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Induction of vitellogenesis by glass bottom boat in the female banana shrimp, *Fenneropenaeus merguiensis* de Man

Ponsit Sathapondecha[a], Wilaiwan Chotigeat[a,b,*]

[a] Department of Molecular Biotechnology and Bioinformatics, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand
[b] Center for Genomics and Bioinformatics Research, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, 90112, Thailand

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ABSTRACT

In shrimp aquaculture, eyestalk ablation is the only technique that is widely used to accelerate ovarian development. Alternative methods for producing improved ovarian development in broodstock are currently being investigated. Several factors involved in the regulation of ovarian development in shrimp have been investigated. Among these factors, growth factors in the transforming growth factor beta (TGF-β) superfamily have been implicated as playing potential roles in the regulation of gonad development. In this work, a member of the TGF-β superfamily known as glass bottom boat (GBB), an ortholog of bone morphogenetic protein (BMP), was investigated to uncover its role in ovarian development in the banana shrimp *Fenneropenaeus merguiensis*. Full-length cDNA of FmGBB was obtained from transcriptome data. Phylogenetic analysis indicated that the sequence of FmGBB from banana shrimp was similar to those of other arthropods and vertebrate BMP 5/6/7, but was different from those of decapentaplegic proteins and vertebrate BMP 2/4. The *FmGBB* transcript was found to be widely expressed in shrimp tissues, and its expression in the ovary was dramatically increased in early and late vitellogenic stages during ovarian development and decreased in the mature stage, suggesting its role in vitellogenesis. To study the effects of FmGBB, a soluble recombinant mature FmGBB peptide (His-TF-rgbb) containing both monomers and homodimers was successfully expressed in *Escherichia coli*. The His-TF-rgbb peptide triggered oocyte proliferation in both cultured ovarian explants and in previtellogenic shrimp upon injection. Interestingly, the injection of His-TF-rgbb into previtellogenic female shrimp stimulated an increase in *Vg* expression in their ovaries while suppressing production of 20-hydroxyecdysone. Our results suggest the potential role of FmGBB in oocyte proliferation and vitellogenesis; this novel finding can be utilized to stimulate ovarian development in cultured shrimp.

1. Introduction

In shrimp aquaculture, traditional eyestalk ablation technique has been widely used to stimulate ovary development in crustaceans, such as *Penaeus monodon* (Uawisetwathana et al., 2011), *Litopenaeus vannamei* (Kumlu et al., 2011) and *Panulirus argus* (Quackenbush and Herrnkind, 1981). Since the eyestalks of crustaceans are a source of gonad-inhibiting hormone (GIH), which plays an inhibitory role in vitellogenesis and ovarian development (Feijo et al., 2016; Treerattrakool et al., 2008), the removal of the eyestalk leads to a reduction in GIH that results in the stimulation of ovarian maturation. However, since eyestalk ablation can be performed only once per one female broodstock, this technique should be avoided through the use of other methods, such as the improvement of shrimp nutrition (Wouters et al., 2001); the injection of stimulating hormones, such as serotonin (Makkapan et al., 2011); and the investigation of the genes involved in gonad development (Nagaraju, 2011).

In crustaceans, several factors that are involved in the regulation of gonad development have been reported, including GIH, putative gonad-stimulating factors (GSFs) in the brain and/or thoracic ganglia, serotonin, dopamine, methyl farnesoate, and steroids (Nagaraju, 2011). Over the past few decades, there have been attempts to identify the GSFs involved in ovarian development in shrimp. For example, it was found that extracts derived from the brain or thoracic ganglia of vitellogenic shrimp can induce vitellogenesis in *Paratya compressa* (Takayanagi et al., 1986) and *Penaeus vannamei* shrimp (Yano et al., 1988). Serotonin induces the release of putative GSFs from the brain or thoracic ganglia in Procambarus clarkii (Fingerman, 1997).

* Corresponding author at: Department of Molecular Biotechnology and Bioinformatics, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand.
E-mail address: wilaiwan58@hotmail.com (W. Chotigeat).

