Vitellogenin Receptor (VgR) Mediates Oocyte Maturation and Ovarian Development in the Pacific White Shrimp (Litopenaeus vannamei)

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Oocyte maturation and ovarian development are sequentially coordinated events critical to reproduction. In the ovaries of adult oviparous animals such as birds, bony fish, insects, and crustaceans, vitellogenin receptor (VgR) is a plasma membrane receptor that specifically mediates vitellogenin (Vg) transport into oocytes. Accumulation of Vg drives sexual maturation of the female crustaceans by acting as a pivotal regulator of nutritional accumulation within oocytes, a process known as vitellogenesis. However, the mechanisms by which VgR mediates vitellogenesis are still not fully understood. In this study, we first identified a unique VgR (Lv-VgR) and characterized its genomic organization and protein structural domains in Litopenaeus vannamei, a predominant cultured shrimp species worldwide. This newly identified Lv-VgR phylogenetically forms a group with VgRs from other crustacean species within the arthropod cluster. Duplicated LBD/EGFD regions are found exclusively among arthropod VgRs but not in paralogs from vertebrates and nematodes. In terms of expression patterns, Lv-VgR transcripts are specifically expressed in ovaries of female shrimps, which increases progressively during ovarian development, and rapidly declines toward embryonic development. The cellular and subcellular locations were analyzed by in situ hybridization and immunofluorescence, respectively. The Lv-VgR mRNA was found to be expressed in the oocytes of ovaries, and Lv-VgR protein was found to localize in the cell membrane of maturing oocytes while accumulation of the ligand Vg protein assumed an even cytoplasmic distribution. Silencing of VgR transcript expression by RNAi was effective for stunting ovarian development. This present study has thus provided new insights into the regulatory roles of VgR in crustacean ovarian development.

Keywords: vitellogenin receptor, crustacean, phylogenetic analysis, vitellogenesis, mRNA expression, ovarian development, oocyte maturation
INTRODUCTION

Ovarian development of oviparous animal comprises the stages of oogonia, previtellogenesis and vitellogenesis (Charniaux-cotton, 1985). During development of oocytes, vitellogenesis occurs as a key process for accumulation of proteins, fats and other nutrients. Unlike the case of viviparous animals, embryos of oviparous animals develop and mature independently outside the parental body, implying that most of the developmentally requisite nutrients need to be supplied from the yolk. The quality of yolk is consequently an important factor in the successful reproduction of oviparous animals (Parma et al., 2015). Evidently, vitellogenesis is necessary for oocyte development in oviparous animals, as a critical preparatory stage to build up energy reserves and biomolecules for subsequent embryonic development (Tsukimura, 2001).

In crustaceans, the size of oocytes expands rapidly by several hundred times after the inception of vitellogenesis, with a mass of nutrients accumulated (Chen et al., 2018a). Vitellin is the most abundant protein component of yolk, which constitutes 60–90% of the total yolk protein in the eggs of crustaceans (Eastman-Reks and Fingerman, 1985). Vitellogenin (Vg) is a protein precursor of vitellin, and it is considered to be synthesized, processed, transported, absorbed and finally forms the yolk (Wahl et al., 1981). Interestingly, the sites of Vg synthesis are reported to be both endogenous and exogenous, which vary among different species, for example: the intestine of nematodes (Kimble and Sharrock, 1983), liver of fish (Hiramatsu et al., 2005; Jia et al., 2013; Chen et al., 2018b), etc. In the case of exogenous Vg, the protein is transported through blood or hemolymph and absorbed into the oocytes through endocytosis mediated by vitellogenin receptor (VgR) (Li et al., 2001; Warrier and Subramoniam, 2002).

The process of Vg/VgR endocytosis has been mainly demonstrated in oviparous amphibians (Wall and Patel, 1987), fish (Hara et al., 2016), and insects (Sappington and Raikhel, 1998). In general, exogenous Vg in the circulation binds to VgR located at the plasma membrane of oocytes to form a complex (Wall and Patel, 1987). The Vg/VgR complex is internalized into the cytoplasm as coated vesicles (Sappington and Raikhel, 1998). Finally, Vg is processed to become the mature yolk protein, while VgR is recruited to the cell membrane through the tubular vesicles (Sappington and Raikhel, 1998). Thus, VgR plays an essential role in the ovarian development of oviparous animals, which is further supported by evidence that genetic ablation or mutations of VgR could result in impaired or abnormal ovarian development, and even female sterility in birds (Bujo et al., 1995) and insects (Lin et al., 2013).

Vitellogenin receptor belongs to the low-density lipoprotein receptor (LDLR) family (Hussain et al., 1999). Structurally, it consists of five highly conserved domains, namely, the ligand-binding domain (LBD), EGF-precursor homology domain (EGFD), O-like sugar domain (OLSD), transmembrane domain (TM) and cytosolic domain that contains an internalization motif (IM) (Tufail and Takeda, 2009). VgR CDNAs have been amply found in oviparous invertebrates and lower vertebrates, including birds (Stifani et al., 1990), fish (Tao et al., 1996; Hiramatsu et al., 2004; Mizuta et al., 2013), amphibians (Okabayashi et al., 1996), arthropod insects (Schonbaum et al., 1995; Lin et al., 2013; Zhang H. et al., 2019) and crustaceans (Tiu et al., 2008; Roth and Khalaila, 2012; Bai et al., 2016). In crustaceans including Penaeus monodon (Tiu et al., 2008), Macrobrachium nipponense (Bai et al., 2016), and Macrobrachium rosenbergii (Roth and Khalaila, 2012; Guo et al., 2019), transcript expression of VgR is specifically detected in the ovaries, with the maximal expression levels being reached during the mid-ovarian developmental stages. RNA interference (RNAi) of VgR reportedly suppressed Vg accumulation in the ovary of P. monodon (Tiu et al., 2008) and delayed the maturation of the ovary in M. nipponense (Bai et al., 2016). However, the mechanistic details of VgR regulation of ovarian development is still incompletely understood and at times shown to be controversial in different crustacean species (Tiu et al., 2008; Bai et al., 2016).

