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Molecular cloning and expression of *progestin membrane receptor component 1* (*Pgmrc1*) of the giant tiger shrimp *Penaeus monodon*

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ABSTRACT

Knowledge on molecular mechanisms of steroid hormonal induction on oocyte development may lead to the possible ways to effectively induce ovarian maturation in shrimp. In this study, *progestin membrane receptor component 1* (*Pgmrc1*) of the giant tiger shrimp (*Penaeus monodon*) initially identified by EST analysis was further characterized. The full-length cDNA of *Pgmrc1* was 2015 bp in length containing an ORF of 573 bp corresponding to a polypeptide of 190 amino acids. Northern blot analysis revealed a single form of *Pgmrc1* in ovaries of *P. monodon*. Quantitative real-time PCR indicated that the expression level of *Pgmrc1* mRNA in ovaries of both intact and eyestalk-ablated broodstock was greater than that of juveniles ($P < 0.05$). *Pgmrc1* was up-regulated in mature (stage IV) ovaries of intact broodstock ($P < 0.05$). Unilateral eyestalk ablation resulted in an earlier up-regulation of *Pgmrc1* since the vitellogenic (II) ovarian stage. Moreover, the expression level of *Pgmrc1* in vitellogenic, early cortical rod and mature (II–IV) ovaries of eyestalk-ablated broodstock was greater than that of the same ovarian stages in intact broodstock ($P < 0.05$). *Pgmrc1* mRNA was clearly localized in the cytoplasm of follicular cells, previtellogenic and early vitellogenic oocytes. Immunohistochemistry revealed the positive signals of the Pgmrc1 protein in the follicular layers and cell membrane of follicular cells and various stages of oocytes. Taken the information together, *Pgmrc1* gene products seem to play the important role on ovarian development and may be used as the bioindicator for monitoring progression of oocyte maturation of *P. monodon*.

1. Introduction

The giant tiger shrimp, *Penaeus monodon*, is one of the most economically important penaeid species in South-East Asia (Rosenberry, 1997). Difficulties in sexual maturation of captive *P. monodon* have limited the ability to genetically improve this important species (Preechaphol et al., 2007; Visuthiphole et al., 2009). As a result, the domestication of *P. monodon* has been remarkably slow in Thailand (Withyachumnarnkul et al., 1998).

Molecular mechanisms involving gonadal development of *P. monodon* have long been of interest by aquaculture industries (Benzie, 1998; Preechaphol et al., 2007). An initial step toward understanding molecular mechanisms of ovarian (and oocyte) maturation in *P. monodon* is the identification and characterization of reproduction-related genes that are differentially expressed during ovarian development of this economically important species.

Estrogen-like compounds in invertebrates were first described in the ovaries of an echinoderm (Donahue and Jennings, 1937). In *P. monodon*, the titers of conjugated pregnenolone and unconjugated and conjugated dehydroepiandrosterone (DHEA) were found to be maximal at early and late vitellogenesis. Unconjugated progesterone was found in ovaries at the late vitellogenic and mature stages of ovarian development whereas conjugated testosterone was only detected in the mature ovaries (Fairs and Quinlan, 1990).

Progesterone, P4, and its derivatives (progestins) are sex steroid hormones that play important roles in gametogenesis (Miura et al., 2006). 17α-Hydroxyprogesterone stimulated vitellogenin synthesis in *Marsupenaeus japonicus in vivo* (Yano, 1987). Progesterone stimulated ovarian maturation and yolk protein synthesis of penaeid shrimp (Kulkarni et al., 1979; Yano, 1985; Quackenbush, 2001). It also promoted spawning of *Metapenaeus ensis* (Yano, 1985). Nevertheless, molecular mechanisms of vertebrate-like

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hormones have not been well established in penaeid shrimps at present (Qiu et al., 2005).

Recently, progesterone and 17α-hydroxyprogesterone were extracted from the polychaetes. Their activity in comparison with the synthetic hormones (0.4, 0.7 and 1.0 ng/ml for P4 and 1.0, 2.0 and 3.0 ng/ml for 17α-OHP4) were *in vitro* tested against previtellogenic ovaries of *P. monodon* for 24 h. P4 was more effective in enhancing the final maturation of oocytes while 17α-OHP4 had more effects on vitellogenic oocytes. Interestingly, synthetic steroid hormones at equal hormone concentrations produced similar results to steroid hormones extracted from natural polychaetes (Meunpol et al., 2007). Nevertheless, receptors mediated the activity of progesterone and its derivatives have not been reported in penaeid shrimp.

The actions of P4 are mediated through its nuclear receptor, the progesterone receptor (nPR), as the classical pathway (Rao et al., 1974). However, P4 retains its actions in a variety of nPR negative cells and in mutant mice devoid of classical nPR leading to the finding that progestins elicit their actions through interactions with other proteins (Zhu et al., 2003, 2008).

Subsequently, two totally distinct classes of putative membrane-bound progestin receptors have been reported in vertebrates: membrane progestin receptors (mPR, subtypes α, β, γ, also called progestin or adipoQ receptors; PAQR, VII, VIII and V, respectively; Zhu et al., 2003; Peluso et al., 2006) and progestin membrane receptor component (PGMRC subtypes 1 and 2; Mourot et al., 2006; Cahill, 2007; Thomas, 2008). However, progestin signaling through these families of novel membrane proteins is still unclear.

To investigate the possible non-genomic actions of progestins in ovarian development, the full-length cDNA of *P. monodon progestin membrane receptor component 1* (*Pgmrc1*) was characterized. Expression patterns of *Pgmrc1* gene products during ovarian development of intact and eyestalk-ablated *P. monodon* were examined. Localization of *Pgmrc1* mRNA and protein in ovaries of *P. monodon* was determined using *in situ* hybridization and immunohistochemistry, respectively.

2. Materials and methods

2.1. Experimental animals

Juvenile shrimp (4-month-old) were purchased from a commercial farm in Chachoengsao (eastern Thailand). Female broodstock were wild-caught from the Andaman Sea (west) and acclimated under the farm conditions for 2–3 days. The post-spawning group was immediately collected after shrimp were ovulated ($N = 6$). Ovaries were dissected out from juveniles ($N = 5$) and intact broodstock ($N = 48$) and weighed. For the eyestalk ablation group, shrimp were acclimated for 7 days prior to unilateral eyestalk ablation. Ovaries of eyestalk-ablated shrimp were collected at 2–7 days after the ablation ($N = 30$). The ovarian developmental stages of *P. monodon* were classified into four stages according to gonadosomatic indices (GSI, ovarian weight/body weight × 100): <2, 2–4, >4–6 and >6% for previtellogenic (stage I, $N = 13$ and 5 for intact and eyestalk-ablated broodstock, respectively), vitellogenic (II, $N = 7$ and 5), early cortical rod (III, $N = 8$ and 10) and mature (IV, $N = 14$ and 10) stages, respectively. The ovarian stage of each shrimp was further confirmed by a conventional histological technique (Qiu et al., 2005). For tissue distribution analysis, various tissues of a female and testes of a male broodstock were collected. Shrimp tissues were immediately placed in liquid N_2 and kept at −70 °C until needed.