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Table 1
Oligonucleotide sequences used as primers in this study.

Primer	Nucleotide sequence (5′ → 3′)	Purpose
Oligo-dT	CCGGAATTCAAGCTTCTAGAGGATCCTTTTTTTTTTTTTTT	cDNA synthesis
FmGBB-R2	GCCAACCACTTTCACCAGCG	qPCR
Fmmatgbb-F-NdeI	GCTCATATGGATATAGCAGACAGGCAACC	Protein expression, qPCR
Fmmatgbb-R-XhoI	GGCCTCGAGCTACTG ACA GCC ACA GGT TTT GAC	Protein expression
Vg-F	TCCATCTGCAGCACCAATCTTCGC	qPCR
Vg-R	GCAACAGCCTTCATTCTGATGCCA	qPCR
EF1α-F	GAACTGCTGACCAAGATCGACAGG	RT-PCR, qPCR
EF1α-R	GAGCATACTGTTGGAAGGTCTCCA	RT-PCR, qPCR

Additionally, vertebrate gonadotropin-releasing hormone (GnRH) was shown to stimulate vitellogenesis in *P. monodon* (Ngernsoungnern et al., 2008). Recently, bursicon, a member of glycoprotein hormone family, was shown to induce vitellogenin (*Vg*) mRNA expression both *in vitro* and *in vivo* in *P. monodon* (Sathapondecha et al., 2015). Although these factors were demonstrated to have gonad-stimulating activity, the key gonad stimulating factors have not been identified so far.

In vertebrates, several growth factors drive the mechanisms underlying the stimulation of gonad development, particularly members of the transforming growth factor beta (TGF-β) superfamily, which include TGF-β, bone morphogenetic protein (BMP), growth differentiating factor (GDF) and activin (Knight and Glister, 2006). For instance, TGFB1 was shown, in combination with follicle stimulating hormone, (FSH) to induce follicle development in rats (Rosairo et al., 2008). BMP15 and GDF9 have been reported to promote ovarian follicular development at very early and later stages, while BMP 2/4/7 and BMP 2/5/6 were shown to induce theta and granulose cell proliferation, respectively (Knight and Glister, 2006). In addition, BMPs can induce estradiol production and inhibit progesterone production in combination with FSH in granulosa cells (Miyoshi et al., 2007; Pierre et al., 2005; Shimasaki et al., 1999), thus suggesting that members of the TGF-β superfamily are involved in oogenesis.

In insects, glass bottom boat (GBB), also known as TGFβ-60A, is an ortholog of vertebrate BMP 5/6/7 that plays several significant roles in embryonic development and cellular function. These roles include the regulation of metamorphosis via juvenile hormone biosynthesis (Ishimaru et al., 2016), the development of the wing imaginal disk (Bangi and Wharton, 2006; Khalsa et al., 1998; Ray and Wharton, 2001), and the maintenance of energy homeostasis (Ballard et al., 2010) and germline stem cells in *Drosophila* testis (Kawase et al., 2004). GBB can bind to other proteins, such as decapentaplegic protein (DPP), to form a heterodimer that regulates the self-renewal of intestinal stem cells in adult *Drosophila* (Tian and Jiang, 2014). Although several functions of GBB have been reported, its role in the gonadal development of crustaceans has not yet been characterized.

Therefore, this study aimed to characterize the role of GBB in gonadal development in the banana shrimp, *Fenneropenaeus merguiensis* (FmGBB). A full-length cDNA of FmGBB was obtained from transcriptome data and its expression profile during ovarian maturation in shrimp was determined. Additionally, the function of FmGBB during the stimulation of gonad development was studied in female shrimp using recombinant FmGBB peptides.