The Pacific white shrimp (Litopenaeus vannamei), belonging to Arthropoda, Crustacea, and Decapoda in taxonomy, is the most important cultured shrimp species in the world. In captivity, the ovaries of cultured L. vannamei are often unable to mature as they would naturally (Chen et al., 2014). Following artificial unilateral eyestalk ablation and nutritional supplementation, however, the ovaries of L. vannamei can mature and allow spawning within 3–5 days (Chen et al., 2018a). In L. vannamei, the precise source of Vg production during vitellogenesis remains obscure, for which the ovary as an endogenous site and the hepatopancreas as an exogenous site have both been suggested (Raviv et al., 2006; Ventura-Lopez et al., 2017). The regulatory mechanisms for Vg gene expression have also been explored in the ovary (Tsutsui et al., 2007, 2013; Bae et al., 2017; Chen H. Y. et al., 2018; Kang et al., 2019) and hepatopancreas (Chen et al., 2014, 2018a; Luo et al., 2015). In addition to Vg synthesis, efficient absorption and accumulation of Vg by the oocytes in the ovary is another vital process for vitellogenesis in oviparous animal (Stifani et al., 1990). However, information regarding how VgR-mediated Vg accumulation and ovarian development is achieved in L. vannamei is still limited. In this study, we established the genetic basis and functional importance of VgR in L. vannamei ovarian maturation by (1) identifying the full-length cDNA of VgR in L. vannamei (Lv-VgR) and analyzing its corresponding gene and protein structures; (2) determining the expression patterns of Lv-VgR in different tissues, across ovarian developmental stages including embryonic and larval periods; (3) visualizing the Lv-VgR mRNA and protein positive cells in the ovaries; and (4) evaluating the effects of Lv-VgR RNAi on the ovarian development in morphological, anatomical and histological contexts. Overall, we study has provided new information for understanding the mechanisms underlying oviparous ovarian development, which may help to improve artificial culture of an economically valuable penaeid shrimp species.
MATERIALS AND METHODS

Animals
For molecular cloning, tissue distribution and ovarian development, healthy Pacific white shrimp (L. vannamei) were collected from the Haihao Shrimp Culture Center, (Zhanjiang, China) and they were maintained in filtered seawater [30 parts per thousand (ppt), pH 8.2] at 30°C under a 12-h dark:12-h light photoperiod with a feeding schedule of twice a day. The shrimp were anesthetized on ice and killed by decapitation. The animal experiments were conducted in accordance with the guidelines and approval of the ethics committees of South China Sea Institute of Oceanology, Chinese Academy of Sciences.

Molecular Cloning of Lv-VgR cDNA
Total RNA was extracted from the ovaries of sexually mature female shrimp using TRIzol reagent (Invitrogen, Carlsbad, CA, United States) and reversely transcribed into first-strand cDNA with PrimeScript II™ 1st strand cDNA Synthesis Kit (Takara, Dalian, China). Based on a unigene that was obtained from a L. vannamei Illumina transcriptome constructed by our lab previously and shares high sequence homology with the P. monodon VgR, gene-specific primers (Table 1) were designed to amplify four overlapped partial fragments of VgR in L. vannamei (Lv-VgR), and the corresponding full-length sequence was obtained by 5′- and 3′-rapid amplification of cDNA ends (RACE) as described previously (Chen et al., 2008).

Bioinformatics Analysis
The gene organization of Lv-VgR was generated by comparing the obtained cDNA sequence in this study and the gene sequence which was obtained from the L. vannamei genome (Zhang X. J. et al., 2019a). The open reading frame (ORF) of Lv-VgR was determined by ORF finder and the corresponding amino acid (a.a.) sequence was deduced by using ExPaSy translate tool. The molecular weight and theoretical isoelectric point (pI) of Lv-VgR were calculated by ExPaSy ProtParam tool. Signal peptide and transmembrane helices were predicted by SignalP 4.0 Server and TMHMM Server v.2.0, respectively. N- and O-linked glycosylation sites were identified by NetNGlyc 1.0 Server and YinOYang 1.2 Server, respectively. Structural domains were analyzed by using SMART program and ScanProsite program. Multiple a. a. sequences alignment was performed using Clustalx 1.8 and presented by GeneDoc. Phylogenetic tree was conducted by a Neighbor Joining method using MEGA6 with bootstrap of 1000 replicates.

