CHAPTER IV

DISCUSSION

Isolation and characterization of functionally important genes in *P. monodon*

Different biotechnological approaches, for example; injection of vertebrate steroid hormones, neurotransmitters and ecdysteroids (Benzie 1998; Okumura, 2004) and the use of specially formulated feed (Harrison, 1990) have been applied to induce the ovarian maturation of female shrimp but results are inconsistent owing to limited knowledge on genetic and hormonal control of penaeid species.

The development of oocytes consists of a series of complex cellular events, in which differential genes express temporally and spatially to ensure the proper development of oocytes and to store transcripts and proteins as maternal factors for early embryogenesis (Qiu *et al.*, 2005). To provide an insight into molecular aspects governing reproductive processes of *P. monodon* for future functional genomic studies, an initial step toward understanding molecular mechanisms of ovarian (and oocyte) maturation and sex differentiation cascades in *P. monodon* is the identification and characterization of sex-related genes expressed in ovaries of this economically important species.

Gene expression and tissue distribution analysis are important and provide the basic information to set up the priority for further analysis of functional genes. Based on the fact that a particular genes may express in several tissues and possesses a different function in different tissues, primer pairs for 85 gene homologues were designed along with those from ovaries (71 primer pairs) and testes (2 primer pairs). All primers were tested against the first strand cDNA synthesized from ovaries and testes.

A total of 110 gene homologues (63.29% of investigated gene homologues) were successfully amplified. A large number of primers (36.71%) did not generate the

amplification product. This may be resulted from the use of EST originally found in hemocytes (e.g. *mitocondrial oxodicarboxylate*, *fructose 1,6-bisphosphate aldolase*, *TRAP-like protein precursor*). On the other hand, several genes originally found from the ovarian EST library (e.g. *phospholipase C, adenosylhomocysteinase, ras interacting protein RIPA, high mobility group protein DSP1* and *translationally controlled tumor protein*) did not generate the amplification product in both ovaries and testes. This reason for this is that they may be rare transcribed in gonads of *P. monodon*. Increasing of the first strand cDNA template and number of amplification cycles may provide the positive amplification product. The other possibility is that several primers used may not be appropriate and new primers should be re-designed. The example of this case is a homologue of *high mobility group protein DSP1* where the positive product is obtained after the newly designed primer pair is used.

The expression patterns of investigated genes can be classified to those specifically expressed in ovaries but not other tissues (A), those expressed in ovaries and some tissues but not testes (B), those preferentially expressed in ovaries than testes and other tissues (C), those preferentially expressed in ovaries than testes and also displayed abundantly expressed in the other tissues (D) and those did not show differential expression between ovaries and testes of *P. monodon* (E).

The first group was composed of homolguess of *ovarian lipoprotein receptor.* and *female sterile*. These genes are regarded as those essential for ovarian development of *P. monodon*.

In penaeid shrimps, ovarian development is characterized by the accumulation of a major yolk protein (vitellin) and the formation of cortical rods in the oocytes (Meusy and Payen, 1988). The precursor of vitellin (vitellogenin, VTG) is synthesized in the ovary and hepatopancreas, transported to the oocytes, and accumulated in the ooplasm as vitellin for utilization as a nutritional source during embryogenesis. After the completion of yolk accumulation, the cortical rods are formed radially around the periphery of the oocyte plasma membrane, and mature oocytes are spawned.

Cholesterol is the precursor of steroid hormones and steroid hormones stimulate ovarian development. Lipoprotein particles secreted from the synthesized tissues are absorbed to cells via receptor-mediated pathways and a key regulator of

steroidogenesis, was found to be regulated by lipoprotein at the transcriptional level (Argov, et al, 2004). In ovaries of *P. monodon*, estrogen such as estrone and 17-β-estradiol were detected by GC-MS (Fiars et al., 1990)

Ovarian lipoprotein receptor which belongs to the low density lipoprotein receptor (LDLR) superfamily plays an important role in receptor-mediated endocytosis of LDL (Schneider, 1992). Apparently, the full length cDNA and gene organization of *VTG receptor* (*VTGR*) or *LDLR* in shrimps have not been reported. Accordingly, a homologue of ovarian lipoprotein receptor found in the present study should be further characterized whether it is a *VTGR* or not.

