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RESEARCH PAPER

Characterization and Expression of Cytochrome b5 Gene and Protein in Ovaries of Giant Tiger Shrimp *Penaeus monodon*

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Abstract

The full-length transcript of *cytochrome b5* (*PmCytb5*) in the giant tiger shrimp (*Penaeus monodon*) was characterized. It was 1539 bp with an open reading frame (ORF) of 432 bp deducing to 143 amino acids. The deduced PmCytb5 protein contained predicted cytochrome b5-like heme/steroid binding domain (Cytb5, positions 17-91) and a transmembrane domain (positions 120-139). In non-ablated wild adults, *PmCytb* transcript was significantly increased in mature ovaries while it was earlier upregulated in late vitellogenic stage in eyestalk-ablated shrimp ($P<0.05$). Progesterone injection (0.1 µg/g) induced the expression of *PmCytb5* at 48 hours post injection (hpi, $P<0.05$) but serotonin (50 µg/g) did not alter its expression ($P>0.05$) in domesticated adults. The cytoplasmic PmCytb5 protein was upregulated from immature ovaries in the early vitellogenic stage and comparably expressed in subsequent stages in non-ablated wild broodstock. In eyestalk-ablated wild broodstock, a similar expression profile was observed but its expression was further increased in late vitellogenic, and mature stages. The membrane PmCytb5 protein was upregulated in late vitellogenic and mature stages in the former but expressed at a similar level in different ovarian stages in eyestalk-ablated broodstock. Results suggested the important role of PmCytb5 in ovarian development of *P. monodon*.

Introduction

The giant tiger shrimp (*Penaeus monodon*) is one of the economically important penaeid species in Southeast Asia (Benzie, 1998; Pongtippatee et al., 2018). Since 1994, shrimp production (mainly *P. monodon*) in Thailand was approximately 200,000 metric tons annually (Limsuwan, 2004). Owing to problems from shrimp diseases, its aquacultural production has decreased since the last two decades and the production of *P. monondon* was 13,000 tons in 2019 (U.S Soybean Export Council, 2020).

The aquaculture production of *P. monodon* is largely constrained by the lack of domesticated broodstock due to difficulties on reproductive maturation of shrimp in captivity (Withyachumnarnkul et al., 1998; Ibara et al., 2007). Consequently, the farming production of *P. monodon* in Asia has significantly declined and its production is replaced by farming of the introduced Pacific white shrimp,

Litopenaeus vannamei. Therefore, breeding programs for production of high quality domesticated broodstock of *P. monodon* need to be developed.

Reduced reproductive maturation of captive *P. monodon* females has been reported (Makinouchi & Hirata, 1995; Benzie et al., 1998; Withyachumnarnkul et al., 1998; Preechaphol et al., 2007). Oogenesis and meiotic maturation of oocytes are complicated processes which are composed of complex cellular phenomena where various genes are differentially expressed for proper development of oocytes (Qiu et al., 2005). An understanding on expression of genes/proteins involving ovarian development of *P. monodon* are useful for domestication of this species (Clifford & Preston, 2006; Coman et al., 2006). The information could be applied to resolve the bottleneck on reduced ovarian development and spawning of captive *P. monodon* (Okumura et al., 2004 and 2006; Preechaphol et al., 2010a, 2010b and 2010c).

Domestication and breeding programs of *P. monodon* requires the basic information on molecular mechanisms controlling the ovarian development and maturation processes (Benzie et al., 1998; Withyachumnarnkul et al., 1998; Ibara et al. 2007; Preechaphol et al., 2007, 2010a, 2010b and 2010c). Several genes functional contributed in molecular reproduction of *P. monodon* have been reported for example, vitellogenin (*PmVtg*, Hiransuchalert et al., 2013a), vitellogenin receptor (*PmVtgr*, Klinbunga et al., 2015), catechol *O*-methyltransferase (*PmComt*, Buaklin et al., 2013), farnesoic *O*-methyltransferase (*PmFAMeT*, Buaklin et al., 2015) and small androgen receptor interacting protein (*PmSarip*, Hiransuchalert et al., 2013b). Cytochrome b5 protein has been shown to play the important role on modulation of 17α-hydroxylase and 17–20 lyase (CYP17) activities in a steroid hormone biosynthesis (Akhtar et al., 2005). Typically, it contains a Cytb5 domain which is important for its function and single transmembrane domain which is not involved with its interaction with the redox partners (Sergeev et al., 2014). However, the functional contribution of Cytb5 gene/protein in ovarian development of penaeid shrimp has not been reported.

Previously, *progestin membrane receptor component 1* of *P. monodon* (*PmPgmrc1*; 2015 bp with an open reading frame (ORF) of 573 bp deducing to 190 amino acids) which contained a cytochrome b5 like heme/steroid binding (Cytb5) domain at the N-terminus (positions 68–166) was characterized and showed functional involvement of ovarian development in *P. monodon* ($P<0.05$) (Preechaphol et al., 2010c). To resolve problems from reduced degrees of reproductive maturation of captive *P. monodon*, *cytochrome b5* (*PmCytb5*) cDNA was isolated and characterized. Expression of *PmCytb5* gene and protein in different ovarian stages of wild *P. monodon* adults was evaluated. Effects of a neurotransmitter (serotonin, 5-HT) and progesterone injection on expression of *PmCytb5* transcripts in ovaries of domesticated *P. monodon* was determined. The information revealed a possible application for inducing reproductive maturation of *P. monodon* in captivity.

Materials and Methods

Experimental Samples

For determination of gene expression profiles of *PmCytb5*, wild females were collected alive from the Andaman Sea (west of peninsular Thailand). Shrimp were acclimatized in the laboratory (28-30°C and 32 ppt seawater under the natural daylight in 1000-liter fish tanks with aeration). for 1 week. Ovarian stages of wild shrimp were externally visualized during the acclimation period for initial selection of broodstock. Non-ablated shrimp were sacrificed and further classified by the gonadosomatic index (GSI, ovarian weight/body weight x 100) values to previtellogenic, early vitellogenic, late vitellogenic and mature ovaries (GSI < 1.5, 2-4, >4-6 and >6.0%; *N*=10, 7, 7 and 9, respectively) (Rao et al., 1995; Qiu et al., 2005). Ovaries were kept at -80°C until needed. For eyestalk-ablated shrimp, immature wild females were also live-caught from the Andaman Sea and acclimated for 1 week before subjected to eyestalk ablation. Different stages of ovaries of eyestalk-ablated shrimp were collected (*N*=4, 6, 9, and 11, respectively) and kept at -80°C until needed.