Lv-VgR Transcript in Different Tissues, Ovarian Developmental Stages, and Embryonic and Laval Stages
The tissue expression pattern of Lv-VgR mRNA were detected in selected tissues, included the heart, gill, eyestalk, intestine, thoracic nerve, ventral nerve, muscle and hepatopancreas from the sexually immature adult shrimp (7.85 ± 2.58 g), and sexually mature male (31.34 ± 5.36 g) and female shrimp (37.49 ± 6.91 g), respectively, and the testis from the sexually mature male shrimp and the ovary from the sexually mature female shrimp. The mRNA expression profile of Lv-VgR was further detected in the ovaries during maturation. In this case, previtellogenic female shrimp were selected for artificially induced maturation with unilateral eyestalk ablation and nutrition enhancement (Chen et al., 2018b), and ovarian development was defined into four stages, namely, the stages I–IV, based on the classification of predominant oocytes as described previously (Chen H. Y. et al., 2018). For ontogeny, the mRNA levels of Lv-VgR were detected in the embryonic and larval developmental stages included the zygote, blastula, gastrula, limb bud embryo, larva in membrane, nauplius, zoa, mysis, and post-larval. In this case, about thirty individuals were mixed together as a sample and three samples were detected in a developmental stage. The embryonic and larval samples were collected when 80% of the population had reached the objective stage, and the morphologies was determined as described previously (Wei et al., 2014). The tissue, embryonic and larval samples were immediately frozen in liquid nitrogen and stored in 80°C, or directly stored in RNAlater solution (Ambion, Austin, TX, United States) for further analysis. The transcript levels of Lv-VgR were measured by quantitative real-time PCR (qPCR). Briefly, total RNA was extracted with TRizol reagent and reverse transcribed with PrimeScript™ RT reagent Kit containing gDNA eraser (Takara). The cDNA samples obtained were then subjected to a Thermal Cycler Dice® Real

### Table 1 | Primers used for cloning, RT-PCR, DIG-labeled DNA probe and the sequences for siRNA synthesis.

| Primer name | Sequence (5′–3′) | Purpose |
|-------------|-----------------|---------|
| VgR-5′-end-F | GGAAGAAGCTCTTCGCAAGA | Primer for 5′ end fragment |
| VgR-5′-end-R | AATGGGACGCACTTCTGAACCT | Primer for 5′ end fragment |
| VgR-A-F | TGCAGCAAGCGATGTTGATT | Middle fragment A |
| VgR-A-R | TTGCTTTACCTTGAAGGATTGAC | Middle fragment A |
| VgR-B-F | GTGCGAAGAATCTTGGTCTGCA | Middle fragment B |
| VgR-B-R | CTGCAATTAGGGGCTTCCTGC | Middle fragment B |
| VgR-C-F | TAATCTGCGCAACCAAGA | Middle fragment C |
| VgR-C-R | CCAACCTGTCGCAAGGC | Middle fragment C |
| VgR-D-F | CTGGACAGAAACCTTATTGCGC | Middle fragment D |
| VgR-D-R | CTGGCTGTGCTACTATGCGC | Middle fragment D |
| VgR-3′-race-F1 | TCCCTTGAGACCCGTTAGGA | 3′ RACE |
| VgR-3′-race-F2 | TTCTTGATATAGTCGTCGGQGATG | 3′ RACE |
| VgR-3′-race-F3 | ACTGACTACTACCTGTAAGTGAG | 3′ RACE |
| VgR-al1-F | GGAAGAAGCTCTTCGCAAGA | Full length |
| VgR-al1-R | TGCAGCTCTCAGAAGGATTGAC | Full length |
| VgR-al2-F | CCAATGATACCTCGAATTACCC | Full length |
| VgR-al2-R | TGTTCTGCTCGTTGTCGTTG | Full length |
| VgR-q-F | GGTTGTCGAGCTTGCGTGTG | RT-PCR |
| VgR-q-R | CCACTTACCTGCGTGTG | RT-PCR |
| GAPDH-F | CCACTTACCTGCGTGTG | RT-PCR |
| GAPDH-R | AGAACAAGCTGACTGCAAGA | RT-PCR |
| VgR-PF | TAATCTGCGCAACCAAGA | Synthesis of DIG-labeled DNA probe |
| VgR-PR | CCAACCTGTCGCAAGGC | Synthesis of DIG labeled DNA probe |
| VgR-3′-F1 | GCGAAGAAGCTCTTCGCAAGA | VgR siRNA |
| VgR-3′-R1 | UAAUGUUGUUGGUAUGCUCCU | VgR siRNA |
| EGFP-482-F | GCAUCUGUAGUGUAGUCCAU | NC siRNA |
| EGFP-482-R | UUGAAAGUUCUACUCGGUAGC | NC siRNA |
Time System III (Takara) for quantitative analysis with the TB Green™ Premix EX Taq™ II Kit (Takara) and specific qPCR primers for target genes (Table 1). In this case, GAPDH was used as an internal control based on its stable expression levels observed.

**In situ Hybridization**

The cellular localization of Lv-VgR mRNA in the ovaries during different developmental stages were performed by *in situ* hybridization (ISH). Briefly, samples from the ovaries were removed quickly and fixed overnight in 4% Paraformaldehyde Fix Solution (BBI, Sangon Biotech, Shanghai, China) for overnight. After a series of concentrations of alcohol dehydration, and paraffin embedding, the ovarian samples were cut into 5-µm sections and mounted onto slides. The sections were then digested by Proteinase K (20 µg/ml) at 37°C for 25 min, prehybridized at 42°C for 2 h and hybridized with a digoxigenin (DIG)-labeled DNA probe (8 ng/µl, Roche, Basel, Switzerland, *Table 1*) against *Lv-VgR* mRNA at 42°C for overnight. The ISH signal was developed by a diaminobenzidine (DAB) method by incubation with horseradish peroxidase (HRP)-conjugated anti-DIG antibody, and the nucleus was stained with hematoxylin.

