Fish and Shellfish Immunology 84 (2019) 259–268

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Identification and characterization of two novel vascular endothelial growth factor genes in *Litopenaeus vannamei*

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ARTICLE INFO

Keywords:
VEGF signaling pathway
Shrimp
White spot syndrome virus
RNA interference
Protein interaction

ABSTRACT

Vascular endothelial growth factor (VEGF) signaling pathway induces endothelial cell proliferation, promotes cell migration, and inhibits apoptosis. Although three VEGF and two VEGF receptor genes have been identified in *Litopenaeus vannamei* and demonstrated their roles in WSSV infection, another two novel VEGF genes (*LvVEGF4*, *LvVEGF5*) were isolated and their involvements in the WSSV infection of shrimp were studied in the present study. The deduced amino acid sequences of both *LvVEGF4* and *LvVEGF5* contained a signal peptide, a typical PDGF/VEGF domain and a cysteine knot motif (CXCXCX). Tissue distribution analysis showed that *LvVEGF4* was predominantly expressed in gill and hemocytes, while *LvVEGF5* was mainly detected in hemocytes and intestine. WSSV infection could cause up-regulation of the transcriptional levels of *LvVEGF4* and *LvVEGF5*. Their functions were studied by double-strand RNA interference. The results showed that knock-down of *LvVEGF4* and *LvVEGF5* led to a decrease of the viral copy number in WSSV infected shrimp. Yeast two-hybrid analysis showed that both LvVEGF4 and LvVEGF5 could interact with LvVEGFR1 rather than LvVEGFR2. In addition, knock-down of *LvVEGF4* and *LvVEGF5* could reduce the expressional levels of downstream genes *FAK* and *PI3K*. The present study provides new clues in demonstrating that the VEGF signaling pathway is involved in the process of WSSV infection in shrimp.

1. Introduction

White spot syndrome virus (WSSV), which usually causes 100% mortality of the shrimp in three to five days, is a great threat in shrimp aquaculture [1,2]. As a kind of invertebrates, shrimp generates immune responses to pathogens mainly through innate immune system [3]. When infected by microorganism, they mainly rely on the pattern recognition receptor (PRR) to identify pathogens and then activate cell signaling pathways. Signal transduction induces specific antimicrobial peptides (AMPs) to kill the pathogens [4,5]. Toll, IMD and JAK/STAT pathways are regarded as important pathways regulating the innate immune responses of invertebrates including shrimp [6–8].

VEGF signaling pathway is involved in a variety of biological processes including vascular proliferation, cell differentiation, cell migration and so on [9]. As reviewed by Neufeld et al. there are five VEGFs and three VEGFRs in mammals [10]. Different VEGF binds to specific receptors and exerts different biological functions. VEGF (VEGF-A) binds to VEGFR1 and VEGFR2 to perform as a highly specific mitogen for vascular endothelial cells [11]. VEGF-B binds to VEGFR-1 to regulate the activity of plasminogen activator in endothelial cells [12]. VEGF-C is proved to bind with VEGFR-2 or VEGFR-3 to regulate vascular development and maintenance of the lymphatic vessels [13]. VEGF-D is a ligand for VEGF receptor 2 (Flk-1) and VEGF receptor 3 (Flt-4) and it could promote tumor angiogenesis and growth [14]. VEGFRs belong to tyrosine-kinase receptor family by the presence of seven immunoglobulin-like loops in their extracellular part and a split tyrosine-kinase domain in their intracellular part. When performing functions, VEGF dimer might bind and link two VEGF receptors together to form homo- or heterodimers of receptors [10]. Moreover, VEGF could regulate other innate immune signaling pathways such as MAPK/ERK, PI3K, JAK/STAT etc. [15–17], which are involved in pathogen infection [18–20].

Recently, the VEGF signaling pathway has also been reported directly participating in pathogen infection. When the human pancreatic

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https://doi.org/10.1016/j.fsi.2018.10.019
Received 19 July 2018; Received in revised form 5 October 2018; Accepted 7 October 2018
Available online 09 October 2018

Table 1
Primer sequences and corresponding annealing temperature.

Primer name	Primer sequence(5'-3')	Expected size (bp)	Annealing temperature (°C)
LvVEGF4-F	ATTAGAATTCTTACGCTCGTG	594	55
LvVEGF4-R	TTAAGATTCCATCACGTAACG		
LvVEGF5-F	TCGGCGACGCTCATTATC	1029	55
LvVEGF5-R	CAGCAAGGAACAGTTGT		
LvVEGF4-qF	TGGAGGCTCACAGATTGA	137	57
LvVEGF4-qR	GAAGTTGCGGTTGAACAC		
LvVEGF5-qF	AATATGAGCCAGACGACAG	134	57
LvVEGF5-qR	CACCACAAGTGAGCAGAA		
18S-F	TATACGCTAGTGGAGCTGGAA	136	56
18S-R	GGGGAGGTAGTGACGAAAAAT		
LvVEGF4-dsF	TAATACGACTCACTATAGGGAGCTGTGGCATAAACAAGGTG	545	59
LvVEGF4-dsR	TAATACGACTCACTATAGGGTTGTGAGGAGTCGTGGTGTCT		
LvVEGF5-dsF	TAATACGACTCACTATAGGGCGGCGAATAATTGAGTATAAGAGTA	556	59
LvVEGF5-dsR	TAATACGACTCACTATAGGGTGCAAATGCCAGTGTAAGGA		
EGFP-dsF	TAATACGACTCACTATAGGGCAGTGCTTCAGCCGCTACCC	289	59
EGFP-dsR	TAATACGACTCACTATAGGGAGTTCACCTTGATGCCGTTCTT		
VP28-qF	AAACCTCCGCATTCCTGTGA	141	55
VP28-qR	TCCGCATCTTCTTCCTTCAT		
LvFAK-qF	ATTACTCAACACCAGCAACC	172	57
LvFAK-qR	GTTCCCTCGGACTCCACCTT		
LvPI3K-qF	TATGAAGTAACCCGTAGTGCCA	187	57
LvPI3K-qR	TGCCCACATCTCCTGACTGA		
LvVEGF4-YF	GGATCCATTAGAATTCTTACGCTCGTG	591	55
LvVEGF4-YR	GAATTCTTAAGATTCCATCACGTAACG		
LvVEGF5-YF	GGATCCTCGGCGACGCTCATTATC	1029	56
LvVEGF5-YR	GAATTCCAGCAAGGAACAGTTGTAGC		
LvVR1(2–5)-YF	ACTTTGACCACATACAAGTA	1127	57
LvVR1(2–5)-YR	TCCATGTTCATTGGAGGTTT		
LvVR2(1–4)-YF	TTCAACCCAGTGCATGTTGAGGAG	1473	58
LvVR2(1–4)-YR	CACAACTACTAGCCTCAGTTTGCG		

Note: T7 promoter sequences were underlined. *BamH* I and *EcoR* I sites were wave-underlined.

carcinoma cell lines were infected by *Vaccinia virus* (VV), increased expression of VEGF-A could enhance VV gene expression, replication, and cytotoxicity [21]. *Bovine papular stomatitis virus* (BPSV) could encode a novel VEGF homologue which could bind to VEGFR-2 with high affinity and contribute to the proliferation and highly vascularized nature of BPSV lesions [22]. In the genome of *Orf virus*, a gene encoding a homology to mammalian VEGF was discovered [23], which was proved to activate VEGFR-2 and participated in virus infection [24]. In *Drosophila*, the expression of one VEGF, *pvf2*, was up-regulated after infected by *E. coli* [25]. In the crustacean *Eriocheir sinensis*, a VEGF homologous gene, *EsPVF1* could respond to the infection of *Vibrio anguillarum* and *Pichia pastoris* [26]. Two VEGF homologous genes, *MjVEGF-1* and *MjVEGF-2* were proved to participate in the process of immune response in *Marsupenaeus japonicas* [27].