2.2. Isolation of total RNA and mRNA

Total RNA was extracted from shrimp tissues using TRI Reagent (Molecular Research Center). Ten micrograms of the extracted total RNA was treated with DNase I (0.5 U/1 μg of RNA, GE Healthcare) at 37 °C for 30 min and subjected to phenol–chloroform extraction. For RACE-PCR, mRNA was further purified from total RNA using a QuickPrep Micro mRNA Purification Kit (GE Healthcare). RNA was kept in the absolute ethanol at −70 °C prior to reverse transcription.

2.3. Rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR) and primer walking of the 3′ terminus of Pgmrc1

A gene-specific primer (5′-RACE; 5′-TGTCCTGTCGTTTCATCTTGG GCAC-3′) was designed. 5′-RACE-PCR was carried out using a SMART RACE cDNA Amplification Kit following the protocol recommended by the manufacturer (BD Bioscience Clontech). The amplified fragment was electrophoretically analyzed, eluted from the gel, cloned into pGEM-T Easy vector and sequenced. The 3′ direction of *Pgmrc1* was sequenced from the original cDNA clone by a primer walking approach. Nucleotide sequences of EST and 5′-RACE-PCR were assembled and searched against previously deposited sequences in the GenBank using BlastX (Altschul et al., 1990; available at http://ncbi.nlm.nih.gov). The transmembrane and protein domain were identified using TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) and SMART (http://smart.embl-heidelberg.de/), respectively. Molecular weight and p*I* and hydrophobicity of the deduced Pgmrc1 protein were examined using Protparam (http://www.expasy.org/tools/protparam.html) and ProtScale (http://www.expasy. org/tools/protscale.html), respectively.

2.4. Phylogenetic analysis of P. monodon Pgmrc1

Deduced protein sequences of Pgmrc1 and 2 from various species were retrieved from the GenBank and phylogenetically compared with that of *P. monodon*. Multiple alignments were carried out using ClustalW (Thompson et al., 1994). A bootstrapped neighbor-joining tree (Saitou and Nei, 1987) was constructed to illustrate phylogenetic relationships among sequences of Pgmrc from different taxa using PHYLIP (Felsenstein, 1993) and appropriately illustrated using Treeview (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

2.5. Northern blot analysis

Five micrograms of total RNA extracted from various stages of ovaries and from other tissues of intact and eyestalk-ablated shrimp were electrophoresed through a 1.2% agarose gel containing 3.7% formaldehyde, transferred onto a Hybond N membrane and UV-crosslinked. The membrane was hybridized with the digoxigenin-labeled anti-sense cRNA probes (5′-TAATACGACTCACTATAGGGCTTTGGTGTCCTTTGCTTCCTC-3′ where a T7 promoter sequence is underlined) in EasyHyb (Roche) at 68 °C overnight. The membrane was washed and detected according to the protocol recommended by the manufacturer.

2.6. Sequence alignments of ovarian and testicular forms of Pgmrc1 and expression analysis

Three full-length cDNAs of *Pgmrc1*; *Pgmrc1-Ts* (1980 bp), *Pgmrc1-Tm* (2848 bp), and *Pgmrc1-Tl* (2971 bp) previously identified in testes of *P. monodon* (GenBank Accession No. EU440763–EU440765) were retrieved from the GenBank and multiple aligned with that isolated from ovaries of *P. monodon* using ClustalW (Thompson et al., 1994). An ovary/testis forward primer (5′-CTCCTCTATGCAGATGGACTCTGTC-3′) and ovary-specific (5′-GACCACCTTCGTCGTCAACA-3′; the expected product of 493 bp) and testis-specific (5′-TGCTGTGTTTCAATGGGACC-3′; 727 bp) reverse primers were designed. RT-PCR was carried out in a 25 μl volume

containing 1× PCR buffer, 2 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 μM each primer, 100 ng first strand cDNA template of ovaries or testes and 1 U DyNazyme™ II DNA Polymerase (Finnzymes). *EF-1α*$_{500}$ (F: 5′-ATGGTTGTCAACTTTGCCCC-3′ and R: 5′-TTGACCTCCTTGATCACACC-3′) was included as the positive control. PCR was performed by predenaturation at 94 °C for 3 min followed by 35 cycles of a 94 °C denaturation for 30 s, a 55 °C annealing for 45 s and a 72 °C extension for 1 min. The final extension was carried out at 72 °C for 7 min. Amplicons were electrophoretically analyzed by 1.5% agarose gels and visualized with a UV transilluminator after ethidium bromide staining (Sambrook and Russell, 2001).

2.7. RT-PCR and tissue distribution analysis of total Pgmrc1

Expression of *Pgmrc1* in ovaries and testes of cultured juveniles and wild broodstock ($N = 5$ for each group) was analyzed by RT-PCR with primers Pgmrc1$_{147}$-F (5′-TTAATGGCAAGATCTTTGATGTCAC-3′) and Pgmrc1$_{147}$-R (5′-CACTGAGGTCATCGTACTCTTCCTT-3′) using conditions described above with the exception that the amplification was only carried out for 25 cycles with the extension time of 30 s. Tissue distribution analysis of *Pgmrc1* was performed for 35 cycles using the same conditions.

2.8. Quantitative real-time PCR

Standard curves representing 10^3–10^8 copies (in triplicate) of *Pgmrc1* (primers Pgmrc1$_{147}$-F/R) and the internal control, *EF-1α*$_{214}$ (F: 5′-TCCGTCTTCCCCTTCAGGACGTC-3′ and R: 5′-CTTTACAGACACGTTCTTCACGTTG-3′), were constructed. One microgram of DNAse I-treated total RNA of each specimen was reverse-transcribed using an Impromt-II Reverse Transcription System (Promega). *Pgmrc1* and the control (*EF-1α*$_{214}$) transcripts in different shrimp groups were examined in a 10 μl reaction volume contained 5 μl of 2× LightCycler 480 SYBR Green I Master (Roche), 25 ng the first strand cDNA template, 0.4 μM each gene-specific primer. The thermal profile were 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 58 °C for 30 s and 72 °C for 30 s. Real-time PCR of each specimen was carried out in duplicate. The relative expression level (copy number of *Pgmrc1* and that of *EF-1α*) between shrimp possessing different stages of ovarian development were statistically tested using one-way analysis of variance (ANOVA) and Duncan's new multiple range test. Significant differences were considered when $P < 0.05$.

2.9. In situ hybridization (ISH)

Ovaries of intact and eyestalk-ablated *P. monodon* broodstock were fixed in 4% paraformaldehyde prepared in 0.1% phosphate-buffered saline (PBS, pH 7.2) overnight at 4 °C. The fixed ovarian tissue was washed four times with PBS at room temperature and stored in 70% ethanol at −20 °C until used. Conventional paraffin sections (5 μm) were carried out. The sense and anti-sense cRNA probes Pgmrc1$_{435}$-T7/F (5′-TAATACGACTCACTATAGGGTGTGTTCCTCTTGGGTGTCTGTA-3′) and Pgmrc1$_{435}$-R/SP6 (5′-*ATTTAGGTGACACTATAGAA*TCTGTGGGCTGTTCTCCTGGT-3′), containing a T7 (underlined) and SP6 (italicized) promoter sequences, respectively; were synthesized using DIG RNA labeling mix (Roche). Tissue sections were dewaxed with xylene and dehydrated in absolute ethanol. The sections were prehybridized with 2× SSC containing 50% deionized formamide, 1 μg/μl yeast tRNA, 1 μg/μl salmon sperm DNA, 1 μg/μl BSA and 10% (w/v) dextran sulfate at 50 °C for 30 min and hybridized with either the sense or anti-sense probe in the prehybridization solution overnight at 50 °C. After hybridization, the tissue sections were washed twice with 4× SSC for 5 min each and once with 2× SSC containing 50% formamide for 20 min at 50 °C. The sections were immersed in prewarmed RNase A buffer (0.5 M NaCl, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA) at 37 °C for 5 min and treated with RNase A (20 μg/ml) at 37 °C for 30 min. Tissue sections were washed four times with the RNase A buffer (37 °C, 10 min each) and 2× SSC (50 °C, 15 min each). High stringent washing was carried out twice in 0.2× SSC at 50 °C for 20 min each. Detection of the positive hybridization signals was carried out with a DIG Wash and Block Buffer kit (Roche) (Qiu et al., 2005; Qiu and Yamano, 2005).