**Immunofluorescence**

The antigens of *Lv-VgR* (a.a. 73–398) and *Lv-Vg* (a.a. 19–366) were generated by an *Escherichia coli* recombinant protein expression system. The polyclonal antibodies against VgR and Vg were purified from the serum of a rabbit with antigen injection of Freund's adjuvant and 300 µg rVgR/rVg for four times at 12 days intervals (Genecreate Biological Engineering Company, Wuhan, China). Complete Freund's adjuvant was used for the first immunization and incomplete Freund's adjuvant were used for the three more immunizations. The VgR and Vg antibodies were labeled with the FITC dye (488 nm, Invitrogen) and Cy3 dye (532 nm, Invitrogen), respectively, for visualization of the endogenous proteins, and DAPI reagent (405 nm, Invitrogen) was used for staining of the cell nucleus. The immunofluorescence (IF) was performed as described previously (Pan et al., 2018), and fluorescent images were observed and recorded with a DM-IRB fluorescence microscopy (Leica, Frankfurt, Germany).

**RNA Interference for Inhibition of Ovarian Development**

The roles of VgR in the ovarian development of female shrimp were investigated by using an RNA interference (RNAi) approach. Based on the *Lv-VgR* cDNA sequence, the potential small interfering RNA (siRNA) targeting sites were identified with siDirect version 2.0 and DSIR online program, and the specificity of potential siRNAs was assessed with a global Blast. In this study, EGFP-482 siRNA was used to be non-targeting siRNA (NC siRNA), and the *Lv-VgR* and NC siRNA were synthesized by Sangon Biotech Company (Shanghai, China).

Before injection of siRNA, 80 female shrimps with intact eyestalks were kept in a 15-m³ culture pond for nutrition enhancement for a week. After that, the shrimp were applied for unilateral eyestalk ablation and the ovaries began to develop subsequently. At 36 h later, 28 shrimp at the previtellogenic ovarian developmental stage were picked out and randomly transferred to seven tanks (4 individuals per 1-m³ tank). Shrimp in one tank were killed and sampled immediately for the 0 time point and in other six tanks were injected with siRNA as described before (Cai et al., 2017). Shrimp in each two tanks were set as one group and injected with 375 µl of DEPC-treated PBS, NC siRNA [200 ng/g body weight (bwt)] and *Lv-VgR* siRNA (200 ng/g bwt), respectively. The siRNA was diluted in DEPC-treated PBS. The shrimp in each group were killed and sampled at 24 and 48 h after injection. The GSIs were weighed and calculated after sampling, and the ovarian developmental stages were confirmed by the observation of paraffin sections with hematoxylin and eosin (H/E) staining. In parallel experiment, the efficiencies of *Lv-VgR* siRNA was assessed by qPCR as described above.

**Data Transformation and Statistical Analysis**

For qPCR, the relative expression levels of *Lv-VgR* were calculated using the comparative Ct method with the formula $2^{-\Delta \Delta Ct}$. The raw data were simply transformed as a percentage of the mean values in the control group, and the statistical analysis were performed with SPSS (IBM Software, Seattle, WA, United States). For qPCR and GSI measurement, the data expressed as the mean ± SE (standard error) were analyzed by using Student's *t*-test or one-way ANOVA followed by Fisher’s least significant difference (LSD) test.
RESULTS

Molecular Cloning and Structural Characterization of Lv-VgR

In this study, the full-length cDNA of vitellogenin receptor (Lv-VgR) was identified from the ovary of Pacific white shrimp. The Lv-VgR cDNA is 6019 bp in length, consisting of a 61-bp 5′ untranslated region (UTR), a 5832-bp ORF and a 126-bp 3′-UTR with a 28-bp poly-A tail (GenBank accession No. MN807241, Supplementary Figure S1). By screening the *L. vannamei* genomic data, the Lv-VgR gene was found to be located in the LOC113811783 of the LV AN scaffold 2260, and the Lv-VgR gene is 6019 bp in length with 42 exons that were separated by 41 introns (Figure 1A).

The Lv-VgR precursor protein is 1943-amino acid (a.a.) in size, and it is composed of a 34-a.a. signal peptide and a 1909-a.a. mature protein with a deduced molecular weight of 213.58 kDa and pI of 5.21 (Supplementary Figure S1). Similar to the VgRs from other oviparous animals, Lv-VgR contains the five typical conserved modular elements for the LDLR superfamily (Figure 1B), namely, LBD, EGFD, OLSD, TM, and IM. Lv-VgR has two LBD and two EGFD. LBD 1 consist of five LDLR class A repeats and the LBD 2 consist of eight class A repeats. EGFD 1 contains eight YWTD repeats and four epidermal growth factor (EGF)-like repeats, while EGFD2 contains two YWTD repeats and four EGF-like repeats. A Thr- and Ser-rich OLSD are located in front of the TM region, followed by a cytosolic tail contains two IM motifs with the conversed NPX(Y/F) characteristics. Moreover, 10 putative *N*-linked glycosylation motifs and 2 putative *O*-linked glycosylation sites were observed in the Lv-VgR precursor.