Previously, three types of VEGF genes and two types of VEGFR genes were identified in *L. vannamei* and they all participated into WSSV infection [28–31]. In the present study, another two members of VEGF family (*LvVEGF4*, *LvVEGF5*) was identified from *L. vannamei* and their functions during WSSV infection were explored. Furthermore, the interaction between LvVEGF4, LvVEGF5 and LvVEGFRs was also studied by the yeast two-hybrid system. The current study will provide new evidence for understanding the role of the VEGF signaling pathway during WSSV infection in shrimp.

2. Materials and methods

2.1. Experimental animals

Healthy adult Pacific whiteleg shrimp cultured in our lab, with a body length of 12.5 ± 0.4 cm and a body weight of 25.1 ± 2.5 g, were used for tissue distribution analysis and WSSV challenge experiments. Shrimp with a body length of 8.80 ± 2.30 cm and a body weight of 6.55 ± 3.19 g were used for dsRNA injection and WSSV replication detection after gene silencing. All shrimp were acclimated in air-pumped circulating sea water at 25 °C before experiments.

2.2. Tissues collection and WSSV challenge experiments

In order to detect the tissue distribution of *LvVEGF4* and *LvVEGF5*, different tissues from 12 individuals were dissected. Firstly, hemolymph was obtained by using a sterile syringe preloaded with equal volume of modified anticoagulant Alsever solution and then centrifuged immediately at 800 g at 4 °C for 10 min [32]. The supernatant was discarded and the hemocytes were collected and preserved in liquid nitrogen. Then the hepatopancreas, heart, eyestalk, muscle, lymphoid organ, intestine, stomach, gill, epidermis, testis and ovary were collected and kept in liquid nitrogen.

For virus challenge experiment, WSSV was purified from the cephalothoraxes of infected *Exopalamon carincauda* and quantified according to the method previously described [33]. Healthy adult shrimp were divided into two groups including WSSV group and PBS group, with 80 individuals in each group. In WSSV group, each shrimp was injected with 20 μl WSSV solution containing 10^4 copies of WSSV, while in PBS group each was injected with 20 μl PBS as the negative control. At 0.5, 3, 6, 12, 24 and 48 hours (h) post WSSV infection, gill, intestine and hemocytes of 12 shrimp in each group were collected separately and frozen in liquid nitrogen.

2.3. Total RNA extraction and cDNA synthesis

To detect the transcriptional level of *LvVEGF4* and *LvVEGF5*, total RNA of each sample was extracted using RNAiso Plus reagent (TaKaRa, Japan) following the manufacturer's protocol. The concentration of extracted RNA was detected by Nanodrop 2000 (Thermo Fisher Scientific, USA) and the RNA quality was assessed by electrophoresis on 1% agarose gel. The cDNA samples were synthesized using PrimeScript RT Reagent Kit (TaKaRa, Japan) with 1 μg total RNA. Genomic DNA was firstly removed by gDNA Eraser. And then the first-strand cDNA

was synthesized by PrimeScript RT Enzyme following the manufacturer's instructions.

2.4. Gene cloning and phylogenetic analysis

The assembled nucleotide sequences encoding *LvVEGF4* and *LvVEGF5* were obtained from the Illumina-based transcriptome database of *L. vannamei* [34]. Primers LvVEGF4-F/LvVEGF4-R and LvVEGF5-F/LvVEGF5-R (Table 1) were designed to validate the two sequences. The PCR program for *LvVEGF4* and *LvVEGF5* amplification was the same as follows: 1 cycle of denaturation at 95 °C for 5 min, 32 cycles of denaturation at 95 °C for 45 s, annealing at 57 °C for 30 s, and extension at 72 °C for 45 s, followed by an extension at 72 °C for 7 min. The specific products were assessed by electrophoresis on 1% agarose gel. Then the amplified products were purified using TIANgel Midi purification kit (Tiangen, China), cloned into pMD19-T vector (TaKaRa, Japan) and then transformed into *Escherichia coli* Trans 5α competent cells for sequencing (TransGen Biotech, China).

According to sequencing results, the complete ORF regions and amino acid sequences of *LvVEGF4* and *LvVEGF5* were deduced using ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Protein domains were predicted with InterProScan software (http://www.ebi.ac.uk/interpro/). Signal peptides were predicted by SignalP 3.0 Server software (http://www.cbs.dtu.dk/services/SignalP/). The VEGF homologous sequences from other species (Table 2) were subjected to perform multiple sequences alignment using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and then the phylogenetic tree was constructed by the neighbor-joining algorithm using the MEGA 4 software. The reliability of the tree was tested by bootstrapping using 1000 replications.

2.5. Quantitative real-time PCR analysis of LvVEGF4 and LvVEGF5 in different tissues

The relative transcriptional levels of *LvVEGF4* and *LvVEGF5* in different tissues were detected by SYBR Green-based quantitative real-time PCR. Primers LvVEGF4-qF/LvVEGF4-qR, LvVEGF5-qF/LvVEGF5-qR (Table 1) were used to detect the expression levels of the target genes, while primers 18S-F/18S-R (Table 1) were designed to detect the expression of the reference gene, 18S rRNA. The program for *LvVEGF4* and *LvVEGF5* detection was as follows: denaturation at 94 °C for 2 min; 40 cycles of 94 °C for 15 s, annealing at 57 °C for 20 s, and 72 °C for 20 s. The PCR product was denatured to produce melting-curve to check the specificity of the PCR product. The programs for 18S rRNA were almost the same as that for *LvVEGF4* and *LvVEGF5* but the annealing temperature was 56 °C. Detection on the transcripts of *LvVEGF4* and *LvVEGF5* for all samples was repeated in triplicate. The relative expression level of the target gene was calculated using $2^{-\Delta\Delta Ct}$ method [35].

In order to explore the involvement of *LvVEGF4* and *LvVEGF5* in WSSV infection on shrimp, we also detected the expression of *LvVEGF4* and *LvVEGF5* at different time points during WSSV infection using quantitative real-time PCR. Primers and program used were the same as those used in the tissue distribution detection.

2.6. Preparation of dsRNA and optimization of injection dosage for gene interference

The double-strand RNAs of *LvVEGF4* and *LvVEGF5* were synthesized using TranscriptAid T7 High Yield Transcription Kit (Thermo Fisher Scientific, USA). Two pairs of primers with T7 promoter sequences, LvVEGF4-dsF/LvVEGF4-dsR, LvVEGF5-dsF/LvVEGF5-dsR (Table 1) were designed for the synthesis of dsRNA of *LvVEGF4* and *LvVEGF5*. The PCR program for template amplification was as follows: denaturation at 94 °C for 5 min; 40 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s and extension at 72 °C for 30 s; final extension at 72 °C for 10 min. The PCR products were assessed by electrophoresis on 1% agarose gel and purified using Gel Extraction Kit (OMEGA Bio-tec, USA). The purified products were used as template to synthesize the corresponding dsRNAs of *LvVEGF4* and *LvVEGF5*. Redundant single-strand RNA was digested by RNaseA (TaKaRa, China). The concentrations of synthesized dsRNAs were assessed by Nanodrop 2000 (Thermo Fisher Scientific, USA) and their qualities were assessed by electrophoresis on 1% agarose gel. The same method was used to synthesize the dsRNA according to sequence of EGFP gene, with the primers EGFP-dsF and EGFP-dsR (Table 1) which was used as unrelated interference control.