2.10. Production of anti-Pgmrc1 polyclonal antibody and Western blotting

Rabbit polyclonal antibody (PAb) was raised against KLH MBS-linked synthetic peptide (**C**EAKDTKAKTDD). The serum containing anti-Pgmrc1 PAb was purified by affinity chromatography using the synthetic peptide as the coupling ligand following the protocol recommended by the manufacturer (GE Healthcare). Ovarian tissues were homogenized in the SDS sample buffer supplemented with 1 mM PMSF (Qiu et al., 2005). The protein concentration was measured by a dye binding assay (Bradford, 1976). Proteins were resolved in a 12.5% gradient polyacrylamide gel under reducing conditions (Laemmli, 1970) and electroblotted onto a PVDF membrane (Towbin et al., 1979). The membrane was treated in the blocking solution (Roche) and incubated with the primary antibody at 1:1000 for 1 h at room temperature. After washing with TBS (20 mM Tris–HCl, 0.9% NaCl, pH 7.4) containing 0.1% Tween 20, the membrane was incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase (Bio-Rad) at 1:10,000 for 1 h. The hybridized signal was detected using NBT/BCIP (Roche) as a substrate.

2.11. Immunohistochemistry (IHC)

Ovaries were dissected out from *P. monodon* broodstock and fixed in Davidson's fixative. Standard paraffin sections (5 μm) were carried out. IHC was carried out essentially described by Qiu and Yamano (2005). Briefly, deparaffinized sections were immerged in 0.01 M citric acid buffer (pH 6.0) containing 0.1% Tween 20 and autoclaved for 5 min. After treatment in a blocking solution (Roche), the sections were incubated with anti-Pgmrc1 PAb (1:300) for 1 h and rinsed with PBS three times for 5 min each. The tissue sections were incubated with goat anti-rabbit IgG conjugated with peroxidase (simple stain PO, Nichirei) for 30 min and rinsed with PBS. Immunoreactive signals were visualized using diaminobenzidine (Wako Pure Chemical Industries) as the substrate. Tissue sections were also incubated with preimmune rabbit serum and the blocking solution as the negative control.

3. Results

3.1. Characterization of the full-length cDNA and primary structure of P. monodon Pgmrc1

The full-length cDNA of *P. monodon Pgmrc1* was 2015 bp in length containing an open reading frame (ORF) of 573 bp corresponding to a polypeptide of 190 amino acids with the 5′ and 3′ UTRs of 19 and 1423 bp, respectively (Accession No. GQ505293, Fig. 1A). The deduced protein with closest similarity to this sequence was *Pgmrc1* of the medaka, *Oryzias latipes* (*E*-value = 3e−47). The predicted p*I* value and MW of the deduced Pgmrc1 protein were 4.6 and 21.0 kDa, respectively. A cytochrome b5 like heme/steroid binding (cyt-b5) domain was found at positions 68–166 (*E*-value = 2.7e−20). The N-terminus of the deduced Pgmrc1 protein was highly hydrophobic (Fig. 1B). A predicted transmembrane domain (IFTSPLNVFLLGVCTVLIY) was found at positions 23–41 of the deduced Pgmrc1 protein of *P. monodon* (Fig. 1C).