Comparative and Phylogenetic Analysis of Lv-VgR

Multiple alignment was performed with the VgR a.a. sequences from various oviparous species (Figure 2). In this case, the newly obtained Lv-VgR only share high sequence identities of its counterpart in other crustaceans (*P. monodon*, 84%), but not other VgR from insects (*Aedes aegypti*, 24%; *Spodoptera exigua*, 22%; and *Drosophila melanogaster*, 22%) or vertebrates (*Gallus gallus*, 11%; *Xenopus laevis*, 11%; and *Morone americana*, 11%). The number of amino acid residues of VgRs from arthropods are almost twice of that from vertebrates, based on the fact that duplicated LBD/EGFDs are presented in the arthropod VgRs while only a single LBD/EGFD is found in the vertebrate VgRs. The VgR a.a. sequences were further analyzed comparatively by dividing into eight structural regions including SP, LBD 1, EGFD 1, LBD 2, EGFD 2, OLSD, TM, and cytoplasmic tail (Table 3). Although the identities for the whole sequences are low, the structural regions of VgRs are reasonably conserved between different species. The LBD/EGFD regions of vertebrate VgRs show relatively high conservation in both the former and latter
FIGURE 2 | Amino acid sequence alignment of VgR from different oviparous animal, including Gallus gallus (P98165.1), Xenopus laevis (BAA22145.1), Morone americana (AAO92396.1), Spodoptera exigua (AOX13593.1), Aedes aegypti (AAK15810.1), Drosophila melanogaster (AAB60217.1), Penaeus monodon (ABW79798.1), and Litopenaeus vannamei (MN807241). The conserved amino acid residues are boxed in purple, yellow, and blue, respectively, based on the percentages of their identities. The sequence identities between Lv-VgR and the VgR from other species are also showed.

LBD/EGFD regions (27–37%/15–18%) of the vertebrate VgRs, and are more similar to the latter one based on lengths and the identities of the a.a. sequence (Table 3).

The Genbank accession numbers of protein used in this study are shown in Table 2. A phylogenetic analysis was conducted with the VgRs from multiple oviparous animal species (Figure 3A). The phylogenetic tree is divided into two three clusters, namely, the VgRs from vertebrates, arthropods and nematodes. The newly identified Lv-VgR is grouped into a sub-branch of crustacean VgRs that is closed to the branch of insect VgRs. The structure domains for the corresponding VgRs were also analyzed comparatively (Figure 3B). The characteristic domains, such as LBD, EGFD, OLSD, TM, and IM, are observed in most of LDLR superfamily members. However, the duplicated LBD/EGFD regions are only found in the arthropod VgRs but not in their counterparts from vertebrates and nematodes. In addition, OLSD is missing in a larger number of arthropod VgRs but remains in Lv-VgR.

Transcript Expression of Lv-VgR in Different Tissues, Ovarian Developmental Stages, and Embryonic and Laval Stages

The transcript expression of Lv-VgR were detected in multiple tissues from sexually immature shrimp, and sexually mature male and female shrimp by quantitative qPCR. As shown in Figure 4A, the Liv-VgR mRNA was specifically expressed in the ovary of sexually mature female shrimp, but hardly detected in any other tissues form sexually immature and mature shrimp. During the ovarian development, the expression levels of Lv-VgR increased continuously from stage I to stage IV, and reached the highest abundance in the stage IV (Figure 4B). During the embryonic and larval developmental stages, the highest expression level of Lv-VgR was observed at the stage of zygote and reduced sharply at the stages of blastula and gastrula. The expression of Lv-VgR was further decreased at the stage of zoea and kept at very low levels in the larval (Figure 4C).

**In situ Hybridization for Lv-VgR mRNA During Ovary Development**

The cellular expression of Lv-VgR mRNA were detected by ISH in the ovaries in different developmental stages. Compared with the negative control group without digoxigenin probe, the experimental group showed strong positive signals for Lv-VgR expressed cells. As shown in Figure 5A, Lv-VgR mRNA was abundant in the cytoplasm of the oocytes. During development of the oocytes, the intensity of hybridization signals increased, reaching a maximum during stages III–IV, which is consistent with the mRNA expression profile by qPCR.
TABLE 3 | The identity between the Pacific white shrimp and other species in individual domain.

| Region | SP | LBD 1 | EGFD 1 | LBD 2 |
|--------|----|-------|--------|-------|
| Special | Location | Length | Identity | Location | Length | Identity | Location | Length | Identity | Location | Length | Identity | Class A |
| G. gallus | 1–46 | 46 | 23% | 50–375 | 326 | 28% | 376–770 | 395 | 15% | 50–375 | 326 | 37% | 8 |
| X. laevis | 1–27 | 27 | 17% | 31–356 | 326 | 30% | 358–750 | 393 | 15% | 31–356 | 326 | 36% | 8 |
| M. americana | 1–25 | 25 | 13% | 29–354 | 326 | 27% | 356–749 | 394 | 16% | 29–354 | 326 | 37% | 8 |
| S. exigua | 1–17 | 17 | 8% | 29–217 | 189 | 28% | 218–938 | 725 | 21% | 942–1283 | 342 | 32% | 7 |
| A. aegypti | 1–30 | 30 | 19% | 44–251 | 208 | 32% | 253–951 | 699 | 27% | 955–1301 | 347 | 34% | 8 |
| D. melanogaster | NA | NA | NA | 89–306 | 218 | 31% | 309–1026 | 718 | 25% | 1030–1377 | 348 | 32% | 8 |
| P. monodon | 1–20 | 20 | 44% | 77–279 | 203 | 88% | 280–978 | 699 | 87% | 981–1327 | 347 | 80% | 8 |
| L. vannamei | 1–34 | 34 | 100% | 93–295 | 203 | 100% | 296–991 | 696 | 100% | 994–1332 | 339 | 100% | 8 |