The dsRNA silencing efficiency of *LvVEGF4* and *LvVEGF5* was optimized prior to the genetic interference experiment. Totally 36 shrimp were divided into three groups, dsVF4 group (injected with dsRNA of *LvVEGF4*), dsVF5 group (injected with dsRNA of *LvVEGF5*) and dsEGFP group (injected with dsEGFP). Each group was divided into three subgroups injected with 2 μg, 4 μg and 6 μg dsRNA for each shrimp. The dsRNAs for different genes were injected into the last abdominal

Table 2
Information of VEGFs used for phylogeny analysis.

Gene symbol	Annotation	Accession number	Species
LvVEGF1	Vascular endothelial growth factor 1	No. KT598503	*Litopenaeus vannamei*
LvVEGF2	Vascular endothelial growth factor 2	No. KT598504	*Litopenaeus vannamei*
LvVEGF3	Vascular endothelial growth factor 3	No. KU886195	*Litopenaeus vannamei*
LvVEGF4	Vascular endothelial growth factor 4	MH475138	*Litopenaeus vannamei*
LvVEGF5	Vascular endothelial growth factor 5	MH475139	*Litopenaeus vannamei*
PlPvf1	PDGF and VEGF related factor 1	ASU10867	*Pacifastacus leniusculus*
HsVEGF-A	Vascular endothelial growth factor A	No. P15692	*Homo sapiens*
HsVEGF-B	Vascular endothelial growth factor B	No. P49765	*Homo sapiens*
HsVEGF-C	Vascular endothelial growth factor C	No. P49767	*Homo sapiens*
HsVEGF-D	Vascular endothelial growth factor D	No. O43915	*Homo sapiens*
DmPvf1	PDGF/VEGF factor-1	No. CAC24699	*Drosophila melanogaster*
DmPvf2	PDGF/VEGF factor-2	No. CAC42213	*Drosophila melanogaster*
DmPvf3	PDGF- and VEGF-related factor 3	No. AAF52485	*Drosophila melanogaster*
MdVEGF-A	vascular endothelial growth factor A-like	No. XP_005192020	*Musca domestica*
CcVEGF-D	vascular endothelial growth factor D-like	No. XP_004535321	*Ceratitis capitata*
BcVEGF-D	vascular endothelial growth factor D isoform X2	No. XP_011182059	*Bactrocera cucurbitae*
NvVEGF-C	vascular endothelial growth factor C isoform X2	No. XP_003428015	*Nasonia vitripennis*
AmVEGF-A	vascular endothelial growth factor A	No. XP_006568971	*Apis mellifera*
CbPDGF-B	Platelet-derived growth factor subunit B	No. EZA50606	*Cerapachys biroi*
ApVEGF-1	PDGF- and VEGF-related factor 1-like precursor	No. NP_001156259	*Acyrthosiphon pisum*
TcVEGF-A	vascular endothelial growth factor A	No. XP_001814264	*Tribolium castaneum*
EsPDGF	PDGF/VEGF-related factor 1	No. ADF87936	*Eriocheir sinensis*

segment of each shrimp separately. In each group, four individuals were injected. At 48 h after injection with different dsRNA, four individuals in each group were collected and their cephalothoraxes were dissected and frozen in liquid nitrogen for RNA extraction and cDNA synthesis according to the method described in Section 2.3. The transcription levels of *LvVEGF4* and *LvVEGF5* were detected using quantitative real-time PCR according to Section 2.5. After detection of the interference efficiency, 6 μg dsRNA of *LvVEGF4* and *LvVEGF5* for one individual was used in the following RNAi experiment.

2.7. Detection on the WSSV copy number in WSSV infected shrimp with LvVEGF4 or LvVEGF5 interference

After optimization for the dsRNA dosage, healthy shrimp were divided into three groups including dsVF4 group, dsVF5 group and dsEGFP group with 30 individuals in each group. For dsVF4, dsVF5 and dsEGFP group, 6 μg of dsLvVEGF4, 6 μg dsLvVEGF5 or dsEGFP dissolved in 20 μl PBS was injected into each individual, respectively. At 48 h after dsRNA injection, 3000 copies of WSSV dissolved in 20 μl PBS was injected into each individual. The pleopods of 15 individuals from each group were collected at 24 h and 48 h after WSSV injection. The samples collected from each group at each time were divided into three duplicate samples and frozen in liquid nitrogen for DNA extraction.

Genomic DNA was extracted from the frozen pleopods using Genomic DNA Kit (Tiangen, China) following the manufacturer's instructions. The WSSV copy number in each DNA sample was quantified by a quantitative real-time PCR with primers VP28-qF and VP28-qR (Table 1) and standard plasmid containing *VP28* gene from WSSV [33]. Briefly, the plasmid DNA containing a 281 bp fragment of *VP28* gene from WSSV was constructed and transfected into *E. coli* cells. The plasmid was then extracted and quantified to calculate the copy number. The plasmid was then diluted into serial concentration corresponding to 1×10^8 to 1×10^3 copies WSSV/μl and used for generating a standard curve. The qPCR was performed with the diluted plasmid DNA and the pleopods DNA under the following program: denaturation at 94 °C for 2 min; 40 cycles of 94 °C for 20 s, 55 °C for 20 s, and 72 °C for 20 s. The WSSV copy number per ng pleopods DNA was calculated based on the standard curve by Eppendorf Mastercycler® ep realplex (Eppendorf, Germany).

2.8. Yeast two-hybrid assay

2.8.1. Bait and prey protein expression plasmid construction

Primers LvVEGF4-YF/LvVEGF4-YR and LvVEGF5-YF/LvVEGF5-YR with *BamH* I and *EcoR* I digested sites (Table 1) were designed to amplify the ORF of *LvVEGF4* and *LvVEGF5*. The PCR program for amplifying *LvVEGF4* was as follows: 1 cycle of denaturation at 95 °C for 5 min, 32 cycles of denaturation at 95 °C for 45 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s, followed by an extension at 72 °C for 7 min. The PCR program for amplifying *LvVEGF5* was the same but the annealing temperature was 56 °C. The specific products were assessed by electrophoresis on 1% agarose gel. The amplified products were purified using TIANgel Midi purification kit (Tiangen, China) and was inserted into the vector encoding the *GAL4* transcriptional activation domain of *BamH* I and *EcoR* I digested pGADT7. Then plasmids pGADT7-VF4 and pGADT7-VF5 were constructed and used as prey protein expression plasmid.

In addition, the same method was used to construct bait protein expression plasmid. Primers LvVR1(2–5)-YF/LvVR1(2–5)-YR and LvVR2(1–4)-YF/LvVR2(1–4)-YR with *BamH* I and *EcoR* I digested sites (Table 1) were designed to amplify the ORF of *LvVEGFR1* and *LvVEGFR2*. Purified amplified products were inserted into the vector encoding the *GAL4* transcriptional binding domain of *BamH* I and *EcoR* I digested pGBKT7. Plasmids pGBKT7-VR1(2–5) and pGBKT7-VR2(1–4) were constructed and used as bait protein expression plasmid.