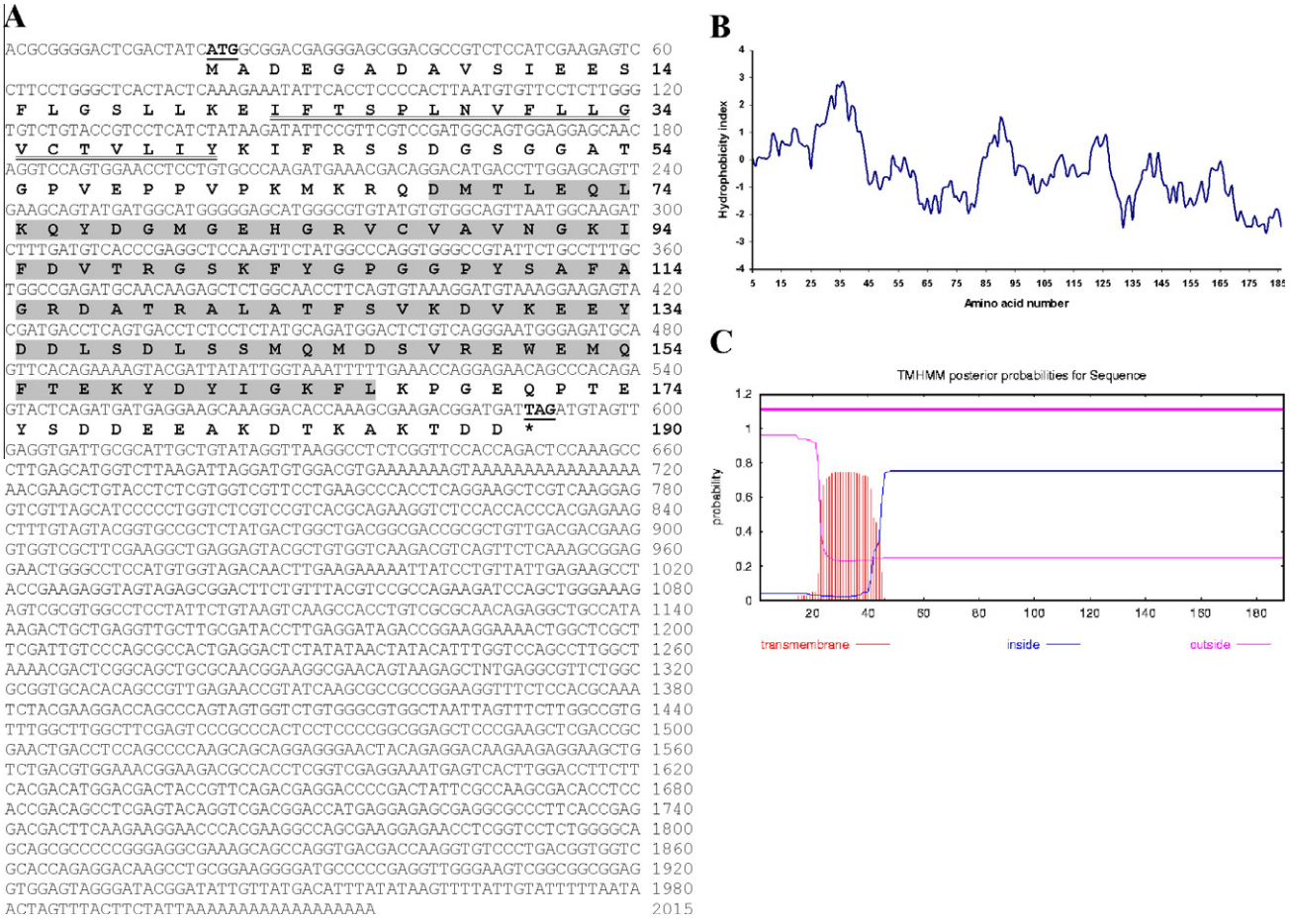

Fig. 1. (A) The full-length cDNA and deduced protein sequences of *Pgmrc1* (2015 bp, ORF of 573 bp corresponding to a deduced polypeptide of 190 aa) further characterized by 5′-RACE-PCR and 3′ primer walking of the original EST clone. The putative start (ATG) and stop (TAG) codons are illustrated in boldface and underlined. The predicted cytochrome b5-like heme/steroid binding domain (positions 68–166) and a transmembrane domain (positions 23–41) are highlighted and double underlined, respectively. Diagrams showing outside, inside and transmembrane probability (B) and hydrophobicity analysis (C) of the deduced Pgmrc1 protein (Kyte and Doolittle, 1982) are also shown.

Northern blot analysis illustrated a single isoform of *Pgmrc1* in ovaries of *P. monodon* broodstock (Fig. 2). No obvious size polymorphism of *Pgmrc1* in different tissues was found.

3.2. Phylogenetic analysis of Pgmrc1

Pgmrc1 protein sequences were conserved across taxa (data not shown). Phylogenetic analysis indicated that different subtypes of *Pgmrc* (*Pgmrc1* and *Pgmrc2*) were arisen from gene duplication process. Ovarian (this study) and testicular (Leelatanawit et al., 2008) forms of *Pgmrc1* was clustered together. They were phylogenetically allocated to the same clade as *Pgmrc1* of the sea urchin (*Strongylocentrotus purpuratus*) but distantly related to *Pgmrc1* of vertebrates and fish (Fig. 3).

3.3. Expression of ovarian and testicular forms of Pgmrc1 in gonads of P. monodon broodstock

Three testicular forms of *Pgmrc1*, sharing an identical ORF of 573 bp deducing to a 190 amino acid polypeptide, but differed in length of the 3′ UTR region were previously identified (Leelatanawit et al., 2008). A single amino acid variant (A to V, position 88) was found in the deduced ovarian Pgmrc1 protein. Its 3′ UTR was identical to that of testicular forms for 125 bp long but the remaining portion was different (Fig. 4A).

Both ovarian and testicular forms of *Pgmrc1* did not show tissue-specific expression but were co-expressed in gonads of *P. monodon*. The former was more abundantly expressed in ovaries whereas the latter was in testis (Fig. 4B). As a result, expression profiles of total *Pgmrc1* in various tissues were subsequently examined.

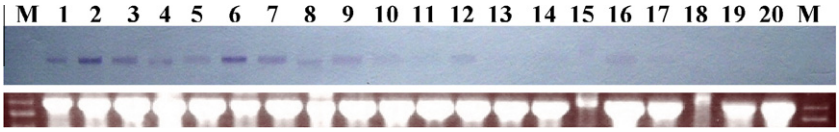

Fig. 2. Northern blot analysis (top) of *Pgmrc1* using total RNA isolated from ovaries of juveniles (lane 1), and intact and eyestalk-ablated broodstock (previtellogenesis, lanes 2 and 6; vitellogenesis, lanes 3 and 7; early cortical rod, lanes 4 and 8 and mature ovaries, lanes 5 and 9) and various tissues (testes, hemocytes, gills, heart, lymphoid organs, intestine, hepatopancreas, stomach, thoracic ganglion, eyestalk, pleopods; lanes 10–20) of *P. monodon* broodstock. Lanes M = RNA marker. *18S rRNA* (5 μg of total RNA, bottom) in various tissues was used as the control.

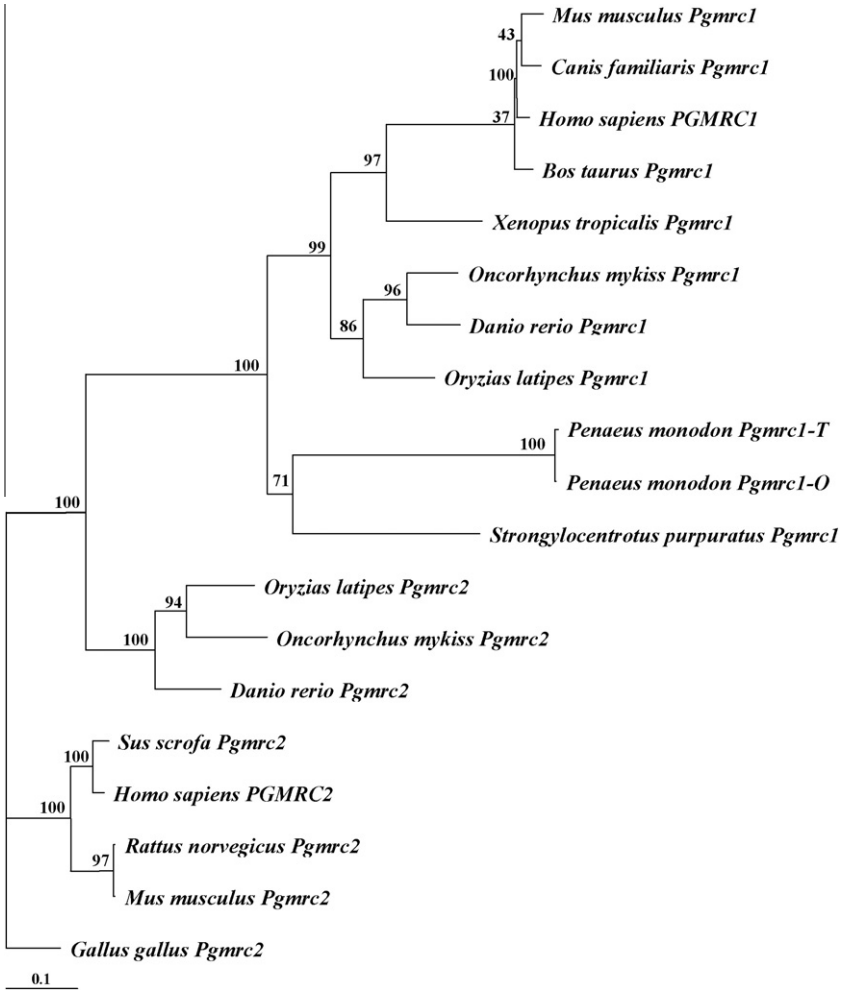

Fig. 3. A bootstrapped neighbor-joining tree illustrating relationships amongst the translated full-length cDNA of *Pgmrc1* of various taxa based on PAM protein distance. Values at the node represent the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original protein sequences. The deduced amino acid sequence of *Pgmrc1* from various species; *Oryzias latipes* (BAE47967), *Danio rerio* (NP_001007393), *Oncorhynchus mykiss* (AAL49963), *Strongylocentrotus purpuratus* (XP_783332), *Canis familiaris* (XP_538151), *Homo sapiens* (NP_006658), *Mus musculus* (AAB97466), *Bos taurus* (NP_001068601), *Xenopus tropicalis* (NP_001006842); and *Pgmrc2* from *Rattus norvegicus* (NP_001008375), *Mus musculus* (AAH44759), *Sus scrofa* (ABX45132) *Homo sapiens* (NP_006311), *Gallus gallus* (NP_001006441), *Oryzias latipes* (NP_001098199), *Oncorhynchus mykiss* (ABD58973), *Danio rerio* (NP_998269) were retrieved from the GenBank and compared with ovarian and testicular forms of *Pgmrc1* of *P. monodon*.