To determine the expression of *PmCytb5* in different ages of domesticated *P. monodon*, 6-, 14- and 18-month-old shrimp (*N*=6 for each group) were collected. Ovaries were dissected out from each shrimp and kept at -80°C until required.

In addition, domesticated 14-month-old *P. monodon* were obtained from Shrimp Genetic Improvement Center (SGIC), Surat Thani, Thailand. Shrimp were acclimated for 1 week (28-30°C and 32 ppt seawater under the natural daylight in 500-liter fish tanks with aeration)). Female shrimp were injected into the first abdominal segment with either 5-HT (50 μg/g body weight, *N*=4 for each group) or progesterone (0.1 μg/g body weight, *N*=4 for each group). Specimens were time-interval collected as described in Hiransuchalert et al. (2013b).

RNA extraction and First-strand cDNA Synthesis

Total RNA was extracted from ovaries of *P. monodon* using TRI Reagent (Molecular Research Center). After DNase I treatment, the first strand cDNA was synthesized from 1 μg of total RNA using an Improm-II™ Reverse Transcription System following the protocol recommended by the manufacturer (Promega).

Isolation of the Full-length cDNA of *PmCytb5*

The cDNA sequence of *PmCytb5* was identified from the ovarian cDNA library of *P. monodon*

(Preechaphol et al., 2007). The full-length cDNA of *PmCytb5* was obtained by sequencing of the EST clone for both directions (OV-N-S01-1852-W). Nucleotide sequence of *PmCytb5* was searched against previously deposited sequences in GenBank using Blast*N* and Blast*X* (Altschul et al., 1990; available at http://ncbi.nlm.nih.gov). The p*I* value and molecular weight of the deduced PmCytb5 protein were examined usingProtParam(http://www.expasy.org/tools/protparam.html). The protein domain and signal peptide in the deduced PmPmCtb5 protein were predicted using SMART (http://smart.embl-heidelberg.de).

Phylogenetic Analysis

The deduced amino acid sequences of PmCytb5 and Cytb5 proteins of various vertebrate and invertebrate species were retrieved from GenBank (http://ncbi.nim.hih.gov) and multiple-aligned using Clustal W (Thompson et al., 1994). A bootstrapped neighbor-joining tree (Saitou & Nei 1987) was constructed using MEGA 7.0 (Kumar et al., 2016).

Quantitative Real-time PCR

Primers for qRT-PCR of *PmCytb5* (Cytb5-F/R, Table 1) were designed using Primer Premier 5.0. Expression levels of *PmCytb5* in different stages of ovaries of intact and eyestalk-ablated wild females, and in ovaries of different ages of domesticated shrimp were analyzed. A single peak from melting curve of the amplification product was examined to ensure specificity of designed primers. As the positive control, *EF-1*α (Leelatanawit et al., 2012) were amplified from the same template. Standard curves representing 10^3 – 10^8 copies of recombinant plasmids of *PmCytb5* (an error for standard curve = 0.00806, efficiency for the amplification = 96.6%) and the internal control, *EF-1*α, were constructed (an error for standard curve = 0.00808, efficiency for the amplification = 97.5% for *EF-1*α). *PmCytb5* and *EF-1*α in each specimen were separately amplified in duplicate in a 10 µl reaction volume containing 5 µl of 2x LightCycler 480 SYBR Green I Master (Roche), 50 ng the first-strand cDNA template, 0.3 µM each primer. The thermal profile for quantitative real-time PCR was 95°C for 10 min followed by 40 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 30 s. Relative expression levels of *PmCytb5* between different groups of *P. monodon* were quantified using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and statistically tested using one way analysis of variance (ANOVA) followed by a Duncan's new multiple range test (P<0.05).

In Vitro Expression and Purification of Recombinant PmCytb5 Protein

Plasmid DNA containing the full-length ORF of *PmCytb5* (EST clone no. OV-N-S01-1852-W; Preechaphol et al., 2007) was used as the template for amplification using the forward rPmCytb5-F-NcoI-F and the reverse rPmCytb5-R-BamHI-R primers (Table 1) using a Bio-Rad MX1000 thermocycler. The amplification product was digested with corresponding enzymes, ligated into pET32a and transformed into *E. coli* JM109. The recombinant plasmid was subsequently transformed into *E. coli* BL21-CodonPlus(DE3)-RIPL. *In vitro* expression of recombinant PmCytb5 protein was performed as previously described in Preechaphol et al. (2010c). The rPmCytb protein was purified under denaturing conditions using a His GraviTrap kit (GE Healthcare) as previously described in Sittikankaew et al. (2010). Each fraction of the washing and eluting step was analyzed by SDS-PAGE and western blotting (Mini-PROTEAN® 3 and Mini Trans-Blot cell, Bio-Rad). The purified proteins were stored at 4°C for immediate use or -20°C for long term storage.

Polyclonal Antibody Production and Western Blot Analysis

Anti-PmCytb5 polyclonal antibody was immunologically produced in a rabbit using the purified rPmCytb5 as an immunogen. For western blot analysis of soluble proteins, ovarian tissues of *P. monodon* were homogenized in 50 mM Tris-HCl; pH 7.5, 0.15 M NaCl supplemented with the proteinase inhibitor cocktail (Roche) and centrifuged at 12000 *g* for 30 min at 4°C. Protein concentrations of the tissue extract were determined by the dye binding method (Bradford, 1976). Thirty micrograms of total ovarian proteins were heated at 100°C for 5 min and immediately cooled on ice. Proteins were size-fractionated on a 15% SDS-PAGE

Table 1. Nucleotide sequences of primers used for characterization and expression analysis of *PmCytb5*

Primer	Primer sequence
Quantitative real-time PCR	
PmCytb5-F:	5'-ACTGGAGCAAGCAGGGATGGACAC-3'
PmCytb5-R	5'-GCAGGGCATACATTCGGTAGATA-3'
EF-1α_{214}-F	5'-GTCTTCCCCTTCAGGACGTC-3'
EF-1α_{214}-R	5'-CTTTACAGACACGTTCTTCACGTTG-3'
Recombinant protein expression	
rPmCytb5-F-NcoI-F	5'-CCGCCATGGGGGAAGAAAGTAAGGATG-3'
rPmCytb5-R-BamHI-R	5'-GGC*GGATCC*TCA**ATGATGATGATGATGATG**AGGGGCATGAACATTGAC-3'

[a]A *Nco* I site is underlined), a *Bam* HI site is italicized and six His encoded nucleotides are boldfaced, respectively.