Prat et. al. (1998) isolated and characterized *lipoprotein receptors* from ovaries of the rainbow trout (*Oncorrynchus mykiss*) and classified these genes to two groups, *rt-LPR and rt-LP[OS]R*. The deduced amino acid sequences of the rt-LPR and rt-LP[OS]R were 75% and 80% identity with very-low-density lipoprotein (*VLDLR*) and vitellogenin receptors (*VTGR*) of other species., Northern blot analysis revealed the expression of lipoprotein receptor mRNA in ovaries but not in somatic tissues (muscle, liver, spleen, heart, and intestine). In contrast, RT-PCR demonstrated that both *rt-LP[OS]R* and *rt-LPR* were generated but the former were expressed in the somatic tissues while the latter showed ovarian-specific expression. Both transcripts were expressed in previtellogenesis to mid-vitellogenic ovaries. In addition, both *rt-LP[OS]R* and *rt-LPR* were found in the ovarian follicular cells by RT-PCR

Both *female sterile* and its related protein, *pole hole* are required for the activation of the Tor receptor which are important for eggshell integrity and embryonic development (Perrimon *et al.*, 1986). The full length of female sterile homologue was first isolated and reported in penaeid shrimp by the present study. The obtained sequence also showed high similarity to *ficolin* (E- value=5e-37) which play an important role in innate immunity of various species (Endoa et al., 2006). Therefore, functional analysis of this isolated gene should be further carried out.

Adipose differentiation related protein, *ATP/GTP binding protein*, *broad complex Z4 isoform*, *aminopeptidase*, *wolf hirschorn syndrome candidate 1 protein*, and *proactivator polypeptide precursor* were regarded as members of the group B. The full length cDNA of several sex-specifically expressed transcripts of *adipose*

differentiation related protein (*ADRP*), *ATP/GTP binding protein*, *aminopeptidase* and *proactivator polypeptide precursor* were also first reported in penaeid shrimp.

During vitellogenesis, neutral lipids were accumulated and formed oil droplets intracellularly therefore this process are important for maturation of ovarian (oocyte) development. Adipogenesis is a complex process controlled by the interplay of intracellular factors and signals from the environment. During this differentiation, a large number of genes have to be regulated in a selective, coordinated manner, and dramatic changes occur in both cell morphology and gene expression (MacDougald and Lane, 1995).

Lipid droplets are formed by a unique monolayer of amphipatic phospholipids surrounding a central hydrophobic core of neutral lipids, mainly consisting of triacylglycerol (TAG) and sterol esters. Two mammalian proteins have been studied for their property to specifically localize at the surface of these organelles: *Perilipin* and *ADRP* (adipocyte differentiation-related protein also known as adipophilin) (Brasaemle et al., 1997). Besides this particular property, *ADRP* and *Perilipin* also show sequence similarity, especially in their *N*-terminal region where they are 40% identical (Lu et al., 2001).

ATP/GTP binding protein plays an important role for the formation of the 3′ end of pre-mRNA for which the pre-mRNA is cleaved endonucleolytically, the upstream cleavage fragment is subsequently polyadenylated and the downstream product is degraded.

The regulation of expression of structural genes is critical for morphogenesis. This requires differential expression of transcription factor, which in turn regulate the tissue-specific expression of structural genes.

Ecdysteroids are known as the moulting hormones in crustacean and insects and, in crustacean, the inactive forms are secreted and converted to 20-hydroxyecdysone by the Y-organ. Ecdysteriods stimulate vitellogenesis in insects and shrimp (Okumura and Aida, 2000).

The *Broad-Complex* (*BR-C*) is an early ecdysone-responsive gene that functions during metamorphosis and encodes a family of zinc-finger transcription factors. It is expressed in a dynamic pattern during oogenesis.

