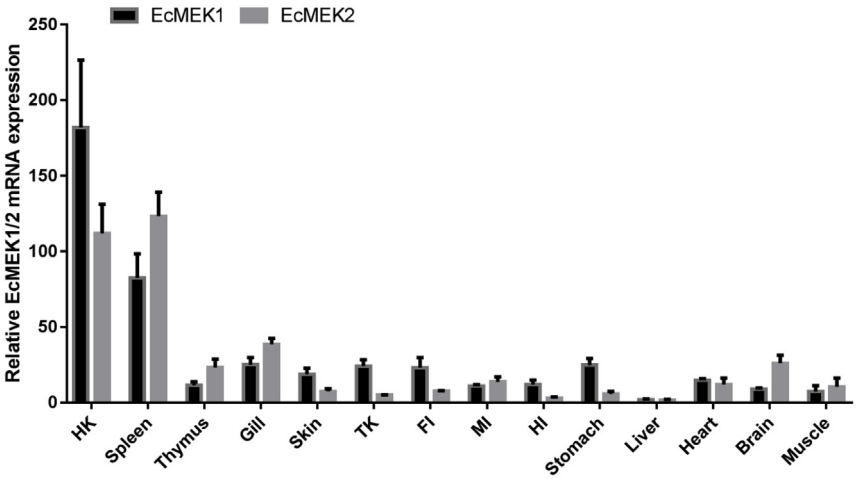

Fig. 4. The expression pattern of *EcMEK1* and *EcMEK2* in healthy grouper tissues. TK: trunk kidney; HK: head kidney; FI: fore intestine; MI: mid intestine; HI: hind intestine. All data are presented as Mean ± SE, n = 3.

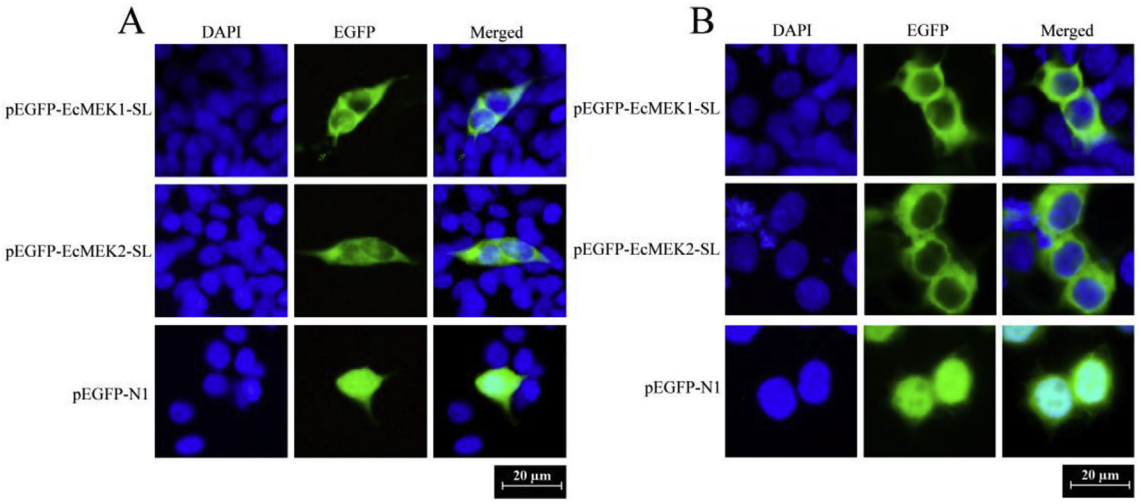

Fig. 5. Subcellular localization of EcMEK1 and EcMEK2 in GS cells (A) or HEK293T cells (B). The ORF of EcMEK1 and EcMEK2 were ligated to pEGFP-N1 translate into GS cells or HEK293T cells (400 ×).