2.8.2. Yeast transformation and galactosidase assays

Prey protein expression plasmids and bait protein expression plasmids were used to identify protein interaction using the yeast two-hybrid system. Fusion plasmid pGADT7-VF4 and pGADT7-VF5 co-transformed into yeast strainY2H Gold with pGBKT7-VR1(2–5) or pGBKT7-VR2(1–4) by the lithium acetate transformation procedure according to Clontech Matchmaker protocol manual (Clontech, USA). Transformants were then plated directly onto selective growth media, DDO plate. DDO is a commercial synthetic defined medium lacking both of leucine and tryptophan for selecting the bait and prey plasmids (Clontech, USA). Clones that grew on DDO plate contained both bait and prey plasmids. In addition, fusion plasmids pGADT7-VF4 or pGADT7-VF5 which co-transformed with pGBKT7 and pGBKT7-VR1(2–5) or pGBKT7-VR2(1–4) with pGADT7 were used to detect the auto-activation. The expression plasmids pGBKT7-p53/pGADT7-Tantigen and pGBKT7-Lam/pGADT7-Tantigen were used as positive and negative controls, respectively. Transformants grew at 30 °C for 3–5 days, until colonies were large enough (diameter 2–3 mm usually) for galactosidase activity assay.

Fast-growing colonies were picked and plated onto QDO/X/A plate. QDO is also a commercial synthetic defined medium lacking adenine, histidine, leucine and tryptophan. X-α-Gal and Aureobasid were added into QDO to make up QDO/X/A. Yeast colonies that expressed *Mel1* would be shown in blue in the presence of the chromogenic substrate X-α-Gal (Clontech, USA).

2.9. Detection on the expression level of LvFAK and LvPI3K in LvVEGF4 or LvVEGF5 silenced shrimp

In mammals, VEGF signaling pathway usually plays biological roles *via* regulating the function of the downstream signal pathways. Thus, we detected the transcriptional levels of downstream genes *LvFAK* and *LvPI3K* in *LvVEGF4* or *LvVEGF5* silenced shrimp by quantitative real-time PCR using the primers LvFAK-qF/qR and LvPI3K-qF/qR (Table 1). The qPCR program and the method of calculating relative expression levels of the target genes are the same as described in Section 2.5.

2.10. Data analysis

The assays described above were biologically repeated for three times. For quantitative real-time PCR, four replicates were set for each sample. The relative transcription level of target genes was obtained using $2^{-\Delta\Delta Ct}$ method and the WSSV copy number per ng DNA was obtained according to the standard curve [35]. The numerical data from each experiment were analyzed to calculate the mean and standard deviation of triplicate assays. An independent sample *t*-test was used to analyze the differences between two groups by SPSS 16.0. $P < 0.05$ was considered statistically significant.

3. Results

3.1. The full length cDNAs and sequence analysis of LvVEGF4 and LvVEGF5

The open reading frames of *LvVEGF4 and LvVEGF5* were confirmed by PCR amplification and sequencing. The ORF of *LvVEGF4* cDNA was 597 base pairs (bp) encoding 198 amino acids (aa). A signal peptide of 17 aa, a conserved VEGF/PDGF domain of 84 aa and a typical cysteine knot motif (CXCXC) were identified in the deduced amino acid sequence of *LvVEGF4* (Fig. 1A, GenBank number: MH475138). The ORF of *LvVEGF5* cDNA was 1035 base pairs (bp) encoding 344 amino acids (aa). A signal peptide of 30 aa, a conserved VEGF/PDGF domain of 89 aa and a typical cysteine knot motif (CXCXC) were identified in the deduced amino acid sequence of *LvVEGF5* (Fig. 1B, GenBank number: MH475139). Phylogenetic analysis showed that LvVEGF4 was clustered with LvVEGF3. Then, they were classified together with other VEGFs from *L. vannamei* and VEGF homologues from other crustacean species

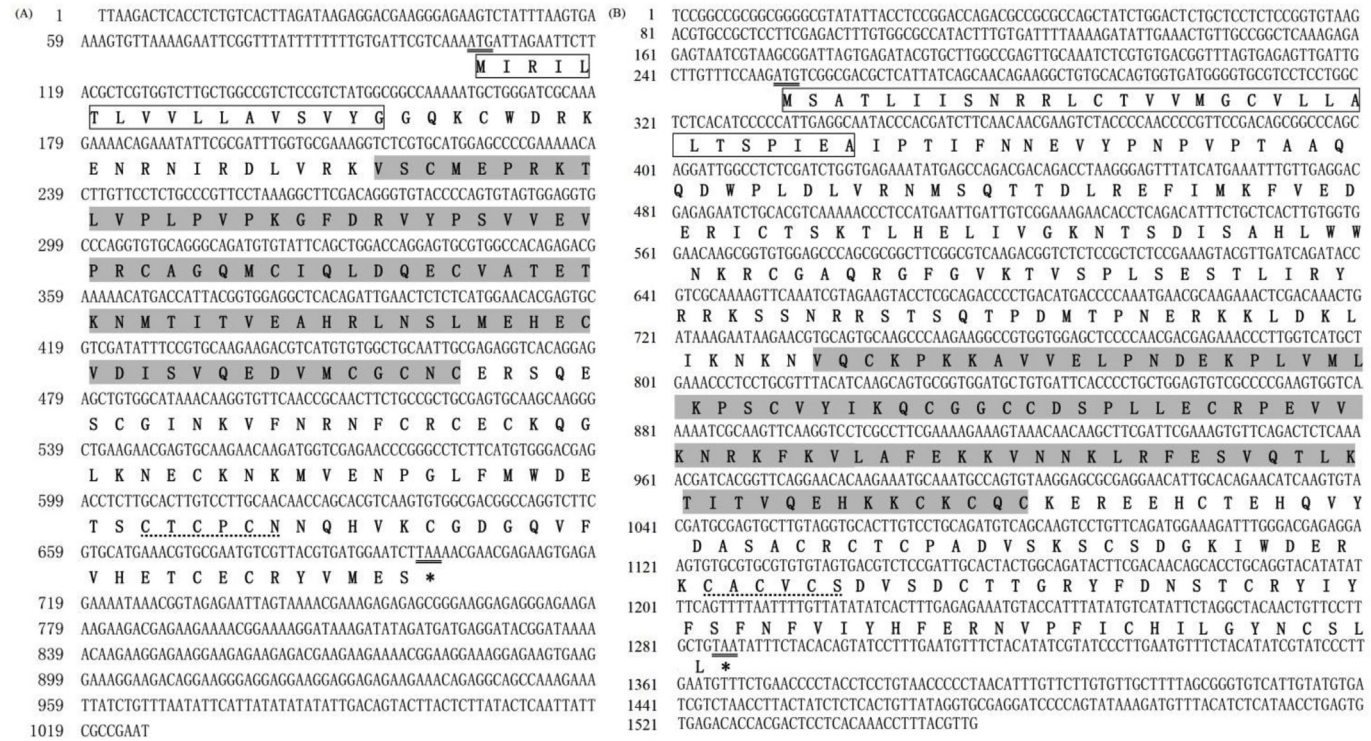

Fig. 1. Deduced amino acid sequences of *LvVEGF4* (A) and *LvVEGF5* (B). Predicted domains were shown with different makers. Start codons and stop codons were marked in double underline. Signal peptide was marked in box. PDGF/VEGF domain was marked in gray. CXCXC repeats were dotted underlined.