2. Materials and methods

2.1. Animal samples

F. merguiensis banana shrimp weighing 40–50 g were caught in the Gulf of Thailand in Nakhon Srithammarat province in Thailand. Shrimp were reared in 30 ppt seawater for three days before being used for experiments. Shrimp sampling was conducted in compliance with the ethical principles (NRCT 1999) and the regulations regarding the use of animals for scientific purposes (Act CE 2015, Thailand).

2.2. RNA extraction and cDNA synthesis

Shrimp tissues, including the eyestalks, brain, thoracic ganglia, hepatopancreas, gills, ovary and testis were extracted, and total RNA was isolated using Trizol™ reagent (Ambion®) according to the manufacturer's extraction protocol. The quality and quantity of the total RNA was determined using a Nanodrop 2000 (Thermo Scientific). One microgram of total RNA was treated with 1 U of DNase I (RQ1, Promega) at 37 °C for 30 min, then 1 μl of 50 mM EDTA was added and the sample was heated at 65 °C for 5 min. After cooling on ice, the sample was mixed with 0.5 μl of 10 μM oligo-dT primer. The sample was heated at 65 °C for 5 min and cooled on ice. 5.5 μl of RNA sample was then added to 7 μl of a mixture containing 1X ImProm-II™ buffer (Promega), 3 mM $MgCl_2$, 0.5 mM dNTP and 1 U of ImProm-II™ Reverse Transcriptase (Promega). The reverse transcriptase reaction was performed at 25 °C for 5 min, 42 °C for 60 min and 70 °C for 10 min. The cDNA was stored at −20 °C until use.

2.3. Determination of FmGBB expression in shrimp tissues

To determine *FmGBB* expression in shrimp tissues, total RNA was isolated from the organs of male and previtellogenic female shrimp. Quantitative RT-PCR (qPCR) was performed to determine the *FmGBB* transcript levels. cDNA from shrimp tissues from the eyestalk, brain, thoracic ganglia, gills, hepatopancreas, testis and ovary was prepared as described in Section 2.2. Two microliters of cDNA was used in the 12.5 μl PCR reaction, which included 1X FastStart Universal SYBR Green master mix (Roche) and 0.2 μM of FmmatGBB-F-Nde I and FmGBB-R2 primers (Table 1). The PCR reaction was performed using a real-time PCR instrument (Mx3000P, Stratagene). The initial denaturation was performed at 95 °C for 3 min, followed by 40 cycles of amplification (95 °C for 10 s, 55 °C for 20 s and 72 °C for 30 s). Subsequently, melting analysis was performed at 95 °C for 1 min, 55 °C for 30 s and 95 °C for 30 s. Each sample was analyzed in duplicate. Elongation factor 1 − α (EF1α) was used as a reference gene to normalize the expression of the target genes. The efficiency of the primers used in this study was 95–100%, as determined using a cDNA dilution curves. The relative quantification was performed using the 2^{-ddCt} method (Livak and Schmittgen, 2001).

2.4. Expression profile of FmGBB during ovarian maturation

The determination of *FmGBB* expression during ovarian development was performed using qPCR. Ovarian development in shrimp is classified into four stages (previtellogenic, early vitellogenic, late vitellogenic and mature stages) and is determined using the gonadosomatic index and histology as described by Wonglapsuwan and colleagues (Wonglapsuwan et al., 2010). Four-to-seven individual shrimp from each stage were used in the study. The determination of *FmGBB* expression during ovarian development was performed in duplicate for each sample using the qPCR procedure described above.

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CAGTCTCTCTCCTGCCTGCCTTGGATACTGCAGCCCCGCGCCTGCTCGCGGTCCCTGTTGCTCCTCCAGCTGCCCGCGGCCTG
CTCTTGCCTCCTGTTGCTGCCTGCTGTCCGCGTGTCCTGCTTGGCTCTGGAGCGCCAGGCATGGGCTAGGGCGACCTGCTGCT
GCGGCCGACGGACCACAAAGGGGAACAGCGCAGCGCCTCCACGAGAAAGTGAGCGCAGGAGGCGTCCAGGTGCTGCGCCGTGC
ATTACGACGATGTAGTGAGTCTCGAGTCCTGCGTGTTCTTGTGTCTCCGCAATCGTTCGTC
311  ATGGGACCAGGGTCGACGAGTGCGCCCAGAAGGGCGGGGGTGTGGGTGTGTGTCGTCGCTGCAGTGGTGT
       M  G  P  G  S  T  S  A  P  R  R  A  G  V  W  V  C  V  V  A  A  V  V