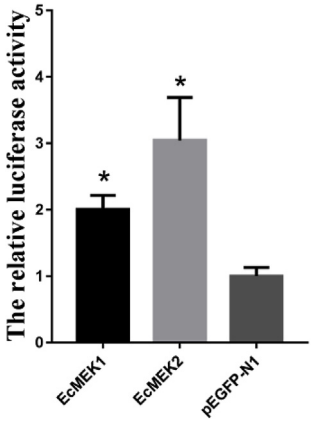

Fig. 6. Detection of EcMEK1 and EcMEK2 in AP1 activity. The relative luciferase activity was calculated as the ratio of firefly to Renilla luciferase activities. Data are the fold changes relative to GS cells transfected with pEGFP-N1. All data are presented as Mean ± SE, n = 3. Significant change is indicated with * ($p < 0.05$).

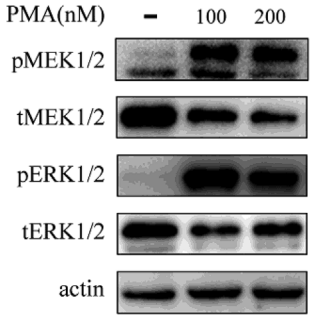

Fig. 7. Western blot analysis of PMA treatment. Grouper HKLs were treated with 100 nM or 200 nM PMA for 15 min and cells lysates were probed with the indicated antibodies. pMEK1/2, phosphorylated MEK1/2; tMEK1/2, total MEK1/2; pERK1/2 phosphorylated ERK1/2; tERK1/2, total ERK1/2.

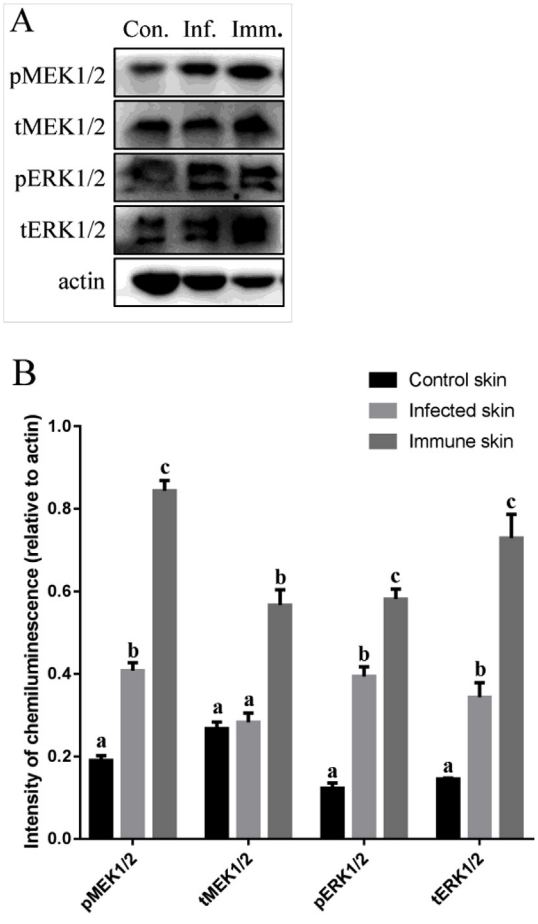

Fig. 8. Western blot analysis of MEK-ERK expression in *C. irritans* infection. Detection of pMEK1/2 (phosphorylated MEK1/2), tMEK1/2 (total MEK1/2), pERK1/2 (phosphorylated ERK1/2) and tERK1/2 (total ERK1/2) in *C. irritans* infected or immune skin using the indicated antibodies, Con.: control skin, Inf.: infected skin; Imm.: immune skin (A). The phosphorylated and total MEK or ERK were measured by densitometric analysis of immunoblots and presented relative to values of β-actin (B). All data are presented as Mean ± SE, n = 3. Different letter in each column denotes statistically significant differences. ($p < 0.05$).

Infection on Marine Fish, CARS-47-18) to Pro. An-Xing Li, and Guangdong Province Science and Technology Projects (grant no. 2013B010404003, no. 2014A010107005) to Dr. Xiao-Chun Luo.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.11.016.