| Region | EGFD 2 | OLSD | TM | Cyto | Total length | Overall identity |
|--------|-------|------|-----|-----|-------------|-----------------|
| Special | Location | Length | Identity | YWTD + EGF | Location | Length | Identity | Location | Length | Identity | Class A |
| G. gallus | 376–770 | 385 | 18% | 5 + 3 | 787–809 | 23 | 17% | 810–863 | 54 | 12% | 863 | 11% |
| X. laevis | 358–750 | 393 | 18% | 5 + 3 | 793–815 | 23 | 26% | 816–869 | 54 | 12% | 869 | 11% |
| M. americana | 356–749 | 394 | 18% | 5 + 3 | 768–790 | 23 | 17% | 791–844 | 54 | 13% | 844 | 11% |
| S. exigua | 1284–1662 | 379 | 19% | 3 + 3 | 1689–1711 | 23 | 12% | 1712–1814 | 103 | 11% | 1814 | 22% |
| A. aegypti | 1302–1689 | 388 | 20% | 3 + 3 | 1722–1744 | 23 | 16% | 1745–1847 | 103 | 11% | 1847 | 24% |
| D. melanogaster | 1379–1770 | 393 | 16% | 3 + 3 | 1799–1820 | 22 | 37% | 1821–1984 | 164 | 8% | 1984 | 22% |
| P. monodon | 1328–1769 | 442 | 81% | 3 + 4 | 1784–1806 | 22 | 86% | 1807–1941 | 135 | 92% | 1941 | 84% |
| L. vannamei | 1333–1774 | 442 | 100% | 2 + 4 | 1788–1810 | 23 | 100% | 1811–1943 | 133 | 100% | 1943 | 100% |
**Immunofluorescence for Localization of VgR and Vg Protein in the Ovary**

By IF, the protein distribution of VgR and Vg were exhibited in the ovarian sections (Figure 5B). In this case, no signals of VgRs were presented in the follicle cells. In the endogenous vitellogenetic oocytes, immunopositive signals of VgRs were only detected in the cytoplasm of, and in the exogenous vitellogenetic oocytes, VgRs were shown to gather from the cytoplasm to plasma membrane. On the contrary, VgR were detected strongly in the plasma membrane and weakly in the cytoplasm of the mature oocytes. In parallel experiment, Vg was exhibited a evenly distribution in the cytoplasm of the exogenous vitellogenetic and mature oocytes.

**Inhibition of Ovarian Development by RNAi of Lv-VgR**

Injection of VgR-siRNA can effectively reduce the transcript expression of *Lv-VgR* in the *L. vannamei* ovaies (Figure 6A). The effect of *Lv-VgR* mRNA silencing on ovarian development was assessed by measuring their GSIs (Figure 6B). In either the blank group injected with PBS or the negative group injected with NC-siRNA, the ovaries of shrimp kept increasing continuously, and reached the corresponding GSIs of stages III and IV at 24 and 48 h after injection, respectively. In contrast, the GSIs of shrimp injected with VgR-siRNA remained at stage II, which was much lower than those injected with PBS or NC-siRNA at both the 24 and 48 h after injection. In summary, silencing of *Lv-VgR* mRNA is effective in inhibiting ovarian development.

By morphological and anatomical observations, the ovaries from shrimp with different treatments have different features (Figure 6C). The ovaries from those without injection were thin and transparent in color of cyan [Figure 6C(O)], and hardly to be observed through the carapace by either dorsal or lateral views [Figures 6C(A,B)]. After that, the ovaries from those injected with PBS or NC-siRNA at 24 h became hypertrophic. Due to accumulation of Vg and other substances, such as carotenoid, the ovaries were became yellow in color and no longer transparent [Figures 6C(P,Q)]. Therefore, the ovaries can be clearly observed through the carapace at this time point [Figures 6C(C–F)]. Finally, the ovaries from those injected with PBS or NC-siRNA at 48 h reached their maximum volume and became fragile [Figures 6C(S,T)]. However, the ovaries form those injected with VgR-siRNA remained thin at either 24 or 48 h [Figures 6C(R,U)]. Interestingly, although the ovaries form the VgR-siRNA injected shrimp kept in the sizes similar to those at stage II, they could be observed through the carapace, since that their color changed to yellow and no longer transparent [Figures 6C(G,H,M,N)].

By further histological analysis with H/E staining (Figure 6D), the ovaries from the 0-h non-injected group mainly comprised of oogonia and previtellogenic oocytes, with a few endogenous vitellogenetic oocytes. The ovaries from the PBS and NC-siRNA injected groups were full of exogenous vitellogenetic oocytes at 24 h after injection, and exogenous vitellogenetic oocytes and mature oocytes with oil droplet deposition at 48 h after injection. On the contrary, the ovaries from the VgR-siRNA injected group still contained a large number of previtellogenic oocytes and endogenous vitellogenetic oocytes at 24 and 48 h after injection. It is notable that the sizes of the exogenous vitellogenetic oocytes found in the ovaries from the VgR-siRNA injected group were significantly smaller than those from the non-injected, and PBS and NC-siRNA injected groups, indicating that the accumulation of Vg was blocked by VgR silencing in the developing oocytes.
**DISCUSSION**