In *Drosophila*, all of the major metamorphic transitions are regulated by changes in the titer of ecdysones. von Kalm et al. (1994) examined a key regulator of metamorphosis and primary ecdysone response gene and illustrated that *BR-C* transmits the hormonal signal to one of its targets, the *Sgs-4 glue gene*. *BR-C* RNAs accumulate in mid third instar larval salivary glands prior to *Sgs-4* induction. The BR-C codes for a family of zinc finger transcriptional regulators. A number of binding sites for these proteins were identified. Some of these binding sites are required *in vivo* for Sgs-4 activity.

In addition, rbp+, a genetically defined Broad-Complex function that is required for Sgs-4 induction, acts through these Broad-Complex binding sites. Thus, the Broad-Complex directly mediates a temporal and tissue-specific response to ecdysone as larvae become committed to metamorphosis (von Kalm et al., 1994).

Differential expression of *cathepsin C* (*dipeptidyl peptidase*) during the final stages of oocyte maturation of *M. japonicus* was recently reported (Qiu *et al.*, 2005). This suggested that various transcripts possessing multifunctions and might perform different roles during oogenesis of *M. japonicus*.

Likewise, the full length cDNA of *aminopeptidase* which was expressed in ovaries but not testes of *P. monodon* broodstock was identified. *Aminopeptidase* is one of biologically active peptides have been proposed to regulate function and differentiation of reproductive organs. The inhibition of this enzyme activity affects steroid hormone production by granulosa and thecal cells affecting regulation of cellular growth and differentiation in reproductive organs by controlling extracellular concentration of peptide factors (Fujiwara,2004).

In *M. japonicus*, several genes including *thrombospondin* (TSP), *peritrophin* (also called *cortical rod protein*, *CRP* and *shrimp ovarian peritrophin*, *SOP*), *cathepsin C* (*dipeptidyl peptidase*) showed differential expression during the final stages of oocyte maturation of that species (Qiu *et al.*, 2005; Qui and Yamano, 2005). Eyestalk ablation promotes levels of both *vitellogenin* transcripts and proteins and

only elevated the protein levels of *TSP* and *CRP* between different stages of ovarian development of *M. japonicus*.

Nuclear autoantigenic sperm protein (*NASP*) which was first described in rabbit and designated a homologue to the *Xenopus* oocyte histone binding protein N1/N2 (Welch *et al.*, 1990) were also characterized. *NASP* binds histone H1 *in vivo* is found in all dividing cells as either a somatic/embryonic (*sNASP*) or a testis/embryonic (*tNASP*) isoforms (Richardson *et al.*, 2000). Overexpression of *tNASP* affects progression through the cell cycle. In mice, *HSP90* acts as a *tNASP*-binding partner (Alekseev *et al.*, 2005). Moreover, *NASP* mRNA expression is tightly regulated during the cell cycle such that mRNA levels in synchronously dividing cells rise steeply during the S phase then become undetectable by late G2/M phase. *NASP* mRNA levels in testis are at least an order of magnitude higher than in any other adult tissue. Unlike somatic cells, primary spermatocytes express *NASP* after DNA synthesis for meiosis has been completed (Richardson et al, 2006).

NASP contains the tetratricopeptide repeat (TPR) domains that are identified in a wide variety of proteins. Examples of important TPR-containing proteins are the anaphase promoting complex (APC) subunits, cdc16, cdc23 and cdc27, hsp90-binding immunophilins, and transcription factors (Das et al, 1998).

PMNASP showed significant differential expression between ovaries and testes of broodstock-sized *P. monodon* ($N = 6$ for each sex, $P < 0.05$; Preechaphol et al., 2007). Abundantly expression level of *NASP* in ovaries but extreamly low expression level in testes of *P. monodon* implied that *PMNASP* should play the important role in ovarian development.

One difficulty in identifying compounds that stimulate crustacean reproduction is the lack of adequate biological markers for reproduction. Vitellogenin is female-specifically expressed and can easily be purified and characterized. As a result, it was popularly applied to follow reproductive maturation of various animals. However, a problem with using the presence of yolk proteins as indicators of reproduction is that their presence in tissues does not clearly distinguish between synthesis, storage, or degradation.