(Laemmli, 1970), transferred onto a PVDF membrane (Hybond P; GE Healthcare) (Towbin et al., 1979) and immunologically detected as described in Sittikankaew et al. (2010).

Membrane proteins from ovaries of wild *P. monodon* broodstock possessing various stages of ovarian development were prepared. A piece of ovaries was homogenized under liquid N_2 and lysed with buffer M (100 mM NaCl, 20 mM Tris–HCl, 2 mM $MgCl_2$, 1 mM EDTA and 1 mM PMSF, pH 7.4). The tissue and cell debris were removed by centrifugation at 600 *g* and at 6000 *g* for 10 min each. After centrifugation at 20000 *g* for 30 min at 4 °C, the membrane pellet was dissolved in buffer M containing 0.2% Triton X-100. Protein concentration was determined (Bradford, 1976). Membrane proteins (5 µg) from each stage of ovaries were loaded on a 12.5% SDS-PAGE. Western blot analysis was carried out (Preechaphol et al., 2010c).

Results

PmCytb5 cDNA and its Primary Structure

The full-length cDNA of *PmCytb5* was 1539 bp in length containing an ORF of 432 bp corresponding to a polypeptide of 143 amino acids and the 5′ and 3′ UTRs of 52 and 1055 bp, respectively (GenBank accession no. MN458489, Figure 1). The closest similar sequence of this transcript was *cytochrome b5-like* of the Pacific white shrimp *L. vannamei* (*E*-value=5 x 10^{-91}).

The deduced PmCytb5 protein had predicted molecular weight and p*I* of 16.20 kDa and 4.51. PmCytb5 protein contained a Cytb5 domain located at positions 17 – 91 and the transmembrane domain (WLVPVGLACLASIIYRMYAL) located at positions 120 – 139 (Figures 1B and 1C). Positive hydrophobicity index was found at the transmembrane domain of the deduced PmCytb5 protein. The putative *N*-linked glycosylation sites were observed at position 12-14 (NTT), 110-112 (NNS), and 113-115 (NQS) and cAMP dependent protein kinase site (K/R-K/R-X-S/T) were observed at positions 95-98 (KKHT) of the deduced PmCytb5 protein.

Phylogenetic Analysis of Deduced of PmCytb5 Protein

A bootstrapped neighbor-joining tree revealed clear separation between Cytb5 proteins from vertebrates and invertebrates (insects and shrimp). Within the former group, Cytb5 of different mammalian species (*Bos taurus*, *Sus scrofa*, *Homo sapiens*, *Mus musculus*, *Rattus norvegicus* and *Canis lupus familiaris*) were closely related. Mammalian and *Gallus gallus* Cytb5 were well phylogenetically separated from piscine Cytb5. Within invertebrates, fruit fly and mosquito Cytb5 exhibited distant relationships with that of penaeid shrimp where PmCytb5 clustered with *L. vannamei* Cytb5 (Figure. 2).

Expression Profiles of *PmCytb5* mRNA During Ovarian Development of Wild and Domesticated *P. monodon*

The expression level of *PmCytb5* in ovaries of intact broodstock was upregulated at stage IV (mature) ovaries in wild intact broodstock ($P<0.05$). In eyestalk-ablated broodstock, its expression was upregulated in stages III (late vitellogenic) and IV (mature) ovaries ($P<0.05$, Figure 3). In domesticated *P. monodon*, the expression level of *PmCytb5* transcript was significantly reduced from 6-month-old juveniles in 14-month-old broodstock. Its expression was subsequently increased in 18-month-old broodstock ($P<0.05$, Figure 4).

Expression Levels of *Pmcytb5* Mrna in Domesticated Adults Following Serotonin (5-HT) and Progesterone Injection

In vivo effects of serotonin and progesterone (50 and 0.1 µg/g body weight) administration on expression of ovarian *PmCytb5* in 14-month-old shrimp were examined. The *PmCytb5* mRNA level was not significantly altered after serotonin injection ($P>0.05$, Figure 5A). Nevertheless, its expression was significantly induced at 48 hours post injection (hpi) following progesterone administration ($P<0.05$, Figure 5B).

Expression of the PmCytb5 Protein During Ovarian Development of Wild *P. monodon*

Recombinant PmCytb5 protein (approximately 16 kDa) was successfully expressed *in vitro* and anti-rPmCytb5 PAb was successfully expressed in rabbit (OD_{650}=1.281 at 1;500 of the serum). Western blot analysis was carried out against total cellular proteins and a positive immunological signal of approximately 16 kDa was found in all developmental stages of wild shrimp. The ovarian soluble PmCytb5 protein was upregulated from juveniles and stage I ovaries in stage II ovaries. Its expression was comparable in subsequent stages in intact broodstock. The PmCytb5 protein was upregulated in stage II and further increased in stages III and IV ovaries of eyestalk-ablated broodstock (Figure 6).

The membrane PmCytb5 protein was also examined. Positive signals of 16 kDa were found in premature ovaries of juveniles and different ovarian stages of broodstock. In intact broodtock, it was upregulated from stages I and II ovaries in stages III and IV ovaries (Figure 7A and 7D). PmCytb5 protein was comparably expressed in different stages of ovarian development in eyestalk-ablated broodstock (Figure 7B and 7C).