References

[1] N.G. Ahn, R. Seger, R.L. Bratlien, C.D. Diltz, N.K. Tonks, E.G. Krebs, Multiple components in an epidermal growth factor-stimulated protein kinase cascade. In vitro activation of a myelin basic protein/microtubule-associated protein 2 kinase, J. Biol. Chem. 266 (1991) 4220–4227.
[2] G. Pearson, F. Robinson, T. Beers Gibson, B.E. Xu, M. Karandikar, K. Berman, et al., Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions, Endocr. Rev. 22 (2001) 153–183.
[3] H.J. Schaeffer, M.J. Weber, Mitogen-activated protein kinases: specific messages from ubiquitous messengers, Mol. Cell Biol. 19 (1999) 2435–2444.
[4] T.G. Boulton, M.H. Cobb, Identification of multiple extracellular signal-regulated kinases (ERKs) with antipeptide antibodies, Cell Regul. 2 (1991) 357–371.
[5] J. Segales, E. Perdiguero, P. Munoz-Canoves, Regulation of muscle stem cell functions: a focus on the p38 MAPK signaling pathway, Front. Cell Develop. Biol. 4 (2016) 91.
[6] S. Vlahopoulos, V.C. Zoumpourlis, JNK: a key modulator of intracellular signaling, Biochem. Biokhimiia 69 (2004) 844–854.
[7] J.M. English, G. Pearson, T. Hockenberry, L. Shivakumar, M.A. White, M.H. Cobb, Contribution of the ERK5/MEK5 pathway to Ras/Raf signaling and growth control, J. Biol. Chem. 274 (1999) 31588–31592.
[8] R. Seger, E.G. Krebs, The MAPK signaling cascade, Faseb. J. 9 (1995) 726–735.
[9] C.F. Zheng, K.L. Guan, Cloning and characterization of two distinct human extracellular signal-regulated kinase activator kinases, MEK1 and MEK2, J. Biol. Chem. 268 (1993) 11435–11439.
[10] J.F. Ohren, H. Chen, A. Pavlovsky, C. Whitehead, E. Zhang, P. Kuffa, et al., Structures of human MAP kinase kinase 1 (MEK1) and MEK2 describe novel non-competitive kinase inhibition, Nat. Struct. Mol. Biol. 11 (2004) 1192–1197.
[11] A. Dang, J.A. Frost, M.H. Cobb, The MEK1 proline-rich insert is required for efficient activation of the mitogen-activated protein kinases ERK1 and ERK2 in mammalian cells, J. Biol. Chem. 273 (1998) 19909–19913.
[12] B-e Xu, J.L. Wilsbacher, T. Collisson, M.H. Cobb, The N-terminal ERK-binding site of MEK1 is required for efficient feedback phosphorylation by ERK2 in vitro and ERK activation in vivo, J. Biol. Chem. 274 (1999) 34029–34035.
[13] M. Fukuda, Y. Gotoh, E. Nishida, Interaction of MAP kinase with MAP kinase kinase: its possible role in the control of nucleocytoplasmic transport of MAP kinase, EMBO J. 16 (1997) 1901–1908.
[14] T. Jelinek, A.D. Catling, C.W. Reuter, S.A. Moodie, A. Wolfman, M.J. Weber, RAS and RAF-1 form a signalling complex with MEK-1 but not MEK-2, Mol. Cell Biol. 14 (1994) 8212–8218.
[15] S. Giroux, M. Tremblay, D. Bernard, J.F. Cardin-Girard, S. Aubry, L. Larouche, et al., Embryonic death of Mek1-deficient mice reveals a role for this kinase in angiogenesis in the labyrinthine region of the placenta, Curr. Biol. : CB 9 (1999) 369–372.
[16] L.F. Belanger, S. Roy, M. Tremblay, B. Brott, A.M. Steff, W. Mourad, et al., Mek2 is dispensable for mouse growth and development, Mol. Cell Biol. 23 (2003) 4778–4787.