The transcripts only (or preferentially) expressed in ovaries but not testes of *P. monodon* found in this study can be additionally used as the responsive indicators for reproductive maturation at the present stages but their involvement for ovarian and oocyte maturation of *P.monodon* and/or differentiation of sexes in *P. monodon* need to be further investigated.

Embryo implantation in the uterus is a critical step in mammalian reproduction, requiring preparation of the uterus receptive to blastocyst implantation. Ovarian steroid hormones estrogen and progesterone (P4) are the primary regulators of uterine receptivity (Yang et al., 2006). The immunophilin FKBP52 serves as a cochaperone for steroid hormone nuclear receptors to govern appropriate hormone action in target tissues. FKBP52 potentiates the function of glucocorticoid receptors (GR), androgen receptors, and progesterone receptor (PR) (Tranguch et,al,, 2005).

Appropriate functioning of nuclear steroid hormone receptors depends on interactions with the molecular chaperone machinery to maintain a functional state competent for hormone binding and subsequent transcriptional activation. The expression of *FKBP52* and *PGR* in preimplantation wild-type embryos of mice from one-cell through blastocyst stages was examined by RT-PCR. The results suggest that *FKBP52* is maternally derived in the oocyte and may have a role in fertilization and preimplantation development independent of PR. Alternatively, oocyte maturation due to follicular deficiency arising from compromised PR function in the absence of FKBP52 may contribute to the reduced fertilization rate (Tranguch et,al,, 2005)..

The fundamental controls of growth in penaeid shrimps are largely unstudied. The full length cDNA of genes encoding vertebrate-like growth factors (*endothelial cell growth factor I*; *PMECGFI*), and cell metabolisms (*3-oxoacid CoA transferrase*, *asparaginyl tRNA synthetase, PMATRS*, *aspatase amino transferase, PMAST*), *dolichyl diphosphooligocharide protein glycotransferaase* (*PMDDPG*) were successfully identified. More importantly, functional analysis of these genes should be further characterized.

Polymorphism of gene homologues analyzed by SSCP

The primary purpose for analysis of cDNA polymorphism was to identify additional isoforms of transcripts during ovarian development of *P. monodon*. In

addition, the discovery of transcripts that showed polymorphism across different individuals (but did not correlate with the increase/decrease of GSI of gonads) is regarded as identitying secondary purpose. Transcripts of the latter group will be further analyzed for the association between single nucleotide polymorphism (SNP) in cDNA or genomic gene fragments with the expression of the corresponding genes. Once the appropriately domesticated families are available, correlations between SNP, gene expression and commercially important phenotypes (e.g. high growth rates, egg or sperm quality) will be extensively examined.

The PCR product of 22 genes was analyzed by SSCP to and. more than one form of *PMATRS* was observed in both ovaries and testes of *P. monodon* broodstock. Female *P. monodon* with relatively low GSI (ovarian weight/body weight x 100 = 0.65, 0.87, 0.92, 1.10 and 1.43%) showed different SSCP patterns with those of shrimps with higher GSI (1.90, 2.02 and 2.13%). *ATRS* is classified to a member of the aminoacyl-tRNA synthetases (AARS) family which exhibits the primary and various secondary functions in different species. This protein family plays a fundamental role in protein synthesis (Kron et al, 2003), Additional isoforms of *PMARTS* found in vitellogenic ovaries of *P. monodon* indicated that this protein is possibly required by a rapid protein synthesis during vitellogenic stages of ovarian development of *P. monodon.*

Highly polymorphic patterns of the amplification product of *ovarian lipoprotein receptor* (*PMOVLR*) and *nuclear autoantigenic sperm protein* (*PMNASP*) were observed. Therefore, correlations between SNP of these genes and their expression level or phenotype (ability to adsorb low density lipoprotein and cell cycle activity through *histone H1 kinase*) should be determined.

Lo et. al. (2003) examined allele-specific gene expression of 1063 transcribed single-nucleotide polymorphisms (SNPs) by using Human SNP oligo arrays. Among 602 genes that were heterozygous, 326 (54%) showed preferential expression of one allele in at least one of seven investigated individuals, and 170 of those showed greater than fourfold difference between the two alleles. The allelic variation has been confirmed by real-time quantitative PCR experiments. Their results demonstrate that variation of gene expression between alleles is common, and this variation may contribute to functions (phenotypes) of the genes.