Discussion

Isolation and Characterization of *PmCytb5* Transcript

Cytochrome b5 (Cytb5) is a small heme protein that plays a role in the modulation of cytochrome P450

A

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CCGGTATTCTGCAACGCCTTGCAAGATCAGTTCCCCGTGACTAGCGGAGACAATGGGGGA 60
                                                  M  G  E  3
AGAAAGTAAGGATGAACAAATTAGCAACACAACTACAAAGGTGTACACCTTAGCGGAGAT 120
 E  S  K  D  E  Q  I  S  N  T  T  T  K  V  Y  T  L  A  E  I 23
TGCGGAGCATAAAACAACAGATTCGTGTTGGATTGTGATTCACGACAAAGTCTATGATGT 180
 A  E  H  K  T  T  D  S  C  W  I  V  I  H  D  K  V  Y  D  V 43
AACCAAATTTTTAGATGAGCATCCTGGAGGAGAGGAAGTGCTACTGGAGCAAGCAGGGAT 240
 T  K  F  L  D  E  H  P  G  G  E  E  V  L  L  E  Q  A  G  M 63
GGACACCACAGAAAGCTTTGAGGATGTTGGTCACTCTACTGATGCTCGGGAGATGATGAC 300
 D  T  T  E  S  F  E  D  V  G  H  S  T  D  A  R  E  M  M  T 83
GGACTATTACCTTGGTGAACTAAGTGAGGAAGACAAGAAGCACACTCAAGATAAGGGTCC 360
 D  Y  Y  L  G  E  L  S  E  E  D  K  K  H  T  Q  D  K  G  P 103
CAAAACCTGGGCATATGATAATAACTCCAATCAGAGTGCATGGAAGTCATGGTTGGTACC 420
 K  T  W  A  Y  D  N  N  S  N  Q  S  A  W  K  S  W  L  V  P 123
TGTAGGCCTAGCATGTCTTGCATCAATTATCTACCGAATGTATGCCCTGCCACAGTCTAG 480
 V  G  L  A  C  L  A  S  I  I  Y  R  M  Y  A  L  P  Q  S  S 143
CTAATGTCAATGTTCATGCCCCTTGATCCATCTGAAGTACATTAGTCATTGTCTCCTTGT 540
 *
TATCCACAGTGGTTTTACTTTTAATTTTTTATATCCTCTTTGCCTACTGTTCTTTTTCCT 600
TCCTTTTCCATGTCTTTGTCAAAATGATAAGTGGAGTCAATAATTTGTTTAAAGAGTGAA 660
ATATTTTACATTGTATTTGTATTTGCACACACACACACACACACACACACACACACAC 720
ACACACACACACACACACACACACACAGAACATGTATATGTATATAAAATCACATA 780
AATTTCCATATAAAATATACATGCAAGTATATTACCAAAACTTGGAATCAATTTTTTTCT 840
ATTTGGCATACAAAGAGACCTATCTTCCAATAATATAAGGAATTTTATGTCTGTCTTCAT 900
TATATTTTAATATATTCTAATGAACTTTTTTTTTTACCATACAAGAGACATTTTTGTAGT 960
AACTAGTATAACTTACATTATCAATAGAACTCGTACATACATGTACTTTGGCAAGGGGAA 1020
TTGTGAGCCTTTATTCTTAGGTAATACTCAGTCTCATTGTAAATTCAGATGCCATAATTC 1080
ATGAACCATTCCTTCTGGCAACTAACAATCAGGCCGTGACTAAATCTTATTTTTACAGTG 1140
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TCACGTGTATCAAGATCAATGTAAAAGTGTTCATACTAATACTATCTTCATTCCATCAAA 1260
ACTGGGAACAATGAGATCAGAATCTGATTTTTTCTTCTTTCCTTTAAATGTATTCCGATT 1320
GCAGAAGCATGTGTTTCGATATATATGGTGGCATTTTGATGGACATACAATGAAGTGCAT 1380
GTAATTTTGCACATGAGTATTACAGACATGTCCTTCAGATGTGACAGGACAGAGTTCTGA 1440
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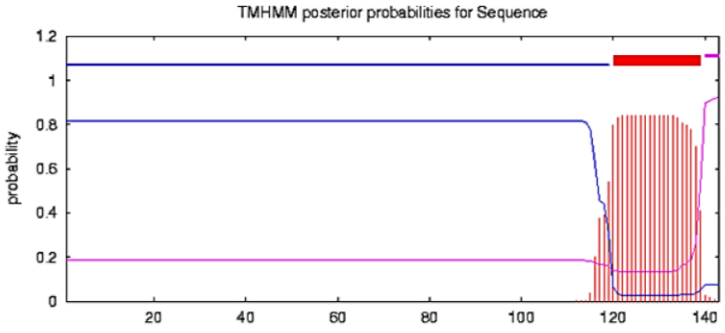
B



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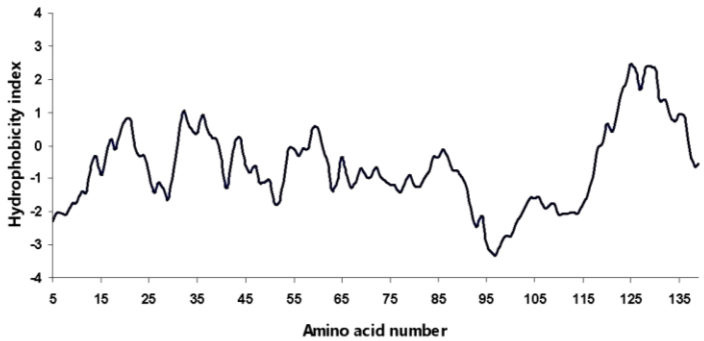

Figure 1. The full-length cDNA and deduced protein sequences of *PmCytb5* (1539 bp, ORF of 432 bp corresponding to a deduced polypeptide of 143 aa; A). The predicted start (ATG) and stop (TAA) codons are shown in boldface and underlined. The predicted Cytb5 domain (positions 17 – 91) is highlighted. The predicted transmembrane domain is indicated by double underlines (positions 120 – 139). Diagrams showing outside, inside and transmembrane probability (B) and hydrophobicity analysis of the deduced PmCytb5 protein (C) according to Kyte and Doolittle (1982) are illustrated.