3.4. Expression of total Pgmrc1 mRNA in various tissues of P. monodon

Tissue distribution analysis indicated that *Pgmrc1* was abundantly expressed in ovaries and hepatopancreas of *P. monodon*. Lower levels of expression were observed in other tissues of a female and testes of a male broodstock (Fig. 5A).

Pgmrc1 was comparably expressed in gonads of juveniles but more preferentially expressed in ovaries than testes of *P. monodon* broodstock. In female shrimp, *Pgmrc1* was more abundantly expressed in ovaries of broodstock than that of juveniles ($P < 0.05$; Fig. 5B and C).

Quantitative real-time PCR revealed that the expression levels of *Pgmrc1* in ovaries of both intact and eyestalk-ablated broodstock were clearly greater than that of juveniles (4-month-old shrimp) ($P < 0.05$). In adults, *Pgmrc1* was up-regulated at the mature stage (stage IV) in intact broodstock ($P < 0.05$). Its expression level was decreased to the normal level after spawning ($P < 0.05$). Interestingly, *Pgmrc1* was up-regulated since vitellogenic (stage II) ovaries in eyestalk-ablated *P. monodon* broodstock. The expression level of *Pgmrc1* in vitellogenic, cortical rod and mature ovaries of eyestalk-ablated broodstock was significantly greater than that of the same developmental stages in intact broodstock ($P < 0.05$) (Fig. 6).

3.5. Localization of Pgmrc1 transcript

The *Pgmrc1* transcript was clearly found to be localized in the ooplasm of previtellogenic and vitellogenic oocytes and in follicular cells in both intact and eyestalk-ablated broodstock. Positive signals were not detected in oogonia and early cortical rod, and mature oocytes. No signal was found with the sense cRNA probe (Fig. 7).

3.6. Western blot and immunohistochemistry

The positive bands corresponding to monomeric and dimeric forms (21 and 42 kDa, respectively) of Pgmrc1 were detected by Western blot analysis. The former was expressed slightly greater in ovaries of juveniles and previtellogenetic ovaries than those of other ovarian stages of shrimp broodstock whereas the latter was more abundantly expressed in the opposite direction (Fig. 8).

Positive immunohistological signals of Pgmrc1 were detected in the follicular layer (FL) and cell membrane of follicular cells and various stages of oocytes. No immunoreactive signals were found in oocytes and other locations of ovaries (Fig. 9).

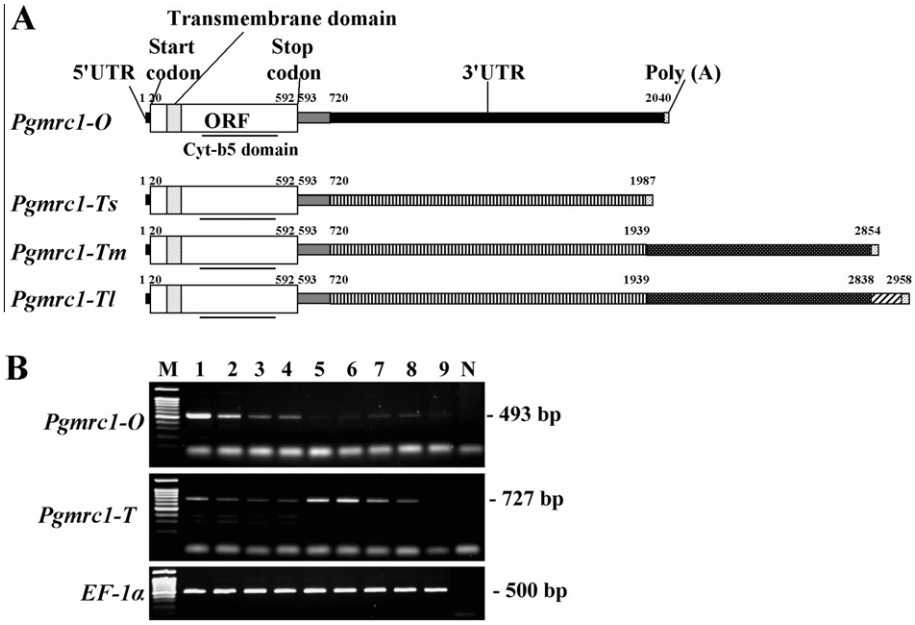

Fig. 4. (A) Schematic diagram illustrating three testicular (short, medium and long) isoforms (Leelatanawit et al., 2008) and a single ovarian isoform (this study) of *Pgmrc1*. (B) RT-PCR of *Pgmrc1* transcript using primers specifically amplified an ovarian form (top) and three testicular forms (bottom) of *Pgmrc1* using the first strand cDNA from ovaries (lanes 1–4) and testes (5–8) of *P. monodon* juveniles (lanes 1–2 and 5–6) and broodstock (lanes 3–4 and 7–8). *EF-1α* (500 bp, C) was used as the positive control. Lanes M and N are a 100 bp DNA marker and the negative control (without the cDNA template), respectively. Lane 9 is genomic DNA of *P. monodon*.

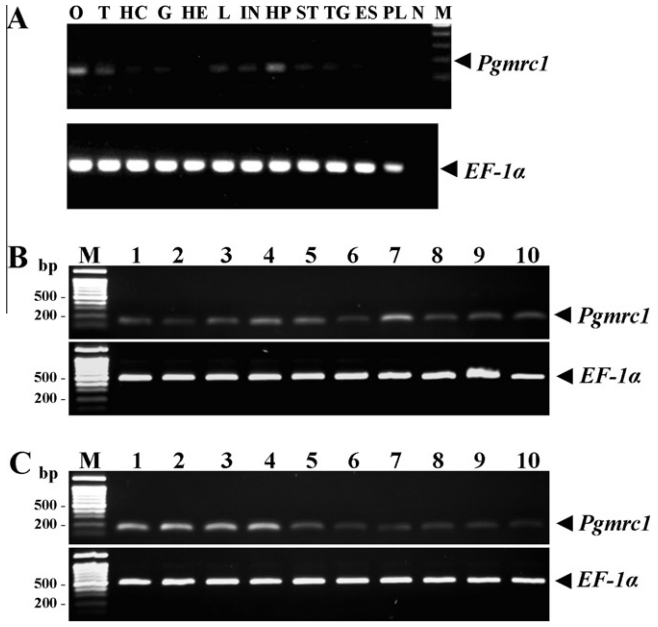

Fig. 5. Tissue distribution analysis of *Pgmrc1* (A) using the first strand cDNA of ovaries (O) and various tissues of *P. monodon* broodstock; testes (T), hemocytes (HC), gills (G), heart (HE), lymphoid organs (L), intestine (IN), hepatopancreas (HP), stomach (ST), thoracic ganglion (TG), eyestalk (ES) and pleopods (PL). *EF-1α* was successfully amplified from the same template. RT-PCR of *Pgmrc1* in ovaries (lanes 1–5, B and C) and testes (lanes 6–10, B and C) of juvenile (B) and broodstock (C) *P. monodon* was also carried out. Lanes M and N are a 100 bp DNA marker and the negative control (without the cDNA template). *EF-1α* (500 bp) was used as the positive control.