Liang *et al.* (2005) studied polymorphism of 5' flanking region in *chicken prolactin* (cPRL) and provided the possibility that polymorphic SNP sites might be related to the broodiness in chickens via modulating the transcriptional level of *cPRL* The dissociation among *cPRL* gene transcription, mRNA storage and hormones were observed.

Trakooljul et al. (2004) showed that *the androgen receptor* (*AR*) is highly polymorphic. Polymorphism affect the predicted amino acid sequence and the consensus transcription factor binding sites and are associated with allele-specific differences of the *AR* mRNA transcript levels in liver of the porcine.

In addition, the amplification product of *TetrasparinD 107* was also highly polymorphic and the product of ovaries exhibited greater variation than that of testes. The high polymorphism of *TetrasparinD 107* may have reflected fluctuation of the quality of oocytes and spermatozoa of *P. monodon* broodstock.

Tetrasparins are conserved proteins found from *Caenorhabditis elegans* to human and often show wide tissue distribution. They are believed to function primarily as organizers of networks of transmembrane and cytoplasmic proteins. Tetraspanins associate in cis-configulation with many other proteins, including immunoglobulin superfamily (IgSF) proteins, integrins, and membrane-anchored growth factors. CD9 which is a member of the tetraspanin protein family presents on the egg surface as well as the surface of many other cell types.(Primakoff et al, 2007). CD9 is thought to act in initial contact of sperm and oocyte plasma membranes and is currently the only oocyte protein known to be required for sperm–oocyte fusion (Runge et al, 2007).

Interestingly, a homologue of *TATA binding protein associated factor 9* revealed a fixed polymorphism between ovaries and testes of *P. monodon* broodstock. TATA-binding protein (TBP) is a central component for transcriptional regulation. It forms complexes with various transcription regulators for example RUVB.

Recently, a 49-kD TBP-interacting protein (TIP49) as isolated in human. The human TIP49 was highly homologous to bacterial RUVB proteins that function as a DNA helicase to promote branch migration of the Holliday junction. (Makino et al, 1998) RUVB is an ATPase transforming chemical energy into mechanical force necessary to

pull DNA through a complex of two RUVA tetramers. The full length cDNA of *RUVB* was recently identified and characterized in *P. monodon*. This gene showed significant correlations between its SSCP genotypes (or SNP) and the growth rates of both domesticated and commercially cultured *P. monodon* juveniles (Prasertlak, 2006). This indirectly suggests that *TATA binding protein associated factor 9* may play an important role on growth of *P. monodon*.

In *P. monodon*, females exhibit approximately 10 - 20% greater growth rate than do males at all stages of development (Browdy, 1998). The diploid chromosome numbers of penaeid shrimps was 86-92 where *P. monodon* possesses $2N = 88$ (Benzie, 1998). Nevertheless, a lack of obvious heteromorphic sex chromosomes in this species has been causing limited knowledge on sex chromosomal system (XY or ZW etc.) and their segregation patterns. In addition, sex determination cascades and genomic sex-diagnostic markers have not been reported in penaeid shrimps. This has prevented the possibility to increase aquacultural production through a monosex culture approach.

Khamnamtong et al. (2006) used bulked segregant analysis (BSA) and AFLP for isolation of genomic sex determination markers in *P. monodon*. A total of 256 primer combinations were tested against 6 - 10 bulked genomic DNA of *P. monodon*. Five and one candidate female- and male-specific AFLP fragments were identified. Female-specific fragments were cloned and further characterized. SCAR markers derived from FE10M9520, FE10M10725.1, FE10M10725.2 and FE14M16340 provided the positive amplification product in both male and female *P. monodon*. Further analysis of these markers using SSCP and genome walk analysis indicated that they were not sex-linked. Therefore, genomic sex determination was not successfully developed in this species.