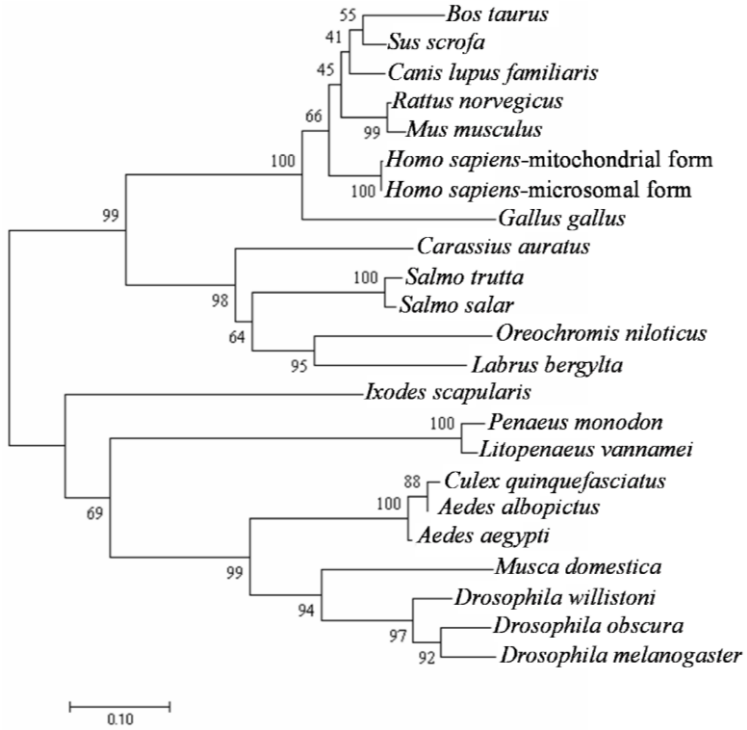

Figure 2. A bootstrapped neighbor-joining tree illustrating relationships between PmCytb5 and cytochrome b5 proteins of various taxa. Values at the node represent the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original sequences. Protein sequences of Cytb5 were retrieved from *Bos taurus* (NP_776458.1), *Rattus norvegicus* (NP_071581.1), *Sus scrofa* (NP_001001770.1), *Homo sapiens* (AAA63169.1 and AAA35729.1), *Mus musculus* (NP_080073.1), *Canis lupus familiaris* (NP_001180227.1), *Gallus gallus* (NP_001001748.1), *Litopenaeus vannamei* (XP_027210420.1), *Ixodes scapularis* (XP_002413177.1), *Aedes aegypti* (XP_001660580.2), *Culex quinquefasciatus* (XP_001867082.1), *Aedes albopictus* (XP_029714313.1), *Drosophila willistoni* (XP_002060982.1), *Drosophila obscura* (XP_022230979.1), *Drosophila melanogaster* (ABW37749.1), *Musca domestica* (NP_001274474.1), *Salmo trutta* (XP_029577376.1), *Salmo salar* (XP_014030741.1), *Labrus bergylta* (XP_020516642.1), *Oreochromis niloticus* (XP_003442320.1), and *Carassius auratus* (XP_026071762.1)

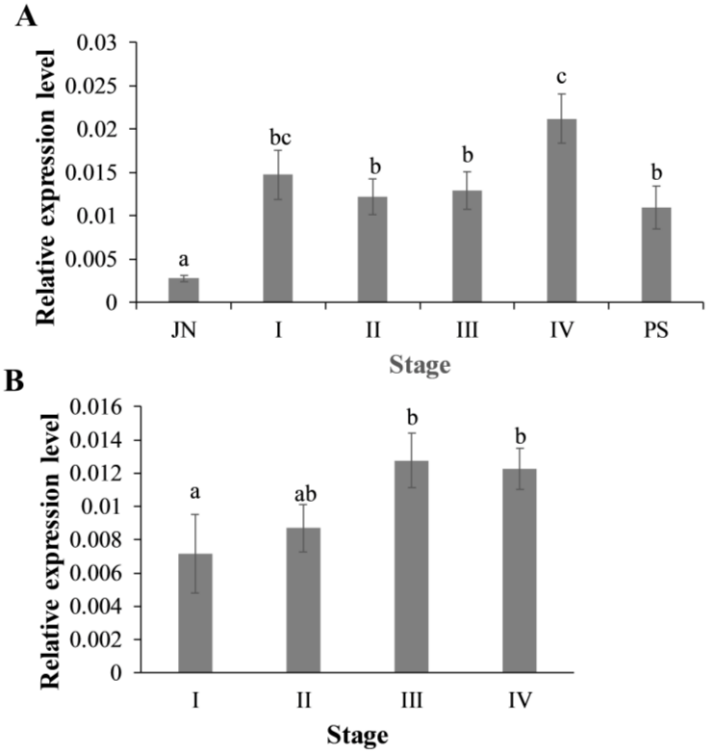

Figure 3. Histograms showing relative expression profiles of *PmCytb5* mRNA in ovaries of domesticated juveniles (JN, A) and different stages of ovarian development (stages I, previtellogenic; II, vitellogenic; III, early cortical rod; and IV, mature ovaries) of intact (A) and unilateral eyestalk-ablated (B) broodstock and intact post-spawning (PS; A) broodstock. Different letters above histograms reveal significant differences between groups of samples (P>0.05).

17a-hydroxylase/17,20-lyase (P450 17A1, CYP17A1) activities (Bhatt et al., 2017; Vergéres & Waskell, 1995). Most of the Cytb5 domain-containing proteins are linked or associated to cell or organelle membranes (Mifsud & Bateman, 2002). The functional involvement of *Cytb5* gene and protein in ovarian development of penaeid shrimp has not been reported.

In the present study, the full-length cDNA of *PmCtyb5* was successfully isolated and reported for the first time in *P. monodon*. The deduced PmCytb5 protein contained an *N*-terminal region (amino acid residues 1-119, one potential transmembrane domain (amino acid residues 120 - 139) and a C-terminal region (amino acid residues 140 -143). The Cytb5 domain which is functionally important for ubiquitous electron transportation in heme-binding protein and progesterone receptor (Mifsud & Bateman, 2002; Meyer et al., 1996; Ozols, J., 1989) was also found. Results from gene and amino acid sequence analysis, transmembrane domain analysis and hydrophobicity

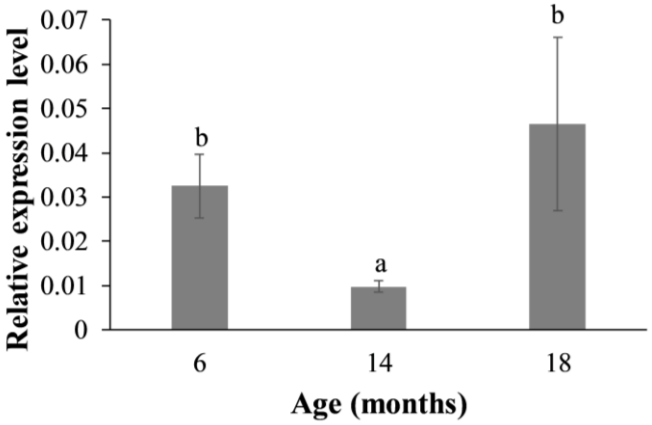

Figure 4. Histograms showing relative expression levels of *PmCytb5* mRNA in ovaries of domesticated 6-, 14- and 18-month-old of *P. monodon* females. Different letters above histograms reveal significant differences between groups of samples (P>0.05).