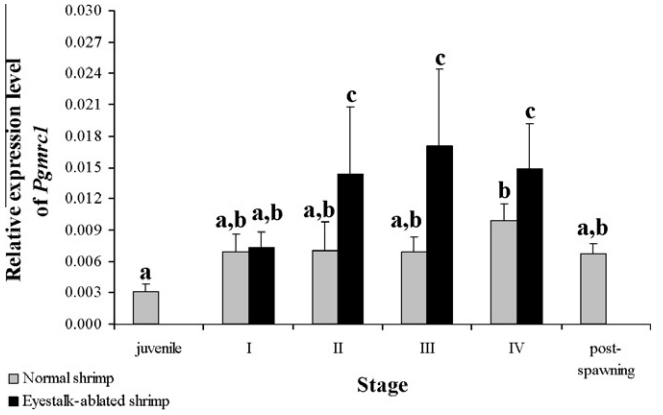

Fig. 6. Histograms showing the relative expression profiles of *Pgmrc1* during ovarian development of intact and unilateral eyestalk-ablated *P. monodon* broodstock. Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P > 0.05$).

4. Discussion

Reduced spawning potential and degrees of maturation of *P. monodon* in captivity crucially prohibits the ability to improve the culture and management efficiency through domestication and selective breeding programs of this species (Withyachumnarnkul et al., 1998). Molecular mechanisms and functional involvement of genes and proteins in ovarian development of *P. monodon* is necessary for better understanding of the reproductive maturation of *P. monodon* in captivity to resolve the major constraint of this economically important species.

In *Xenopus*, progesterone acts as a maturation-inducing hormone (MIH) resulting in meiotic resumption of oocytes from prophase-I arrest (Kishimoto, 2003). The presence of vertebrate-type steroids has been documented in almost all invertebrate groups including crustaceans (Lehoux and Sandor, 1970; Lafont and Mathieu, 2007). The conversion of progesterone into estradiol-17β was reported in *M. japonicus* (Summavielle et al., 2003). Estradiol-17β and progesterone levels in the hemolymph were shown to fluctuate closely with that of the serum vitellogenin level

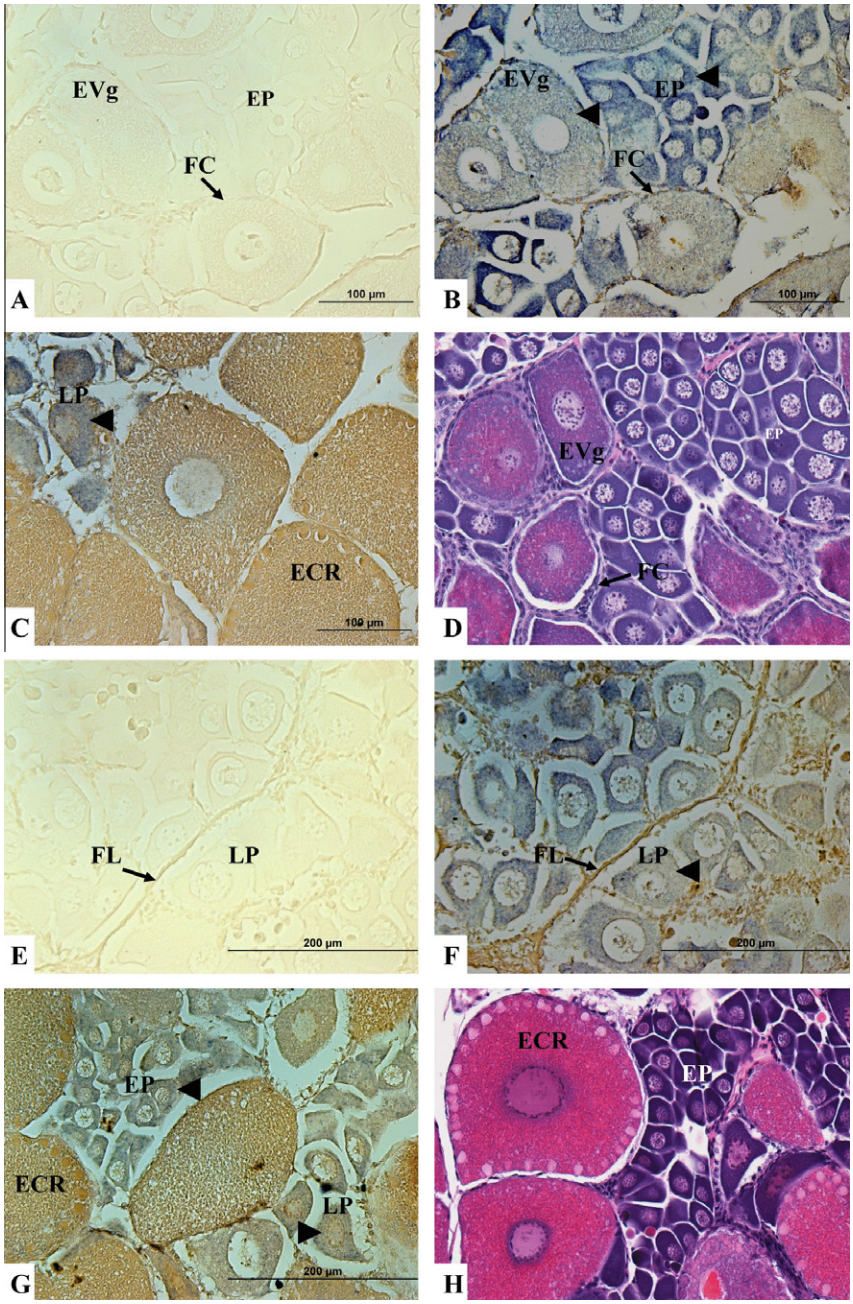

Fig. 7. Localization of *Pgmrc1* mRNA during ovarian development in normal (A–D) and eyestalk-ablated (E–H) *P. monodon* broodstock, visualized by *in situ* hybridization using sense (A and E) and anti-sense (B–C and F–G) *Pgmrc1* probes. Oocyte stages were classified by a conventional hematoxylin/eosin staining (D and H). EP, early previtellogenic oocytes; LP, late previtellogenic oocytes; EVg, early vitellogenic oocytes; ECR, early cortical rod oocytes; FC, follicular cells; FL, follicular layers. Arrowheads indicate positive hybridization signals.

during ovarian maturation stages of *P. monodon* (Quinitio et al., 1994) implying their regulatory roles in vitellogenesis. Nevertheless, progesterone and other sex steroid receptors have not been reported in penaeid shrimp.

The classical steroid receptors are members of the steroid/thyroid hormone receptor superfamily (Evans, 1988). They bind steroids in the nucleus or in the cytosol, dimerize, and migrate to the nuclear genome, where they act as transcription factors. This signal transduction pathway cannot account for the rapid cellular effects of steroids, and a number of different receptors may be involved (Mifsud and Bateman, 2002).