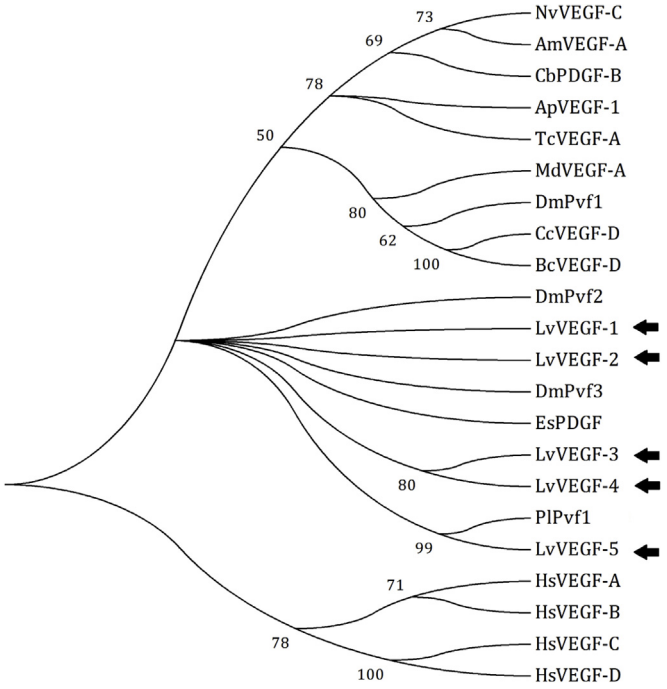

Fig. 2. The neighbor-joining phylogenetic tree of *LvVEGFs* and their homologous genes from other species (The information of VEGFs was shown in Table 2). LvVEGFs were shown with black arrows.

Eriocheir sinensis and *Pacifastacus leniusculus*, and two VEGF homologues (DmPvf2 and DmPvf3) from the insect *D. melanogaster*. VEGF homologues from mammals were clustered into a single branch (Fig. 2).

3.2. Tissue distribution of *LvVEGF4* and *LvVEGF5*

The tissues distribution of *LvVEGF4* and *LvVEGF5* was detected by quantitative real-time PCR. The expression levels of *LvVEGF4* and *LvVEGF5* were normalized against lowest expression tissues of the two genes, respectively. The transcripts of *LvVEGF4* were mainly detected in gill and hemocytes, while relatively lower expression levels were detected in other tissues (Fig. 3A). *LvVEGF5* was mainly distributed in hemocytes and intestine, while it showed very low expression levels in other tissues (Fig. 3B).

3.3. Expression profile of *LvVEGF4* and *LvVEGF5* during WSSV challenge

In order to learn the response of *LvVEGF4 and LvVEGF5* after WSSV infection, the expression profiles of them in different tissues during WSSV challenge were detected. The transcriptional level of *LvVEGF4* was down-regulated in gill while up-regulated at 0.5 h, 48 h post-infection in hemocytes (Fig. 4). The transcriptional level of *LvVEGF5* was up-regulated at 24 h post-infection in hemocytes and at 0.5 h, 6 h post-infection in intestine (Fig. 5).

3.4. Interference of *LvVEGF4* and *LvVEGF5* inhibited WSSV propagation

In order to optimize the *silencing efficiency* of genes by dsRNA interference, the transcriptional levels of *LvVEGF4* and *LvVEGF5* were detected at 48 h after injection of dsLvVEGF4 or dsLvVEGF5. The transcriptional levels of *LvVEGF4* and *LvVEGF5* were significantly decreased by 77% and 81% when compared with dsEGFP group (Fig. 6).

To know the function of *LvVEGF4* and *LvVEGF5* during WSSV infection, the WSSV copy number was detected in shrimp after *LvVEGF4* or *LvVEGF5* interference and WSSV infection. As shown in Fig. 7, there was no significant difference at the early infection stage (24 hpi). However, at 48 h post-infection, the WSSV copy number in shrimp from dsEGFP group increased dramatically, which exceeded 1×10^4 copies/ng DNA. Both in *LvVEGF4* or *LvVEGF5* knockdown shrimp, the WSSV

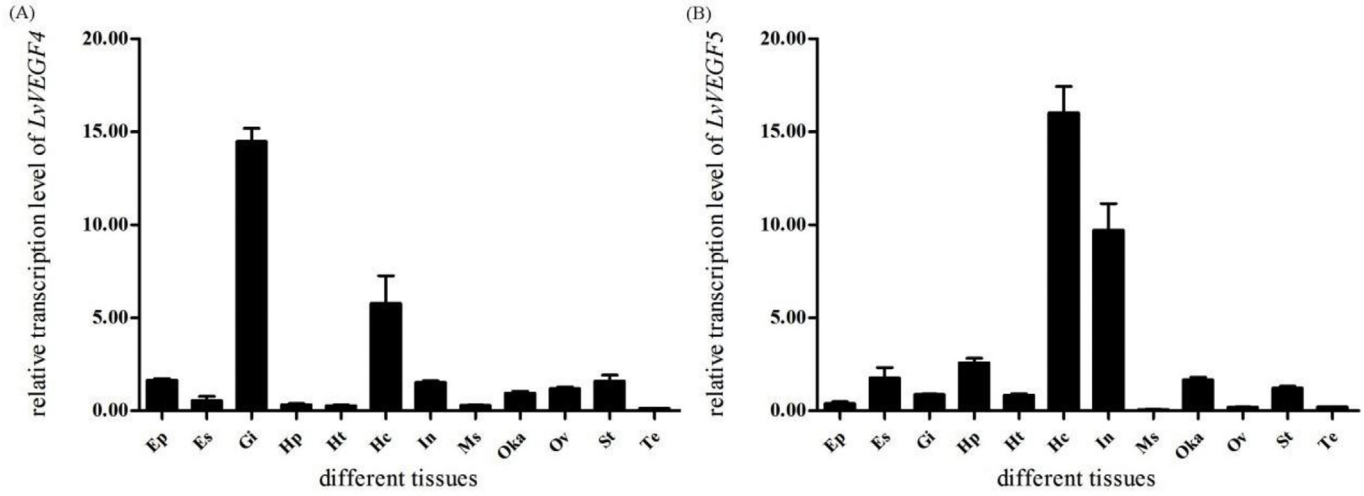

Fig. 3. Tissue distribution of *LvVEGF4* (A) or *LvVEGF5* (B) in *L. vannamei*. Ht, heart; Hp, hepatopancreas; Gi, gill; Oka, lymph organ; Ms, muscle; In, intestine; Hc, hemocytes; Es, eyestalk; St, stomach; Ep, epidermis; Te, testis; Ov, ovary.

copy numbers were less than 1×10^2 copies/ng DNA, which were significantly lower ($P < 0.001$) compared with that of dsEGFP group.

3.5. Detection on the interaction between LvVEGF4, LvVEGF5 and LvVEGFRs

Yeast two-hybrid system was used to investigate the interaction between LvVEGF4 or LvVEGF5 and LvVEGFRs. The positive control, association between pGBKT7-p53 and pGADT7-Tantigen developed a blue color on the plate (Fig. 8A–D, zone 1). Expression of reporter genes was observed and developed a blue color in the yeast cells possessing both pGADT7-VF4 and pGBK-VR1(2–5) plasmids (Fig. 8A, zone 4). Reporter genes were also activated in the yeast cells possessing both pGADT7-VF5 and pGBK-VR1(2–5) plasmids (Fig. 8C, zone 4). However, reporter genes in the yeast cells possessing pGADT7-VF4 and pGBK-VR2(1–4) plasmids (Fig. 8B, zone 4) or pGADT7-VF5 and pGBK-VR2(1–4) plasmids (Fig. 8D, zone 4) did not express. The negative control, pGBKT7-Lam and pGADT7-Tantigen combinations did not activate reporter genes (Fig. 8A–D, zone 5) as well as the self-activation controls, pGADT7-VF4/5 and pGBKT7 (Fig. 8A–D, zone 2) or pGBK-VR1(2–5)/VR2(1–4) and pGADT7 plasmid combinations (Fig. 8A–D, zone 3).