381  GGGCGGCGGGCGGGTGTGCAGCGGGCGTGGGCGTGTGGATGGACGACGGGAATGGGCGCACGGTCCTGGC
     W  A  A  G  G  C  A  A  G  V  G  V  W  M  D  D  G  N  G  R  T  V  L  A

451  TGACAGGCTGGACGCCGAGGAAGCCGCGGATCTGGCGCAGGACATGCTGGACCTCCTGGACCTCCCGGCG
       D  R  L  D  A  E  E  A  A  D  L  A  Q  D  M  L  D  L  L  D  L  P  A

521  CCGCCCGCCATGGTGGGCCACCACCGCCACCACCACCGCGCCCACCGCGCCCACGGCTCGGCGCCCACCT
       P  P  A  M  V  G  H  H  R  H  H  H  R  A  H  R  A  H  G  S  A  P  T

591  GGATGAAGAACATCTACAACACCCTCGACGAGCACGGCCACGCCAACGCGCCCAACATGGACGACGTGCA
     W  M  K  N  I  Y  N  T  L  D  E  H  G  H  A  N  A  P  N  M  D  D  V  H

661  CCGCGAGACCGTCACGGCGGCCGACACCATCATCACCTTCGTCAACAGAGATCCGCCCACGGGCCGCCCA
       R  E  T  V  T  A  A  D  T  I  I  T  F  V  N  R  D  P  P  T  G  R  P

731  GCCCACGGTGCCAACAAGCGGCTTTACTTCGACGTGAATGACGTTCCTCTGGACCACTCGCTTCTCGGGG
       A  H  G  A  N  K  R  L  Y  F  D  V  N  D  V  P  L  D  H  S  L  L  G

801  CGGAAATCCAGGTGCATCGGCAGCCTGGCTTCGACGAGGTGGTCACTCTGCACGTGTATGTGATCACTGA
     A  E  I  Q  V  H  R  Q  P  G  F  D  E  V  V  T  L  H  V  Y  V  I  T  D

871  CGAGCAAGGGAGTGAGTCGAAGGTGGCGCGGGTGAGCCTGAGCGAGCCCGGCTGGGTCACGGTGAACGTC
       E  Q  G  S  E  S  K  V  A  R  V  S  L  S  E  P  G  W  V  T  V  N  V

941  ACGCGGCCCGTCCTCTCGTGGCTCATCTTCCCCGACACCAACTACGGCCTCCGGCTGGCGGTCACGTCGC
       T  R  P  V  L  S  W  L  I  F  P  D  T  N  Y  G  L  R  L  A  V  T  S

1011 CGGGATACAAGCACGAGCGGCATTTCCATGAGGTGGGGATTTCTGGCTCCCATGACGAGGAGGACTACAG
     P  G  Y  K  H  E  R  H  F  H  E  V  G  I  S  G  S  H  D  E  E  D  Y  R

1081 GCCCTTCATGGTGGGCTTCTTCGCTCTCCCGGCCAGCGCCCACAAGAAGAAGAGGATAAGGACGGCGCGA
       P  F  M  V  G  F  F  A  L  P  A  S  A  H  K  K  K  R  I  R  T  A  R

1151 TCGGTGTCCAGGCCCGCCATCAGCAGACCCAGGAAATACAGAGATATAGCAGACAGGCAACCGGGGTGGG
       S  V  S  R  P  A  I  S  R  P  R  K  Y  R  D  I  A  D  R  Q  P  G  W