Female nuclear progesterone receptor (nPR) knockout mice exhibited higher level of *Pgmrc1* than the wild-type controls (Krebs et al., 2000). In contrast, progesterone can mediate its suppressive effect on *Pgmrc1* expression in rat. Accordingly, progesterone (P4) has a specific role in regulation of *Pgmrc1* transcription (Cairns et al., 1991).

Unliganded nPR exists as a heteromeric complex that contains heat shock proteins, hsp 90 and hsp 70, immunophilins, and progesterone receptor-related protein p23 (Johnson and Toft, 1994; Johnson et al., 1996). Recently, the full-length cDNA of *progesterone receptor-related protein p23* of *P. monodon* (*Pm-p23*, 1943 bp with an ORF of 495 bp corresponding to 164 amino acid residues; GenBank Accession No. GU906280) was characterized. *Pm-p23* was up-regulated since stage II (vitellogenic) ovaries of intact and stage III (early cortical rod) ovaries of eyestalk-ablated

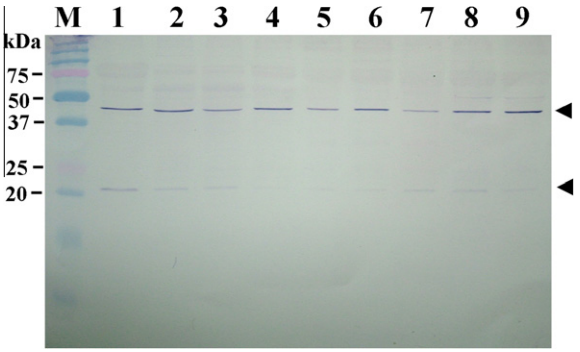

Fig. 8. Western blotting analysis of total ovarian protein (30 μg) of *P. monodon* using purified anti-Pgmrc1 PAb (dilution 1:100; expected MW approximately 21 kDa for a monomer and 42 kDa for a dimer of Pgmrc1). Lane 1 = ovaries of 4-month-old shrimp, lanes 2–3 = previtellogenic ovaries (GSI = 1.19% and 1.75%, respectively), lane 4 = vitellogenic ovaries (GSI = 3.31%), lanes 5–6 = early cortical rod ovaries (GSI = 4.61% and 5.75%, respectively), lanes 7–9 = mature ovaries (GSI = 7.32%, 11.19% and 11.66%, respectively. Lanes M = protein standard.

P. monodon broodstock ($P < 0.05$). *In situ* hybridization indicated that *Pm-p23* was localized in the ooplasm of previtellogenic oocytes. Western blot analysis indicated that the level of Pm-p23 peaked at the vitellogenic stage and decreased as oogenesis progressed. This information suggested possible important roles of *Pm-p23* gene products during ovarian/oocyte development of *P. monodon* (Preechaphol, 2008).

Vertebrate Pgmrc1 (MW 26–28 kDa) was first purified from porcine livers and its cDNA was subsequently cloned from porcine smooth muscle cells and a variety of other tissues (review in Thomas, 2008). A homologue of *Pgmrc1* was identified from suppression subtractive hybridization (SSH) libraries of cDNA from juvenile and broodstock ovaries (Preechaphol, 2008) and those of testes (Leelatanawit et al., 2008) of *P. monodon*. Three isoforms; *Pgmrc1-Ts* (1980 bp), *Pgmrc1-Tm* (2848 bp), and *Pgmrc1-Tl* (2971 bp), with an identical ORF of 573 bp but length polymorphism at the 3′ UTR, were successfully identified in male *P. monodon* (Leelatanawit et al., 2008). Administration of dopamine (10^{-6} mol/shrimp) resulted in a significant increase of *Pgmrc1* level in testes of juvenile *P. monodon* at 3 h post treatment (hpt, $P < 0.05$). However, the expression profiles and localization of *Pgmrc1* gene/protein during ovarian and/or testicular development of this economically important species have not been reported.

In contrast, a single isoform of ovarian *Pgmrc1* of *P. monodon* was observed. Different isoforms of ovarian and testicular *Pgmrc1* shared part of the 3′ UTR suggesting that they should have arisen from alternative spicing of the *Pgmrc1* gene. Therefore, genomic organization and Southern blotting of the *Pgmrc1* gene should be examined to identify whether different isoforms of *Pgmrc1* are transcribed from a single locus or different loci.

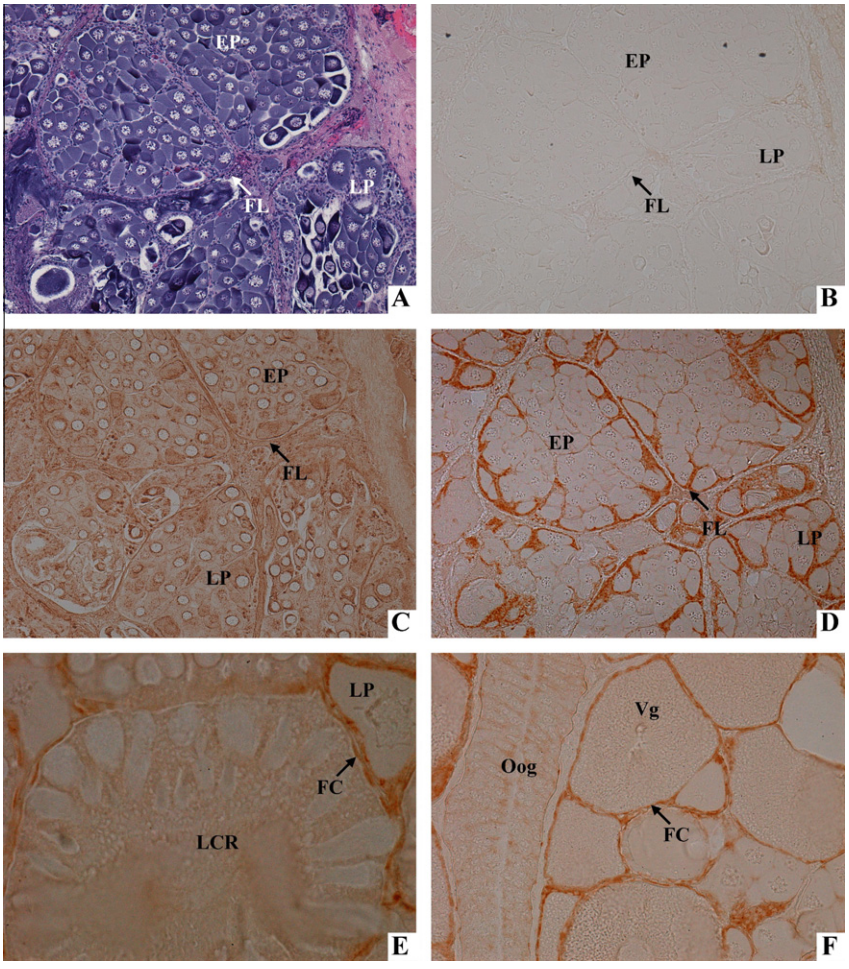

Fig. 9. Immunohistochemical localization of the Pgmrc1 protein in ovaries of wild *P. monodon* broodstock using purified anti-Pgmrc1 PAb (D–F). Hematoxylin and eosin staining (A) of tissue sections was carried out for classification of oocyte stages. The blocking solution (B) and preimmune serum (C) were used as the negative control. EP, early previtellogenic oocytes; LP, late previtellogenic oocytes; Vg, vitellogenic oocytes; LCR, large cortical rod (mature) oocytes; FC, follicular cells; FL, follicular layers; Oog, oogonia.