3.6. Effects of LvVEGF4 or LvVEGF5 interference on the expression of LvFAK and LvPI3K genes

In order to know whether *LvVEGF4* and *LvVEGF5* could regulate downstream genes like other VEGFs genes in *L. vannamei*, the transcriptional levels of *LvFAK* and *LvPI3K* genes were detected in shrimp after *LvVEGF4* or *LvVEGF5* knockdown. After knockdown of *LvVEGF4*, the expression levels of *LvFAK* and *LvPI3K* were significantly down-regulated by 58% and 45%, respectively (Fig. 9A). After knockdown of *LvVEGF5*, the expression levels of *LvFAK* and *LvPI3K* were significant down-regulated by 43% and 38%, respectively (Fig. 9B).

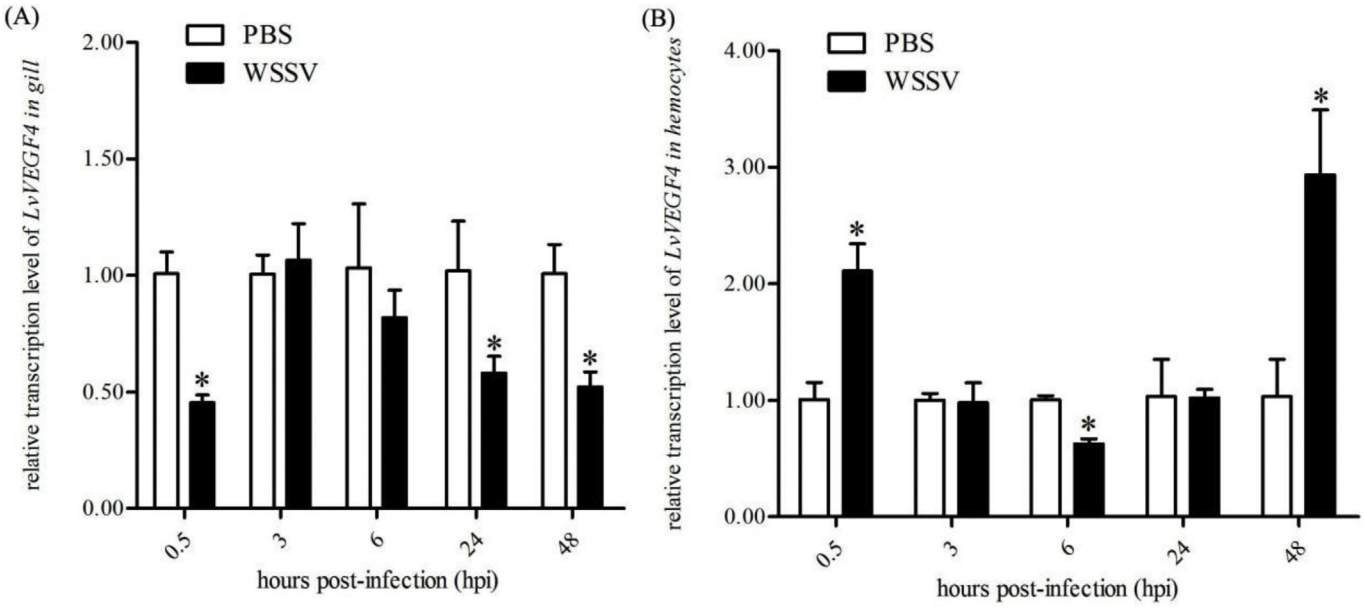

Fig. 4. Expression profile of *LvVEGF4* in gill (A) and in hemocytes (B) at different hours post WSSV infection (hpi). PBS, injected with PBS; WSSV, injected with WSSV in PBS. Stars (*) indicate significant differences ($P < 0.05$) of the gene expression levels between PBS and WSSV groups.

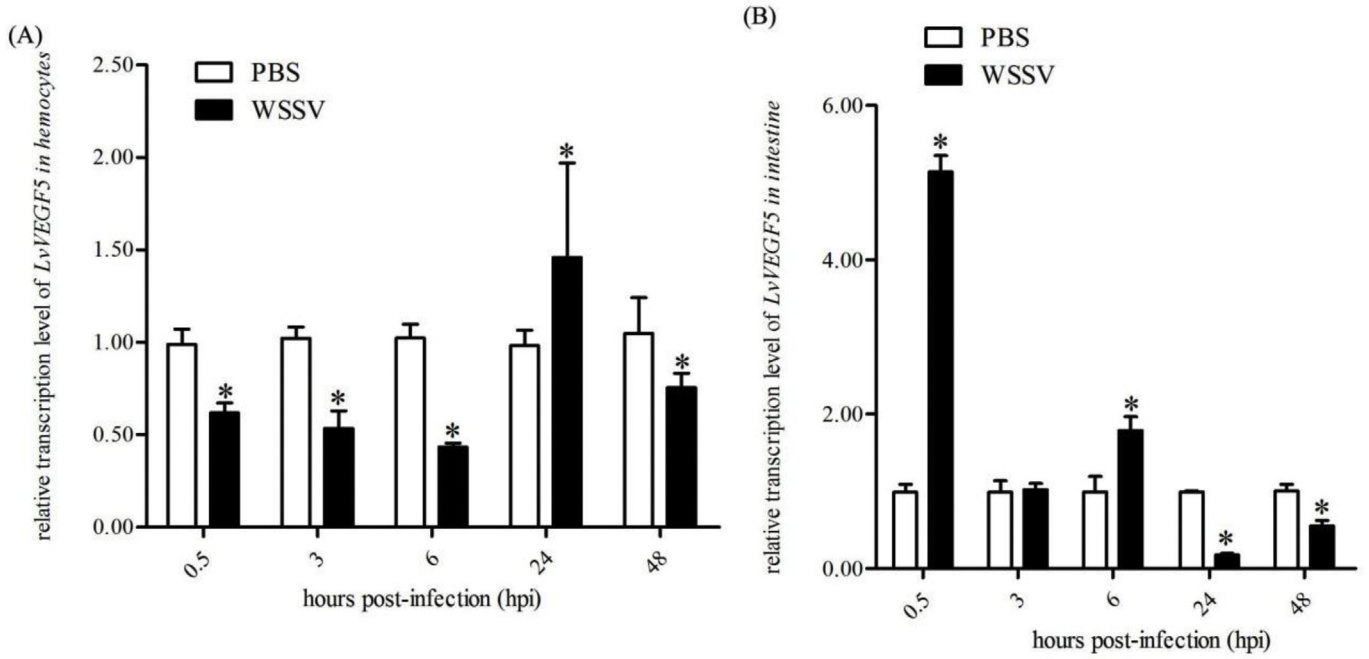

Fig. 5. Expression profile of *LvVEGF5* in hemocytes (A) and in intestine (B) at different hours post WSSV infection (hpi). PBS, injected with PBS; WSSV, injected with WSSV in PBS. Stars (*) indicate significant differences ($P < 0.05$) of the gene expression levels between PBS and WSSV groups.