In this study, the full-length cDNA of VgR has been obtained from Pacific white shrimp *L. vannamei* (Supplementary Figure S1), with predictions of its corresponding gene structure (Figure 1A) and protein structural domains (Figure 1B). Similar to other members in the LDLR superfamily, Lv-VgR contains five high conserved regions including LBD with Class A repeats, EGFD with EGF-like and YWTD repeats, OSLD, TM, and IM (Figure 3B). The Class A, EGF-like and YWTD repeats present in almost all VgRs in oviparous animals, but the specific numbers for these repeats vary in different species (Figure 3B). In general, the LBD of vertebrate VgRs consist of 8 Class A repeats (Okabayashi et al., 1996; Hiramatsu et al., 2004), while the LBD 1 and LBD 2 of arthropod VgRs normally have 4–5 and 6–8 repeats, respectively (Schonbaum et al., 1995; Chen et al., 2004; Tiu et al., 2008; Lin et al., 2013). Similarly, there were 5 YWTD repeats surrounded by EGF-like repeats in the EGFD of vertebrate VgR (Bujo et al., 1994), and 7–9 and 1–4 repeats are shown in the EGFD 1 and EGFD 2 of arthropod VgRs (Chen et al., 2004; Tiu et al., 2008). It has been previously reported that EGFD indirectly affects ligand/receptor binding and dissociation (Davis et al., 1987; Russell et al., 1989), and that variation of the extracellular domains of VgRs reflect the type of potential ligands (Hiramatsu et al., 2015). For example, VgRs in Chicken can recognize Vg, VLDLR and Riboflavin binding protein (Stifani et al., 1990; Mac Lachlan et al., 1994). Similarly, the types and numbers of intracellular motifs as the internalized signals are not identical for different members in the LDLR family, e.g., for the crustacean VgRs, the sequences of IM are normally NP(X)F (Tiu et al., 2008; Roth and Khalaila, 2012; Bai et al., 2016), while for most of the insect VgRs, they are usually L(I/L) (Tufail and Takeda, 2009).

Phylogenetically, major yolk protein precursor (Vg) of crustacean is closely related to the insect apolipopophin II/I (ApoLp-II/I) and vertebrate apolipoprotein B (ApoB), but distant from the Vgs from other oviparous species (Avarre et al., 2007). It is speculated that in the lineage of crustacean, the true ortholog of ancestral Vg gene has been lost, and this group of animals may then utilize either Apo and/or clotting protein (CP) as the major yolk protein (Wu et al., 2013). Thus, the
ancestral Apo gene has been named as Vg in the crustacean lineage. However, there is still a lack of clarity regarding the generation of VgRs in different taxons of oviparous animals. Based on the phylogenetical analysis, our present study has shown that the ancestral arthropod VgR gene remains in both Insecta and Crustacea as the modern insect and crustacean VgR genes. Furthermore, there are two LBD/EGFD regions are present in the arthropod VgRs while only a single LBD/EGFD region is found in the vertebrate VgRs (Figure 3B). Combined with the phylogenetical and structural analyses, it is speculated that the LBD 1/EGFD 1 region is replicated from the LBD 2/EGFD 2 region, and this region duplication occurs after the differentiation of arthropod VgR and before the divergence of insects and crustaceans.

mRNA expression of VgR was specifically detected in the ovary of L. vannamei (Figure 4A), consistent with previous reports in vertebrates (Bujo et al., 1994; Okabayashi et al., 1996; Mizuta et al., 2013) and other crustaceans (Tiu et al., 2008). During ovarian development, expression of Vg in the hepatopancreas of L. vannamei increased continuously and reached a peak in stage III of ovarian development, reducing rapidly at stage IV (Chen et al., 2018b). The current study illustrated that the expression levels of Lv-VgR in the ovary increased continuously from the ovarian developmental stage-I to stage-IV (Figure 4B), indicating that a exogenous Vg absorption process similar to other oviparous animals which exhibit exogenous vitellogenesis, such as fish (Hiramatsu et al., 2015; Hara et al., 2016) and insects (Sappington and Raikhel, 1998). During embryonic and larval stages, the expression levels of Lv-VgR was reduced rapidly, in accordance with the trend of yolk consumption. After breaking out of the egg membrane, the nauplius still consumed the remaining yolk sac nutrients. The expression of Lv-VgR were detectable until the zoæ begin feeding and subsequently no longer need the yolk nutrition.

The cellular localizations of Lv-VgR mRNA and protein were determined by ISH and IF, respectively. In this case, Lv-VgR mRNA is expressed in the oocytes but not the follicle cells of the ovary (Figure 3A), and Lv-VgR protein is located in the membrane of oocytes while the Lv-Vg protein accumulates densely in the cytoplasm of oocytes (Figure 3B). In fish, it is generally considered that Vg enters the growing oocytes via selective endocytosis mediated by the plasma membrane VgR (Hiramatsu et al., 2013). In the process of oocyte development, follicle cells perform many essential physiological functions including supplying glycogen, engulfing cell debris, promoting ovulation, and synthesis of steroid hormones (e.g., androgen, estrone and 17β-estradiol) and cellular transmitters (e.g., insulin-like growth factor, bone morphogenetic proteins and transforming growth factor β) (Young and McNeilly, 2010; Chen et al., 2018a). Distribution of VgR mRNA/protein is absent in the follicle cells of the L. vannamei ovary, similar to that reported in P. monodon (Tiu et al., 2008). However, in P. monodon, VgR was distributed evenly throughout the oocyte cytoplasm (Tiu et al., 2008), which is likely due to the endocytic VgR being detected by IF in cytoplasm. During the ovarian development of L. vannamei, the VgR protein distributed...
**FIGURE 6**

(A) The expression level of *Lv-VgR* mRNA at 0, 24, and 48 h after the injection of PBS, NC-siRNA or VgR-siRNA, and different lowercase letter represented a significant level (*P* < 0.001, ANOVA followed by Fisher’s LSD test). In this study, the data are expressed as mean ± SE (*n* = 6).