[17] D.Q. Li, L. Luo, Z. Chen, H.S. Kim, X.J. Song, S.C. Pflugfelder, JNK and ERK MAP kinases mediate induction of IL-1beta, TNF-alpha and IL-8 following hyperosmolar stress in human limbal epithelial cells, Exp. Eye Res. 82 (2006) 588–596.
[18] S.-K. Jo, W.Y. Cho, S.A. Sung, H.K. Kim, N.H. Won, MEK inhibitor, U0126, attenuates cisplatin-induced renal injury by decreasing inflammation and apoptosis, Kidney Int. 67 (2005) 458–466.
[19] P. Liu, T.P. Zhong, MAPK/ERK signalling is required for zebrafish cardiac regeneration, Biotechnol. Lett. 39 (2017) 1069–1077.
[20] H.Y. Le, Y. Zhang, H. Liu, L.H. Ma, Y. Jin, Q.H. Huang, et al., Eena promotes myeloid proliferation through stimulating ERK1/2 phosphorylation in zebrafish, J. Biol. Chem. 283 (2008) 17652–17661.
[21] B.E. Snaar-Jagalska, S.F. Krens, I. Robina, L.X. Wang, H.P. Spaink, Specific activation of ERK pathways by chitin oligosaccharides in embryonic zebrafish cell lines, Glycobiology 13 (2003) 725–732.
[22] T.A. Hawkins, F. Cavodeassi, F. Erdelyi, G. Szabo, Z. Lele, The small molecule Mek1/2 inhibitor U0126 disrupts the chordamesoderm to notochord transition in zebrafish, BMC Dev. Biol. 8 (2008) 42.
[23] A. Shelly, C. Banerjee, G.K. Saurav, A. Ray, V.S. Rana, R. Raman, et al., Aeromonas hydrophila-induced alterations in cytosolic calcium activate pro-apoptotic cPKC-MEK1/2-TNFα axis in infected headkidney macrophages of Clarias gariepinus, Dev. Comp. Immunol. 76 (2017) 392–402.
[24] A. Jiang, A. Craxton, T. Kurosaki, E.A. Clark, Different protein tyrosine kinases are required for B cell antigen receptor-mediated activation of extracellular signal-regulated kinase, c-Jun NH2-terminal kinase 1, and p38 mitogen-activated protein kinase, J. Exp. Med. 188 (1998) 1297–1306.
[25] J.D. Graves, K.E. Draves, A. Craxton, J. Saklatvala, E.G. Krebs, E.A. Clark, Involvement of stress-activated protein kinase and p38 mitogen-activated protein kinase in mIgM-induced apoptosis of human B lymphocytes, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 13814–13818.
[26] J.I. Healy, R.E. Dolmetsch, L.A. Timmerman, J.G. Cyster, M.L. Thomas, G.R. Crabtree, et al., Different nuclear signals are activated by the B cell receptor during positive versus negative signaling, Immunity 6 (1997) 419–428.
[27] Z.Q. Wang, D.C. Wu, F.P. Huang, G.Y. Yang, Inhibition of MEK/ERK 1/2 pathway reduces pro-inflammatory cytokine interleukin-1 expression in focal cerebral ischemia, Brain Res. 996 (2004) 55–66.
[28] A. Maddahi, L. Edvinsson, Cerebral ischemia induces microvascular pro-inflammatory cytokine expression via the MEK/ERK pathway, J. Neuroinflammation 7 (2010) 14.
[29] Z.Q. Mo, J.L. Wang, R. Han, Q. Han, Y.W. Li, H.Y. Sun, et al., Identification and functional analysis of grouper (*Epinephelus coioides*) B-cell linker protein BLNK, Fish Shellfish Immunol. 81 (2018) 399–407.