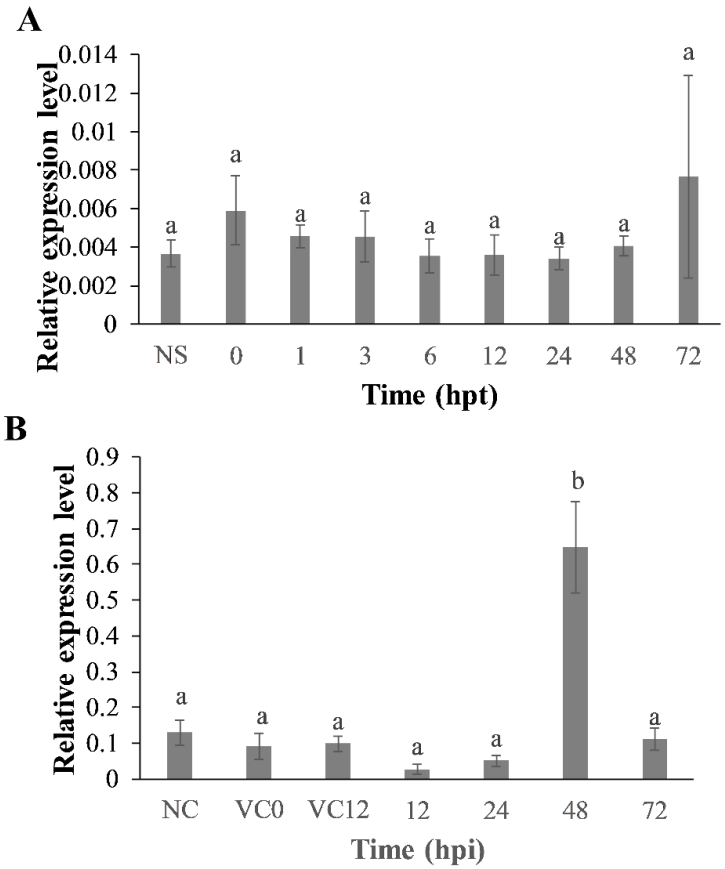

Figure 5. Time-course relative expression levels of *PmCytb5* mRNA in ovaries of domesticated shrimp at 0, 1, 3, 6, 12, 24, 48 and 72 hours post injection (hpi) of 5-HT (50 µg/g body weight; 14-month-old, *N*=4 for each stage, A) and at 12, 24, 48 and 72 hpi of progesterone (0.1 µg/g body weight; 14-month-old, *N*=4 for each stage; B). Shrimp injected with 0.85% saline solution at 0 hpi (NS) or absolute ethanol at 0 and 12 hpi (VC0 and VC12) were included as the controls for respective treatments. Acclimated shrimp with no treatment were included as the negative control (NC). The same letters indicate that the expression levels were not significantly different (P>0.05).

analysis in this study support the suggestion that PmCytb5 is similar to other known Cytb5 proteins previously described in various species.

Phylogenetic analysis indicated close relationships between the mitochondrial (142 amino acids) and microsomal (134 amino acids) forms of human Cytb5. This suggested that different forms of Cytb5 protein were not arisen from a gene duplication process. The topology of a gene tree agrees with classical systematics of examined species. Interestingly, the length of all mammalian Cytb5 proteins were 134 amino acids while that of *G. gallus* was 138 amino acids. They showed clear separation with fish Cytb5 (151-161 amino acids). Within the Phylum Insecta, clear phylogenetic differentiation was observed between insects (128 – 135 amino acids) and penaeid shrimp (142-143 amino acids for *L. vannamei* and *P. monodon*). The insect group could be further differentiated to that of mosquitoes

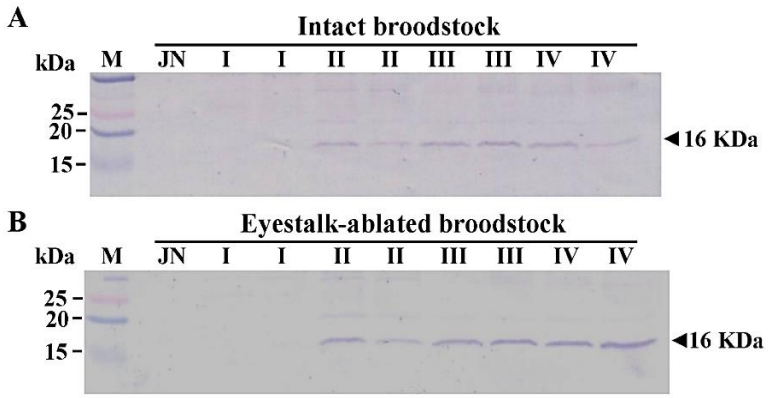

Figure 6. Western blot analysis of total soluble proteins (30 μg) from ovaries of wild intact (A) and eyestalk-ablated *P. monodon* (B) broodstock using purified anti-rPmCytb5 PAb (dilution 1:200; expected MW approximately 16 kDa for non-glycosylated PmCytb5). JN (A and B) = ovaries of 4 month-old shrimp, I = previtellogenic ovaries, II = vitellogenic ovaries, III = late vitellogenic ovaries, IV = mature ovaries. Lanes M = a protein standard.