A cyt-b5 domain functionally important for ubiquitous electron transportation in heme-binding protein and progesterone receptor (Ozols, 1989; Meyer et al., 1996) was found in the deduced Pgmrc1 protein of *P. monodon*. Most proteins with this domain are linked to cell membranes either directly or by forming part of membrane-associated complexes (Mifsud and Bateman, 2002).

Recently, Mourot et al. (2006) characterized the full-length cDNA of Pgmrc1 (ORF of 546 nucleotides deduced to a polypeptide of 181 amino acids with one predicted transmembrane domain; Accession No. AY069921) and mPR-β (1080 nucleotides, 359 amino acids with eight predicted transmembrame domains; Accession No. DQ191163) in the rainbow trout (*Onchorynchus mykiss*). Both membrane-bound receptor transcripts were similarly expressed at the highest levels in the middle of vitellogenesis and down-regulated during late vitellogenetic and mature stages. *In vitro* treatment of ovarian follicles with 17, 20β-progesterone induced only the incomplete oocyte maturation as characterized by the remaining of traces of germinal vesicle.

One potential transmembrane domain (amino acid residues 23–41) was also identified in the deduced Pgmrc1 protein of *P. monodon* as previously reported in mammals (Gerdes et al., 1998) and *O. mykiss* (Mourot et al., 2006). This reflects a short N-terminal extracellular domain, a single transmembrane domain, and a cytoplasm domain of the Pgmrc1 protein. Results on gene and amino acid sequence and hydrophobicity analyses in this study suggesting that *P. monodon* Pgmrc1 is not mPR but was similar to other known Pgmrc1 previously described in various species.

Phylogenetic analysis indicated that different subtypes of *Pgmrc* (*Pgmrc1* and *Pgmrc2*) were arisen from gene duplication process. *Pgmrc1* of *P. monodon* was clustered with *Pgmrc1* of the sea urchin (*S. purpuratus*) but distantly related to *Pgmrc1* of vertebrates and fish. Accordingly, it should be regarded as a new member of the invertebrate *Pgmrc1*.

Tissue distribution analysis revealed that *Pgmrc1* was more abundantly expressed in ovaries than other tissues (e.g. gill, lymphoid organ and eyestalk of female broodstock and testes of male broodstock). This indicated that *Pgmrc1* may play an important role on reproductive development of female shrimp.

The expression profile of *Pgmrc1* suggested its important role in the late stage of ovarian development in intact *P. monodon* broodstock. Therefore, these gene products may be involved in the final oocyte maturation in *P. monodon*. Results from the present study were concordant with the progesterone levels in ovaries of *P. monodon* examined by GC–MS where the highest level was found in mature ovaries (Fairs and Quinlan, 1990).

Levels of the *Pgmrc1* mRNA in eyestalk-ablated *P. monodon* broodstock positively correlated with the progression of oogenesis in terms of earlier up-regulated expression in stage II ovaries and revealed more abundant expression levels during stages II–IV of ovarian development compared to intact broodstock. Accordingly, this transcript may be used as molecular indicators for following the progression of ovarian development and the final maturation of *P. monodon* broodstock. Overexpression of *Pgmrc1* also indicated that appropriate form(s) of progesterone can potentially induce oocyte/ovarian development and maturation of *P. monodon*.

Unilateral eyestalk ablation is used commercially to induce ovarian maturation of penaeid shrimp (Okumura, 2004; Okumura et al., 2006), but the technique leads to an eventual loss in egg quality and death of the spawners (Benzie, 1998). Therefore, predictable maturation and spawning of captive penaeid shrimp without the use of eyestalk ablation is a long-term goal for the shrimp industry (Quackenbush, 2001). Results in this study indirectly reflected that progesterone may directly enhance maturation through vitellogenesis stimulation or act as a precursor of the ovarian vitellogenesis stimulating hormone (VSOH) (Fingerman et al., 1993; Yano and Hoshino, 2006). Therefore, induced reproductive maturation of captive female *P. monodon* may be promoted by administration of appropriate forms of progesterone (progestins).

In situ hybridization was used to determine the location of *Pgmrc1* transcript in ovaries of *P. monodon*. The *Pgmrc1* transcript was localized in ooplasm and the hybridization signals were more intense in previtellogenic than early vitellogenic oocytes in all ovarian stages of both intact and eyestalk-ablated female broodstock. The hybridization signals of *Pgmrc1* were also observed in follicle layers and follicular cells. The finding suggested that *Pgmrc1* seems to be involved in oogenesis and ovarian development of *P. monodon*. Disappearance of hybridization signals of *Pgmrc1* in ooplasm of more mature stages (early cortical rod and mature) of oocytes may be due to significantly increasing oocytes sizes as oogenesis proceeded and low sensitivity of *in situ* hybridization on detecting gene expression per se (Klinbunga et al., 2009).

Non-purified anti-Pgmrc1 PAb generated the positive signals along with non-specific immunoreactive bands following Western blot analysis. After affinity-chromatographic purification, anti-Pgmrc1 PAb revealed two positive bands corresponding to the monomeric and dimeric forms of Pgmrc1 but more intense signals of the dimeric than monomeric forms were observed throughout the reproductive cycle of *P. monodon*. Typically, Pgmrc1 was detected as a 56-kDa dimer accompanying with a 28-kDa monomer in rat ovaries (Selmin et al., 1996) and pig spermatozoa (Meyer et al., 1996).

Pgmrc1 was present at the extracellular surface of the plasma membrane of the immature rat ovaries (Peluso et al., 2006). Furthermore, Pgmrc1 was found in the inner acrosomal membrane of porcine spermatozoa (Losel et al., 2004) and both cytoplasm and the nucleus of human HeLa cell (Beausoleil et al., 2004).

In *P. monodon*, immunohistochemistry revealed positive signals of the Pgmrc1 protein only in the follicular layers and cell membrane of follicular cells and various oocyte stages. Positive reactions were also observed with preimmune serum, but in different structure, i.e. nuclear membrane, of developing oocytes and did not interfere interpretation of results from anti-*P. monodon* Pgmrc1 PAb.

Progestin acts as the maturation inducing factor resulting in resuming meiotic maturation of oocyte (Kishimoto, 1999, 2003). We hypothesize that progestins affects oocyte and ovarian development of *P. monodon* by rapid mediation through Pgmrc1 for activation of intracellular signal transduction pathways (Thomas, 2008).

In the present study, the full-length cDNA of *Pgmrc1* was successfully identified. Expression analysis indicated that *Pgmrc1* seems to be involved in both vitellogenesis and oocyte maturation of *P. monodon*. The further interesting issue is identification of the Pgmrc1 binding partners and the resulting signal transduction pathways mediated physiological responses from appropriate form(s) of progestins in ovaries of *P. monodon*. This basic information would allow better understanding of molecular mechanisms of ovarian development and reproductive maturation of *P. monodon*.

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