[30] Z.Q. Mo, Q. Han, Y.L. Zeng, J.L. Wang, X.Z. Li, Y.W. Li, et al., Molecular characterization and function analysis of grouper (*Epinephelus coioides*) Bruton's tyrosine kinase BTK, Fish Shellfish Immunol. 77 (2018) 91–99.
[31] Z.Q. Mo, M. Yang, H.Q. Wang, Y. Xu, M.Z. Huang, G.F. Lao, et al., Grouper (*Epinephelus coioides*) BCR signaling pathway was involved in response against *Cryptocaryon irritans* infection, Fish Shellfish Immunol. 57 (2016) 198–205.
[32] Z.Q. Mo, Y.W. Li, L. Zhou, A.X. Li, X.C. Luo, X.M. Dan, Grouper (*Epinephelus coioides*) IL-34/MCSF2 and MCSFR1/MCSFR2 were involved in mononuclear phagocytes activation against *Cryptocaryon irritans* infection, Fish Shellfish Immunol. 43 (2015) 142–149.
[33] Y.W. Li, X.M. Dan, T.W. Zhang, X.C. Luo, A.X. Li, Immune-related genes expression profile in orange-spotted grouper during exposure to *Cryptocaryon irritans*, Parasite Immunol. 33 (2011) 679–687.
[34] X.M. Dan, Z.P. Zhong, Y.W. Li, X.C. Luo, A.X. Li, Cloning and expression analysis of grouper (*Epinephelus coioides*) M-CSFR gene post *Cryptocaryon irritans* infection and distribution of M-CSFR(+) cells, Fish Shellfish Immunol. 35 (2013) 240–248.
[35] H.-Y. Sun, M.-Z. Huang, Z.-Q. Mo, L.-S. Chen, G. Chen, M. Yang, et al., Characterization and expression patterns of ERK1 and ERK2 from *Epinephelus coioides* against Cryptocaryon irritans infection, Fish Shellfish Immunol. 74 (2018) 393–400.
[36] Z.Q. Mo, J.L. Wang, M. Yang, L.Y. Ni, H.Q. Wang, G.F. Lao, et al., Characterization and expression analysis of grouper (*Epinephelus coioides*) co-stimulatory molecules CD83 and CD80/86 post *Cryptocaryon irritans* infection, Fish Shellfish Immunol. 67 (2017) 467–474.
[37] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods 25 (2001) 402–408.
[38] Q.W. Qin, T.H. Wu, T.L. Jia, A. Hegde, R.Q. Zhang, Development and characterization of a new tropical marine fish cell line from grouper, *Epinephelus coioides* susceptible to iridovirus and nodavirus, J. Virol Methods 131 (2006) 58–64.
[39] H.J. Cho, J.H. Kang, J.Y. Kwak, T.S. Lee, I.S. Lee, N.G. Park, et al., Ascofuranone suppresses PMA-mediated matrix metalloproteinase-9 gene activation through the Ras/Raf/MEK/ERK- and Ap1-dependent mechanisms, Carcinogenesis 28 (2007) 1104–1110.
[40] S. Pastore, F. Mascia, F. Mariotti, C. Dattilo, V. Mariani, G. Girolomoni, ERK1/2 regulates epidermal chemokine expression and skin inflammation, J. Immunol. 174 (2005) 5047–5056.
[41] S. Takekoshi, Y. Kambayashi, H. Nagata, T. Takagi, Y. Yamamoto, K. Watanabe, Activation of protein kinase C by oxidized diacylglycerols, Biochem. Biophys. Res. Commun. 217 (1995) 654–660.
[42] A. Cohen, C. Brodie, R. Sarid, An essential role of ERK signalling in TPA-induced reactivation of Kaposi's sarcoma-associated herpesvirus, J. Gen. Virol. 87 (2006) 795–802.
[43] Y.W. Li, X.C. Luo, X.M. Dan, X.Z. Huang, W. Qiao, Z.P. Zhong, et al., Orange-spotted grouper (*Epinephelus coioides*) TLR2, MyD88 and IL-1beta involved in anti-*Cryptocaryon irritans* response, Fish Shellfish Immunol. 30 (2011) 1230–1240.