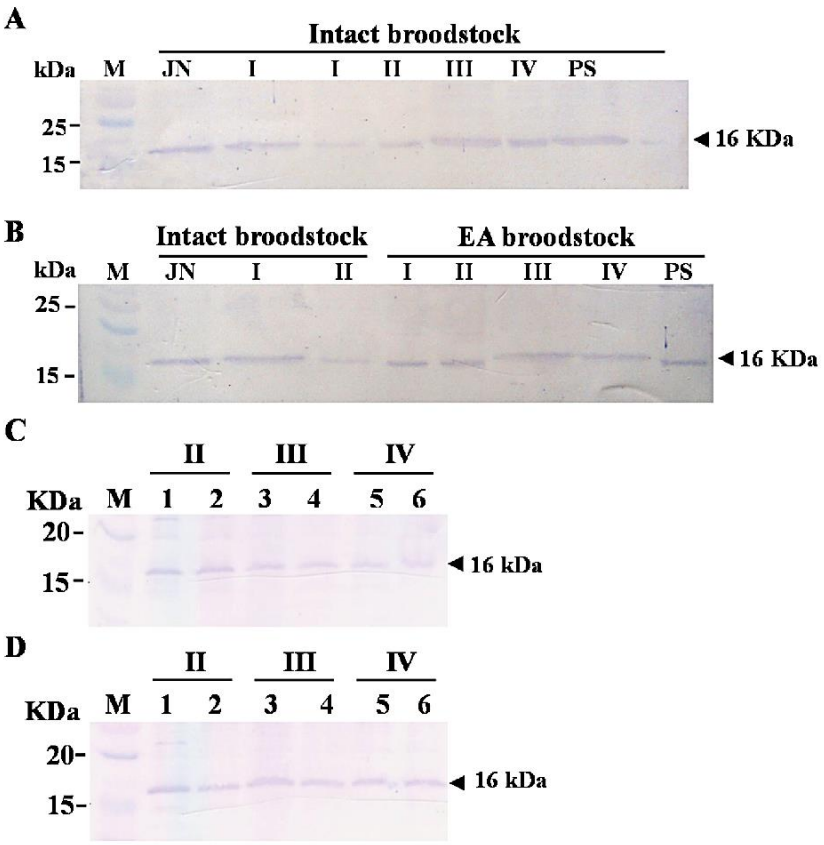

Figure 7. Western blot analysis of membrane proteins of intact (5 μg, A and B) and eyestalk-ablated broodstock (5 μg, B). In addition, ovarian soluble (30 μg, C) and membrane proteins (5 μg, D) extracted from the same individuals of wild intact *P. monodon* broodstock were immunologically examined using purified anti-rPmCytb5 PAb (dilution 1:100). The GSI of shrimp in lanes 1 – 6 (C and D) = 1.8, 1.9, 3.8, 4.8, 5.8 and 6.2%, respectively. Lane M = a protein standard.

(128 – 129 amino acids) and *Drosophila* and *Musca domestica* (133-135 amino acids).

The predicted Cytb5 domain of PmCytb5 protein (positions 17 – 91) was located precede a predicted transmembrane domain (positions 120 – 139). In mammalian cells, two membrane-bound (mitochondrial and microsomal) isoforms of Cytb5 were identified and they were located at outer mitochondrial membrane (146 amino acids) and endoplasmic reticulum membranes (134 amino acids), respectively (Altuve et al., 2001; D'Arrigo et al., 1992; Giordano & Steggles, 1993). The soluble (98 amino acids) and the membrane-bound (134 amino acids) Cytb5 were generated following an alternative splicing of the microsomal isoform (Altuve et al., 2001). The primary structure suggested that the *N*- terminus of PmCytb5 protein should interact with redox partners in cytoplasm of oocytes. In the present study, anti-PmCytb5 polyclonal antibody was successfully produced. As a result, subcellular localization of should be further examined for more detailed studies of PmCytb5 protein.

Expression of *PmCytb5* gene and protein during ovarian development of *P. monodon*

Transcripts/proteins differentially expressed at different stages of ovarian development can be used as indicators for reproductive maturation in *P. monodon* (Preechaphol et al., 2007). Low spawning capacity is usually observed in domesticated *P. monodon*. Previously, we used Sanger-based sequencing of expressed sequence tags (ESTs) for isolation of reproduction-associated genes between ovaries and testes (Leelatanawit et al., 2004), different stages of testes (Leelatanawit et al., 2009), and immature and mature ovaries (Preechaphol et al., 2007 and 2010a) of *P. monodon*. Subsequently, transcriptome analysis of gene expressed heart, muscle, hepatopancreas, and eyestalk for isolation of 165 growth-related genes (e.g. *cathepsin L*, *cyclophilin* and *profilin*) in *P. monodon* was reported (Nguyen et al., 2016). Moreover, comparative transcriptome of different tissues (brain and thoracic ganglia, eyestalks, antennal gland, and ovary) was analyzed to isolate genes associated with ovarian development. In total, 573 differentially expressed genes (DEGs) including neuropeptides and G protein-coupled receptors (*GPCRs*) between previtellogenic and vitellogenic stages were identified (Nguyen et al., 2020). Recently, 11,411 significant DEGs were identified from transcriptome analysis between testis and developed ovaries. Genes related to ovarian development of *P. monodon* were functionally involved in steroid biosynthesis, mRNA surveillance, nucleocytoplasmic transport, oxidative phosphorylation and DNA replication pathways (Li et al., 2022).

After large-scale isolation of candidate genes functionally contributed to ovarian development, characterization of functions and expression analysis of the isolated genes needs to be performed to evaluate their involvement in gonad development of *P. monodon*. We illustrated that genes/proteins in vitellogenesis (*PmVtg* and *PmVtgr*, Hiransuchalert et al., 2013a; Klinbunga et al., 2015), *O*-methyltransferase (*PmFAMeT*, Buaklin et al., 2015), progesterone-mediated oocyte maturation (*PmPgmrc1*, Preechaphol et al., 2010b), signal transduction during oocyte meiotic maturation (*cell division cycle 2*, *PmCdc2*, and *cell-dependent kinase cycle 7*, *PmCdk7*; Phinyo et al. 2013 and 2014) played important roles during ovarian development of *P. monodon*.

In the present study, functional involvement of *PmCytB5* gene in ovarian development of *P. monodon* was evaluated. Eyestalk ablation which is practically used for induction of ovarian maturation of penaeid shrimp was applied. Unilateral eyestalk ablation resulted in an increased mRNA level of *vitellogenin* (*Vtg*) in ovaries of *Marsupenaeus japonicus* (Spence Bate, 1888) (Okumura et al., 2006; Tsutsui et al., 2000). In *P. monodon*, unilateral eyestalk ablation promotes the expression of genes in vitellogenesis (Hiransuchalert et al., 2013a), methyfarnesoate biosynthesis (Buaklin et al., 2015) and signal transduction pathway (Phinyo et al., 2013 and 2014; Ponza et al., 2011). However, eyestalk ablation did not cause strong induction effects on the expression level of genes in the ecdysteroid biosynthesis pathway like *broad-complex* (*PmBrC*, Buaklin et al., 2013) and *progesterone receptor-related protein p23* (*Pmp23*, Preechaphol et al., 2010b).