1221 ACGCGGCGTGCAAGATGAAGCACCTGCACGTCTCCTTCCGCGACCTGGGCTGGGAGGACTGGGTCATCGC
     D  A  A  C  K  M  K  H  L  H  V  S  F  R  D  L  G  W  E  D  W  V  I  A

1291 GCCCGAGGGCTACGACGCCAATTACTGCGAAGGGCGCTGCAGTTTCCCCCTCCACGCCGAGCTGAACGCC
       P  E  G  Y  D  A  N  Y  C  E  G  R  C  S  F  P  L  H  A  E  L  N  A

1361 ACGAACCACGCCCTTGTGCAAACGCTGGTGAAAGTGGTTGGCGACGTCGCGGACGAGACGGAGGTTCCCC
       T  N  H  A  L  V  Q  T  L  V  K  V  V  G  D  V  A  D  E  T  E  V  P

1431 CCAACGCCTGCTGTGCGCCCATCGACCTGGCGACGATACCCGTCTTGTACTACTCTTTTGATAACAATAT
     P  N  A  C  C  A  P  I  D  L  A  T  I  P  V  L  Y  Y  S  F  D  N  N  I

1501 TGTCCTCAAGAAATATCCTATGATGATTGTCAAAACCTGTGGCTGTCAGTAG
       V  L  K  K  Y  P  M  M  I  V  K  T  C  G  C  Q  *
AAAATAGATGTAAAGAACCATTTTATGATGTTGAGTGTAAGATTATATATATATAAATATATGTATACTGCCTTGCATATCCT
ATCTGTCCGTATTTAGGCTTTTGTTATAATAATCACAGATTTTGGGTTATATCTCTTAATCCTAAGTTTTTTTTCTACTTGAC
TACAAAATATATCGAGTTTTTAATTGAATCTATGTAAATAGTTTGGTGTGTTTGTGAGAGACGAGAACAAGAAACAAGAAAGA
AAATAACCGAAAAAGGATTTATTTTGCAAATAGTTTTAATCACCTCCAGCATATATACTTAACTGATAATTTACTCATTCAGA
ATGCATGTGAAACTTTTTTGTATTTCGTTTCCAGATTATTAGATTTAGTTTCTCTTCACTATCAGTGATATGGACTTTCAATG
TTGATCTAATACAGCTGCATCTTCTAAATAATCCAGATATTCCAGAAATATTTAGTGCCTGTACATAAGAAAATGTTTTGTAA
CTAACAGGTCTACATTCATTGACTCAGCTGCATGTGTATAAAATGAAGTTGAGGAAATTACACGCTGTTTTTTCTT
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Fig. 1. Schematic of the full-length cDNA encoding FmGBB showing its deduced amino acid sequence. The translated amino acids corresponding to each codon are shown under the nucleotide sequence of full-length FmGBB (GenBank accession no. KX922856). The signal peptide of FmGBB is represented in gray. The underlined italic letters indicate the furin cleavage sites predicted by ProP 1.0. The underlined bold letters denote conserved sequences contributing to cystine knot formation. The asterisk represents the stop codon.