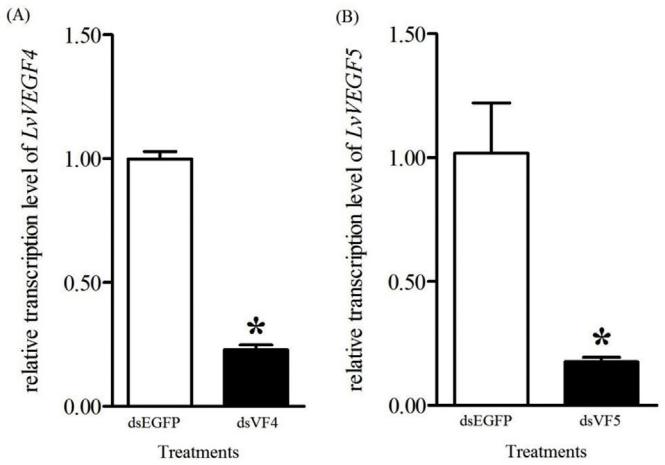

Fig. 6. Expression level of *LvVEGF4* (A) and *LvVEGF5* (B) in shrimp after dsRNA-mediated RNA interference. dsEGFP, injected with dsEGFP; dsVF4, injected with dsRNA of *LvVEGF4*. dsVF5, injected with dsRNA of *LvVEGF5*. Stars (*) indicate significant differences ($P < 0.05$) of gene expression levels between two groups.

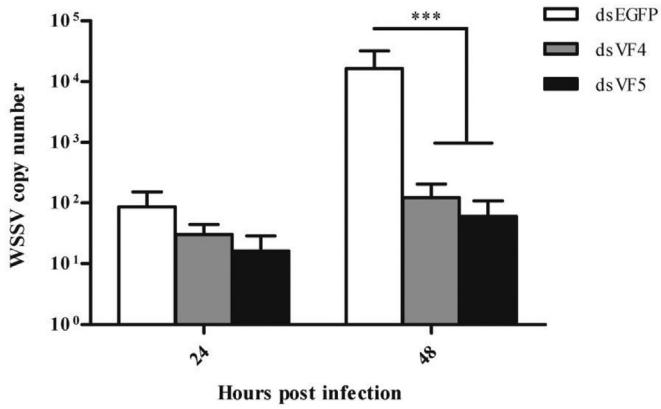

Fig. 7. Amount of WSSV particles in *LvVEGF4*-silenced (A) or *LvVEGF5*-silenced (B) shrimp at different hours post WSSV infection (hpi). dsEGFP group, injected with dsEGFP and WSSV; dsVF4 group, injected with dsLvVEGF4 and WSSV; dsVF5 group, injected with dsLvVEGF5 and WSSV. Stars (*) indicate significant differences ($P < 0.05$) for the gene expression levels between two groups.

4. Discussion

The shrimp VEGF signaling pathway has been reported to participate in WSSV infection recently. Three VEGF genes and two VEGF receptor genes have been identified and their functions have been characterized during WSSV infection in our previous studies [28–31]. In the present study, two other VEGF genes, named *LvVEGF4* and *LvVEGF5*, were identified in the Pacific whiteleg shrimp *L. vannamei*. Both *LvVEGF4* and *LvVEGF5* encoded a typical PDGF/VEGF domain including several conserved cysteine residues forming a cysteine knot motif (CXCXCX), which was similar to the reported VEGF family members [36]. Although phylogenetic analysis showed that five VEGFs from *L. vannamei* were clustered together, multiple alignments of their nucleotide sequences presented low sequence similarity (data not shown), indicating that these VEGFs were encoded by different genes rather than generated through alternative splicing in shrimp. As new members of VEGFs in *L. vannamei*, it was necessary to know whether they participate in WSSV infection as other members in the VEGF signaling pathway.

The high expression of reported VEGF signaling pathway members from *L. vannamei* was confined to several immune-related organs, including hemocytes, lymph organ, gill and intestine [28–31]. Hemocytes are essential for pathogen recognition, phagocytosis, cytotoxicity and cell -cell communication [37]. Lymphoid organ is specific and effective for bacteria and virus clearance [38,39]. Gill directly contacts with the complicated environmental microbes [40]. Intestine, where contains plenty of bacterial communities, also plays important roles in shrimp immune response [41]. Similar to those reported VEGF signaling pathway members, *LvVEGF4* was mainly expressed in gill and hemocytes, and *LvVEGF5* was mainly detected in hemocytes and intestine. Co-expression of the VEGF signaling pathway genes from *L. vannamei* suggested their similar biological functions.

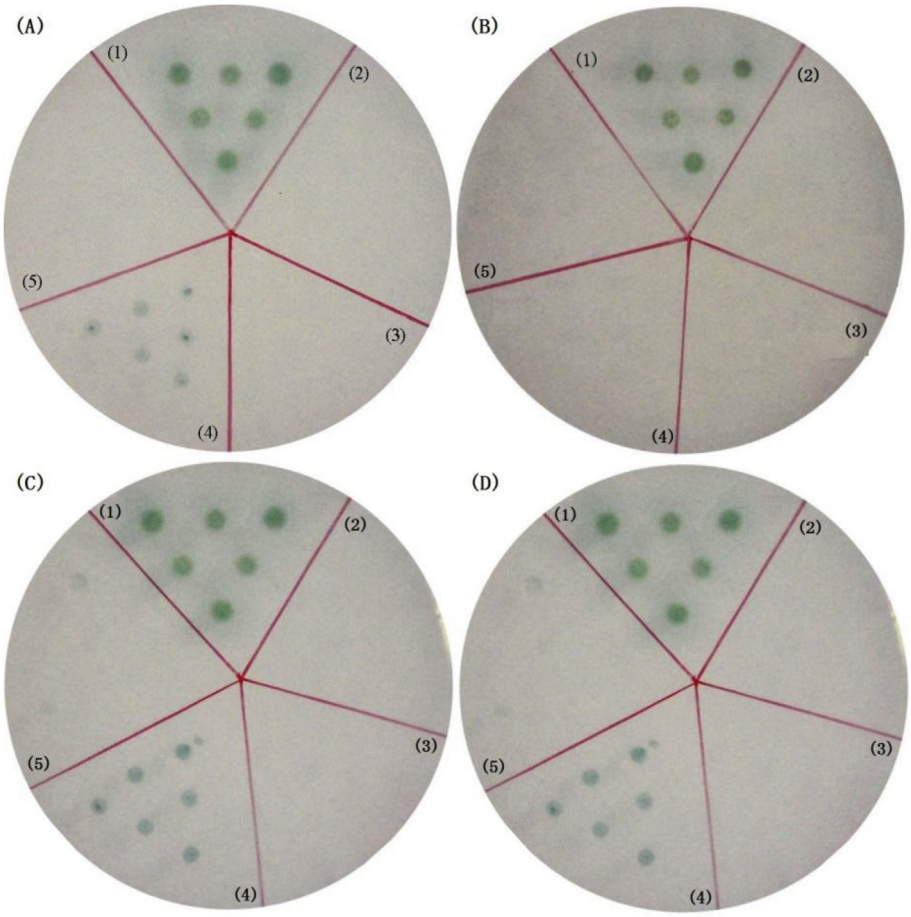

Fig. 8. Yeast two-hybrid analyses on the interaction between LvVEGF4 and LvVEGFR1 (A), LvVEGF4 and LvVEGFR2 (B), LvVEGF5 and LvVEGFR1 (C), LvVEGF5 and LvVEGFR2 (D). Yeast cells were transformed with a combination of the indicated plasmids. (1) pGBKT7-p53 and pGADT7-Tantigen for positive control; (2) pGBKT7-Lam and pGADT7-Tantigen for negative control; (3) pGADT7-VF4 and pGBKT7 plasmids (A and B), pGADT7-VF5 and pGBKT7 plasmids (C and D); (4) pGADT7-VF4 and pGBKT7-VR1(2–5) plasmids (A), pGADT7-VF4 and pGBKT7-VR2(1–4) plasmids (B), pGADT7-VF5 and pGBKT7-VR1(2–5) plasmids (C), pGADT7-VF5 and pGBKT7-VR2(1–4) plasmids (D); (5) pGBKT7-VR1(2–5) and pGADT7 plasmids (A and C), pGBKT7-VR2(1–4) and pGADT7 plasmid (B and D).