In addition, sex-specific (or differential) expression markers in ovaries and testes of *P. monodon* were analyzed by RAP-PCR (150 primer combinations). Twenty-one and fourteen RAP-PCR fragments specifically/ differentially expressed in ovaries and testes of *P. monodon* were successfully cloned and sequenced. Expression patterns of 25 transcripts were tested against the first stranded cDNA of ovaries and testes of 3-month-old and broodstock-sized *P. monodon* ($N = 5$ and $N = 7 - 10$ for females and $N = 4$ and $N = 5 - 7$ for males, respectively). Five (*FI-4*, *FI-44*, *FIII-4*, *FIII-39* and *FIII-58*) and two (*M457-A01* and *MII-51*) derived RAP-PCR markers

revealed female- and male-specific expression patterns in *P. monodon*. Surprisingly, *MII-5* originally found in testes showed a higher expression level in ovaries than did testes of juvenile shrimps but a temporal female-specific pattern in *P. monodon* adults. Results indicated that sex-specific expression markers were successfully developed in *P. monodon*.

The ability to differentiate male and female *P. monodon* by fixed polymorphism of the amplified cDNA of *TATA binding protein associated factor 9* allows classification of this transcript as a newly identified sex-specific expression markers. More importantly, all previously identified markers in this group identified by Khamnamtong et al. (2006) were not sex-linked when tested against genomic DNA of male and female *P. monodon*. It is, therefore, interesting to determine whether polymorphism of *TATA binding protein associated factor 9* are genomic sex-linked in *P. monodon* or not.

Effects of 5-HT on expression of functionally important genes

Biogenic amines (e.g serotonin or 5-HT, epinephrine and dopamine) and peptide neuroregulators are known to modulate the release of neuropeptide hormones from the sinus gland. Injections of serotonin and dopamine antagonist, spiperone (25 μg g^{-1} body weight + 1.5 or 5 μg g^{-1} body weight) induced ovarian maturation and spawning in wild *L. stylirostris* and pond reared *L. vannamei* (Alfaro et al., 2004).

Meeratana et al. (2006) and Wongprasert et al. (2006) illustrated effects of serotonin on ovarian development and synchronous development of vitellogenic and mature oocytes. Nevertheless, molecular mechanisms of 5-HT have not been studied

In the present study, effects of 5-HT on expression levels of various genes in ovaries of juvenile *P. monodon* (approximately 30 g body weight, injected with 50 μg g^{-1} body weight) were examined by semiquantitative RT-PCR. 5-HT significantly elevated the transcription levels of *female sterile*, *nuclear autoantigenic sperm protein*, *ovarian lipoprotein receptor*, *adipose differentiation related peptide*, *aspartate amino transferase* and *3-oxoacid CoA transferase* ($P < 0.05$).

All genes were up-regulated upon single injection of at 50 μg g^{-1} body weight of juvenile shrimp. Based on the mean relative expression levels of the target gene and

the positive control (*EF-1α*), *PMFS* and *PMOVLP* did not require the repeat injection of 5-HT as the second injection was adverse the positive effect of the first injection. Nevertheless, repeat injection of 5-HT extended its effects on the expression level of *PMADRT*, *PMNASP*, *3-oxoacid CoA transferase*, *PMDDPG* and *PMAST*.

Practically, *P. momodon* broodstock rather than juvenile shrimp should be used in the experiment to investigate effects of 5-HT on expression of genes in ovaries of *P. monodon*. However, a large number of individuals are required for the appropriately designed experiments and wild *P. monodon* are extremely expensive. Therefore, juvenile *P. monodon* was used instead.

Yamano *et al.* (2004) illustrated that in most cases ovaries of *M. japonicus* start to develop in the reproductive season but fail to reach full grown requisite for the formation of cortical rods (CRs). Ovaries degenerate without spawning. This is also the major constraint in *P. monodon*. In the present study, a large number of cDNA including those showing sex-specific/preferential expression in ovaries of *P. monodon* were identified and characterized. Molecular mechanisms and expression patterns of genes controlling each step of oocyte maturation and formation of cortical rods (CRs) should be further carried out for better understanding the reproductive maturation of *P. monodon* in captivity.