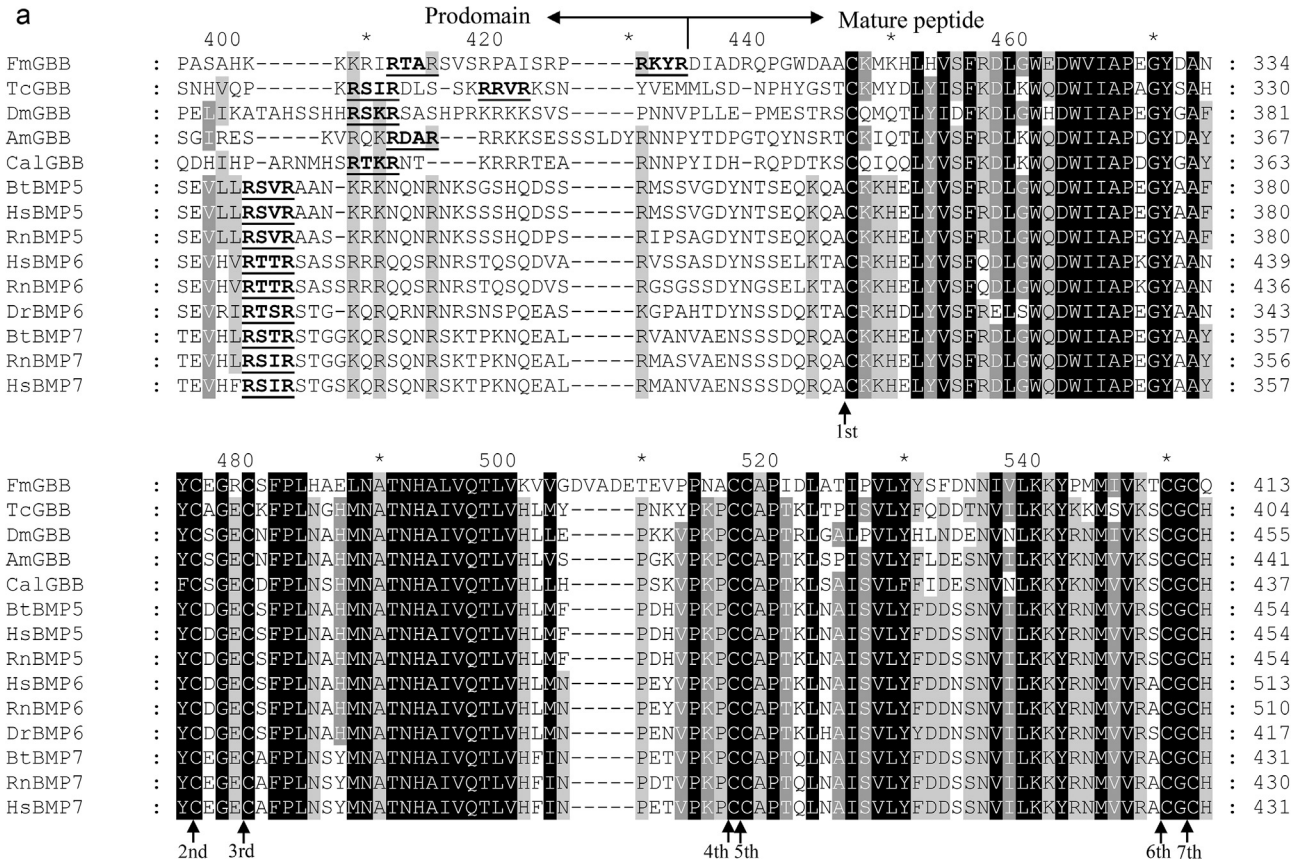

Fig. 2. Alignment of mature FmGBB peptide sequences from insects and crustaceans with those of BMP from vertebrates (a). The pro-domain and mature peptides are separated by furin cleavage sites, as shown by the bold and underlined letters. The arrows indicate the cysteine residues within the sequences. Schematic representing the phylogenetic analysis of amino acid sequences in GBB, DPP, and BMP (b). The amino acid sequences of GBB, DPP, and BMP were aligned using ClustalW, and the phylogenetic tree was generated using a maximum likelihood method based on the JTT matrix-based model in MEGA 7.0 software. The phylogeny test was performed by a bootstrap method using 1000 replicates. The tree is drawn to scale with the branch length representing the number of substitutions per site. The GBB amino acid sequences used in the analysis include those from *Fenneropenaeus merguiensis* (KX922856), *Anopheles gambiae* (AAT07301), *Apis mellifera* (XP_394252), *Bombyx mori* (XP_004929272), *Clogmia albiplunctata* (AHH30785), *Drosophila melanogaster* (NP_477340), *Daphnia pulex* (EFX74626), and *Tribolium castaneum* (NP_001107813). The DPP amino acid sequences include those from *Apis cerena* (ALG65084), *Acropora millepora* (AAM54049), *Bombyx mori* (AKG96797), *Crassostrea gigas* (AHB37078), *D. melanogaster* (NP_477311), and *T. castaneum* (AAB38392). The BMP amino acid sequences include those from *Bos taurus* BMP2 (NP_001092611), *Homo sapiens* BMP2 (AAA5183), *Macrobrachium nipponense* BMP2 (GCVG01053280), *Rattus norvegicus* BMP2 (NP_058874), *B. taurus* BMP4 (NP_001039342), *H. Sapiens* BMP4 (NP_570911), *R. Norvegicus* BMP4 (NP_036959), *B. Taurus* BMP5 (NP_001291945), *H. Sapiens* BMP5 (NP_066551), *R. Norvegicus* BMP5 (NP_001101638), *Denio rerio* BMP6 (NP_001013357), *H. Sapiens* BMP6 (NP_001709), *R. Norvegicus* BMP6 (NP_037239), *B. Taurus* BMP7 (NP_001192944), *H. Sapiens* BMP7 (NP_001710), *R. Norvegicus* BMP7 (NP_001178785), and *Xenopus laevis* BMP7 (CAA45021). The sequence of activin beta from *Mus musculus* (AAC53164.1) was used as an outgroup.

2.5. Expression and purification of recombinant 6X histidine-tagged trigger factor FmGBB (His-TF-rgbb) peptides in Escherichia coli