After WSSV infection, the expression levels of *LvVEGF4* and *LvVEGF5* were apparently affected. Hemocytes were the main target for expression of VEGF signaling pathway genes including *LvVEGF2*, *LvVEGF3*, *LvVEGF4*, *LvVEGF5*, *LvVEGFR1* and *LvVEGFR2* [28–31]. The expression of *LvVEGF5* after WSSV infection showed similar trends as *LvVEGF2* and *LvVEGF3*, which were down-regulated before 3 hpi and up-regulated at 24 hpi [30,31], while *LvVEGF4* showed up-regulation at 0.5 hpi and no apparent up-regulation after 24 hpi. Different from the expression trends of *LvVEGF* genes, the receptor genes *LvVEGFR1* and *LvVEGFR2* showed up-regulation after WSSV infection [28,29]. The expression patterns of *LvVEGF* genes in hemocytes presented complementary features, which might be necessary for sustained activation of VEGF signaling pathway after WSSV infection.

The VEGF signaling pathway is activated through the binding of VEGFs to the extracellular Ig domains of their receptors VEGFRs [42]. In the shrimp *L. vannamei*, LvVEGFR1 might be the receptor of LvVEGF1, LvVEGF2 and LvVEGF3, since knockdown of *LvVEGF1* and *LvVEGF2* caused a down-regulation of the expression of *LvVEGFR1* [30] and LvVEGF3 could interact with the extracellular region of LvVEGFR1 [31]. LvVEGFR2 was also the receptor of LvVEGF2 and LvVEGF3,

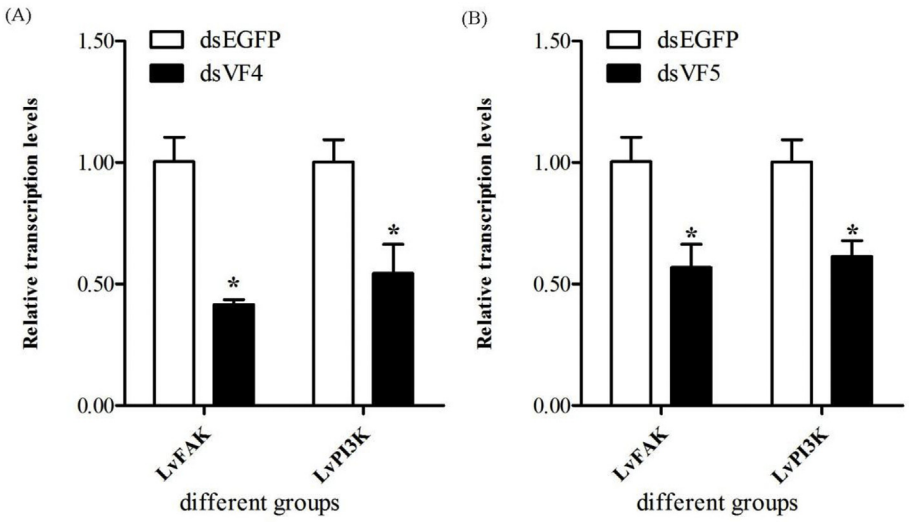

Fig. 9. Expression levels of *LvFAK* and *LvPI3K* in the cephalothoraxes of shrimp after injection of dsLvVEGF4 (A) or dsvVEGF5 (5). dsEGFP, injected with dsRNA of *EGFP*; dsVF4, injected with dsRNA of *LvVEGF4*; dsVF5, injected with dsRNA of *LvVEGF5*. Stars (*) indicate significant differences ($P < 0.05$) for the gene expression levels between two groups.

because LvVEGF2 and LvVEGF3 could interact with the extracellular region of LvVEGFR2 [29]. In the present study, both LvVEGF4 and LvVEGF5 showed interaction with the extracellular region of LvVEGFR1 rather than that of LvVEGFR2, indicating that LvVEGFR1 should be the receptor of LvVEGF4 and LvVEGF5. The multiple interactions between different VEGFs and VEGFRs might enhance the biological function of VEGF signaling pathway in shrimp.

In *L. vannamei*, VEGF signaling pathway genes have been found essential for *in vivo* viral propagation. Knockdown of either *LvVEGF* genes or *LvVEGFR* genes could obviously reduce viral copy numbers in WSSV-infected shrimp [28–31]. Similarly, silencing of *LvVEGF4* and *LvVEGF5* also inhibited *in vivo* viral propagation in WSSV-infected shrimp. Notably, studies show that injection of non-specific dsRNA might protect the hosts against pathogens causing lethal diseases [43,44]. However, our previous study shows that injection of dsEGFP has no influence on WSSV replication and the accumulated death rate of WSSV-infected shrimp when compared with injection of PBS [28]. Therefore, these data support our previous conclusion that the shrimp VEGF signaling pathway facilitates WSSV propagation.

VEGF signaling pathway usually plays its biological functions through regulating intracellular signaling pathways. In the mouse endothelial cells, the focal adhesion tyrosine kinase (FAK) was found as an essential signaling switch of the VEGF signaling pathway [45]. In the human endothelial cells, the phosphoinositide 3-kinase (PI3K) played an essential role in regulating VEGF-inhibited apoptosis [46]. In *L. vannamei*, the VEGF signaling pathway was also found modulating the expression of FAK and PI3K because knockdown of *LvVEGF* genes or *LvVEGFR* genes down-regulated the expression levels of *LvFAK* and *LvPI3K* [28–31]. Similarly, knockdown of *LvVEGF4* and *LvVEGF5* also reduced the expression levels of *LvFAK* and *LvPI3K*. These data suggest that the whole VEGF pathway in shrimp affects the expression of downstream signaling pathway genes *LvFAK* and *LvPI3K*. However, silencing of one of them could only partially reduce the expression of the downstream signaling pathway genes. This might be due to the presence of other VEGF members. Previous studies have shown that the shrimp FAK and PI3K could promote WSSV infection [47,48]. These data suggest that the VEGF signaling pathway in shrimp probably participates in WSSV infection by regulating downstream signaling pathways, which would be elucidated in further studies on the regulation mechanisms.

Acknowledgments

This work was financially supported by the General Program of National Natural Science Foundation of China (41776158, 31772880, 31502161), China Agriculture Research system-48 (CARS-48), and the Blue Life Breakthrough Program of LMBB (MS2017NO04) of Qingdao National Laboratory for Marine Science and Technology.

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