The expression profile of *PmCytb5* mRNA in wild intact *P. monodon* broodstock suggested its important role in the late stage of ovarian development. Earlier upregulated expression of the *PmCytb5* mRNA in eyestalk-ablated *P. monodon* broodstock was observed. Therefore, its gene products may play a role during vitellogenesis in *P. monodon*.

A similar expression profile of PmCytb5 protein were observed. The expression of soluble PmCytb5 protein in premature ovaries of juveniles and stage I ovaries of wild broodstock was extremely limited. Its expression was increased in stage II ovaries of both intact and eyestalk-ablated broodstock. This circumstance suggested that the transcribed *PmCytb5* mRNA in oocytes at the previtellogenic stage was not sufficient for more rapid translation of the PmCytb5 protein during vitellogenesis of *P. monodon*. An increased expression of *PmCytb5* mRNA and soluble protein was found in the final stage of ovarian maturation in eyestalk-ablated adults but not in wild non-ablated broodstock as more rapid development of ovaries are found in the former than that of the latter.

Western blot analysis of membrane-bound PmCytb5 protein was also investigated and the positive immunoreactive signal of approximately 16 kDa was observed in all stages of ovarian development. In intact broodstock, increased expression of membrane PmCytb5 protein was observed in stages III and IV ovaries while comparable expression was found in stages I - IV ovaries of eyestalk-ablated adults. The stable

amount of membrane PmCytb5 protein reflects more rapid ovarian development in eyestalk-ablated broodstock than in non-ablated broodstock where upregulation of the PmCytb5 protein is required during late stages of ovarian development. Considering the expression profiles of PmCytb5 at both transcriptional and protein levels, PmCytb5 gene products should play the functionally important role during late vitellogenic and mature stages of ovaries in female *P. monodon*.

Progesterone but not 5-HT injection induced *PmCytb5* mRNA expression in ovaries of *P. monodon*

Positive effects of serotonin (5-HT) administration on reproductive performance and maturation of various penaeid species were reported (Aktas & Kumlu, 2005; Alfaro et al., 2004; Makkapan et al., 2011; Vaca and Alfaro, 2000; Wongprasert et al., 2006). Results from gene expression analysis revealed significant induction of various reproduction-related genes (e.g. *PmVtg1*, *PmFAMeT* and *PmCdc2*) of *P. monodon* upon injection with 5-HT (Hiransuchalert et al., 2013a; Buaklin et al., 2015; Phinyo et al., 2013 and 2014). However, it did not significantly affect the expression of *PmCytb5* in ovaries of domesticated *P. monodon* broodsotck.

Previously, progestin membrane receptor component 1 of *P. monodon* (*PmPgmrc1*)) which is a putative membrane-bound progestin receptor was isolated and characterized (Preechaphol et al., 2010b). The deduced PmPgmrc1 protein contains a transmembrane domain and Cytb5 domain at positions 23-41 and 68-166 while these predicted domains are located at positions 120-139 and 17-91 of the PmCytb5 protein, respectively. Likewise, *in vivo* effects of 5-HT on the expression of *PmPgmrc1* in ovaries of 6-month-old *P. monodon* juveniles were examined. The level of *PmPgmrc1* transcript in single and twice injection of serotonin (50 µg/g body weight) for 0, 12, 24, 48 and 72 hpi after the first and the repeated infection was not significantly different from the controls ($P>0.05$; Preechaphol, 2008). Accordingly, exogenous 5-HT injection did not affect the expression of Cytb5 domain-containing genes like *PmCytb5* and *PmPgmrc1*.

Progestins (progesterone and derivatives) are sex steroid hormones that play important roles in reproduction maturation of ovaries (Fingerman et al., 1993; Nagahama, 1997; Meunpol et al., 2007). In *Xenopus*, progesterone acts as a maturation-inducing hormone (MIH) resulting in meiotic resumption of oocytes from prophase-I arrest (Kishimoto, 2003 and 2018). Progesterone and its derivative, 17α-hydroxyprogesterone induced maturation and spawning in *Metapenaeus ensis* de Haan, 1844 (Yano, 1985 and 1987). In this study, progesterone injection significantly induced the expression level of ovarian *PmCytb5* implying its functional involvement in ovarian development of *P. monodon*.

Although eyestalk ablation is practically used to induce ovarian maturation of *P. monodon*, an issue about the animal welfare has been concerned. In addition, this technique results in a reduction of egg quality and higher mortality rates of brooders (Benzie, 1998; Okumura, 2004). Therefore, predictable maturation and spawning of captive *P. monodon* without eyestalk ablation is a long-term goal for the industry (Benzie et al., 1998; Quackenbush, 2001). Taken the available information together, induced reproductive maturation of captive female *P. monodon* may be carried out by administration of progesterone. Effects of appropriate forms and doses of proesterone on ovarian development and maturation of *P. monodon* broodstock should be further performed.

Conclusions

In this study, *PmCytb5* cDNA was characterized. Recombinant PmCytb5 protein and its polyclonal antibody was produced. The expression profiles of PmCytb5 mRNA and protein suggested its contribution in ovarian development of *P. monodon* Molecular mechanisms of progesterone induction on overexpression of *PmCytb5* suggested that appropriate form (s) of progesterone (progestins) may potentially induce oocyte/ovarian maturation of *P. monodon* and may be applied to replace the undesirable practice of unilateral eyestalk ablation in the future.

Ethical Statement

All authors declare that the present study was conducted in an ethical, professional and responsible manner following the regulation for animal care and use for scientific research of the National Center for Genetic Engineering and Biotechnology (BIOTEC) Animal Welfare Committee.

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Author Contribution

SK: Conceptualization, Writing – review and editing; KS, SJ, SP, PR OR, WI and PP: investigation, Methodology; BK: Supervision, Writing – Original Draft Preparation.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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