(B) The gonadosomatic index (GSI) of female shrimp at 0, 24, and 48 h after the injection of PBS, NC-siRNA or VgR-siRNA. Different ovarian developmental stages were represented by I, II, III, and IV, respectively. The significant differences were assessed using Student’s *t*-test (*P* < 0.05, **P** < 0.01, ***P** < 0.001). In this study, the data are expressed as mean ± SE (*n* = 4).

(C) Morphological and anatomical analysis for the ovarian development of *L. vannamei*. Panels (A,C,E,G,I,K,M) are dorsal views. (Continued)
from evenly in the cytoplasm to strongly located in the plasma membrane (Figure 5B). Meanwhile, the Vg protein started to be rapidly accumulated in the oocytes. This result indicates that VgR protein transferring from the cytoplasm to plasma membrane predicts the beginning of the endogenous vitellogenetic stage of L. vannamei, and it is also the beginning for a rapid accumulation of yolk in the ovary.

The effects of VgR silencing on shrimp ovarian development were measured by morphological, anatomical and histological methods. The stunting of ovarian development was significantly observed in the shrimp injected with VgR-siRNA. Based on the GSI values, the ovaries in VgR-siRNA treated shrimp remained in stage II until 48 h after the injection, showing the inhibitory effect of VgR blockage on ovarian development (Figure 6B). In general, features of ovaries will appear a series of changes during development, such as from cyan to yellow, from transparent to non-transparent, from thin to hypertrophic, from elastic to fragile. The ovaries from VgR-siRNA injected shrimp were thin and elastic but no longer transparent and yellow [Figures 6C(R,U)]. Remaining thin and elastic showed that the ovaries lacked Vg accumulation. Becoming non-transparent and yellow meant that the hydrolysis of existent Vg protein and the accumulation of carotenoids were not inhibited. By histological level, the sizes of the exogenous vitellogenic oocytes found in the ovaries from VgR-siRNA injected shrimp [marked by black circles in Figures 6D(K,M)] were smaller than those in normal condition [Figures 6D(H,J)], showing that the nutritional deficiencies of oocytes by VgR blockage. As a whole, it is speculated that silencing of Lv-VgR can reduce the transcription and translation levels of Lv-VgR and resulted in a limitation of Vg accumulation but not other processes in ovarian development.

In summary, we have cloned the full-length cDNA sequence of vitellogenin receptor from the Pacific white shrimp, characterized its genomic organization and protein structural domains. A phylogenetical model of VgRs in different taxons of oviparous animals was proposed. Transcripts of Lv-VgR were found to be specifically expressed in the ovaries of female shrimps, with increasing expression levels during ovarian development, and rapidly declines during embryogenesis. In the ovary, VgR protein is located in the cell membrane of maturing oocytes while the accumulated Vg protein is evenly distributed in the cytoplasm. In this vein, we reason that transfer of VgR protein from the cytoplasm to plasma membrane predicts the beginning of rapid yolk accumulation in the ovary of L. vannamei. Silencing of VgR transcript expression by siRNA injection was effective in stunting ovarian development of L. vannamei. The mode of action of VgR was further investigated by observations at the morphological, anatomical and histological levels. While evidently important to the process of Vg accumulation, VgR signaling does not seem to impact other steps of ovarian development. This study has furnished new information for advancing our understanding on the mechanisms of ovarian development underlying crustacean reproduction biology.

**DATA AVAILABILITY STATEMENT**

All datasets generated for this study are included in the article/Supplementary Material.

**ETHICS STATEMENT**

The animal experiments were conducted in accordance with the guidelines and approval of the Ethics Committees of South China Sea Institute of Oceanology, Chinese Academy of Sciences.

**AUTHOR CONTRIBUTIONS**

XuW, CH, and TC conceived and designed the experiments. YR, N-KW, XZ, XiW, and XJ performed the experiments. YR, N-KW, XZ, CZ, CR, PL, XJ, and TC analyzed the data. CZ, CR, XJ, JJ, XuW, CH, and TC contributed reagents, materials, and analysis tools. YR, N-KW, XuW, CH, and TC wrote the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: [https://www.frontiersin.org/articles/10.3389/fphys.2020.00485/full#supplementary-material](https://www.frontiersin.org/articles/10.3389/fphys.2020.00485/full#supplementary-material)

**FIGURE S1 |** Nucleotide and deduced amino acid sequences of Lv-VgR cDNA. The signal peptide (SP) is underlined with red line (a.a. 1–34). The ligand-binding repeats (LBDs) are a.a. 93–285 and a.a. 994–1332, and the Class A repeats are boxed in green. The EGFs are a.a. 296–991 and a.a. 1333–1774, and the EGF-EGF-CA, YWTD repeats are boxed in light blue, dark green and purple, respectively. The O-liked sugar domain (OLSD) are a.a. 1778–1787 underlined with dark point line and the transmembrane region (TM) are a.a. 1778–1810 boxed in yellow. The putative N-linked glycosylation sites (NLGS) are underlined with dark straight line. The internalization motif (IM) is boxed in gray.
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Conflict of Interest: JJ was employed by the company Guangdong Haimao Investment Co., Ltd.
The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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