To express recombinant FmGBB (rgbb), we constructed a recombinant plasmid containing the nucleotides encoding FmGBB. The ovarian cDNA was used as the template to amplify the mature domain of FmGBB using PCR. Each PCR reaction had a total volume of 12.5 µl that was composed of 0.5 µl of cDNA, 1X PrimeSTAR® GXL buffer (Takara®), 0.2 mM dNTPs, 0.2 µM each of the forward primer containing the *Nde* I restriction site and the reverse primer containing the *Xho* I site (Table 1) and PrimeSTAR® GXL DNA polymerase (Takara®). The PCR reaction began with an initial denaturation at 98 °C for 1 min, followed by 35 cycles of amplification (98 °C for 10 s, 55 °C for 15 s and 68 °C for 20 s) and a final extension at 68 °C for 5 min. The pCold® TF (Takara®) plasmid was used as an expression vector. The PCR product and pCold® TF were double digested with *Nde* I and *Xho* I. The restriction enzyme-digested PCR product was ligated with the plasmid, and DNA sequencing of the ligated product was conducted to verify the presence of the FmGBB nucleotide sequence.

The recombinant plasmid was transformed into *E. coli* BL21 DE3. After confirmation that the selected bacterial clones contained the recombinant plasmid, the expression of His-TF-rgbb (6X histidine-tagged trigger factor recombinant gbb) peptide was induced. The bacteria were cultured overnight in LB broth containing 100 µg/ml ampicillin at 37 °C. The starter culture was then inoculated in LB broth and incubated until the OD_{600} reached 0.4–0.8. The bacterial cells were maintained at 4 °C for 15 min, after which the expression of the recombinant protein was induced with 0.1 mM IPTG and the bacteria were cultured at 15 °C for 20–24 h.

After induction, the bacterial cells were harvested and re-suspended in binding buffer (20 mM phosphate buffer (pH 8.0) and 300 mM NaCl), sonicated, and centrifuged at 12,000 rpm for 10 min. The supernatant was collected as a soluble fraction. The recombinant peptide in the soluble fraction was affinity-purified by incubating it overnight at 4 °C in a slurry containing 50% HisPur™ Ni^{2+} resin (Thermo Scientific). After washing several times with binding buffer containing 70 mM imidazole, the His-TF-rgbb peptide was eluted with elution buffer (20 mM phosphate buffer (pH 8.0), 300 mM NaCl and 500 mM imidazole). The purified His-TF-rgbb was then transferred into binding buffer by dialysis. SDS-PAGE of the protein sample was used to detect homo-

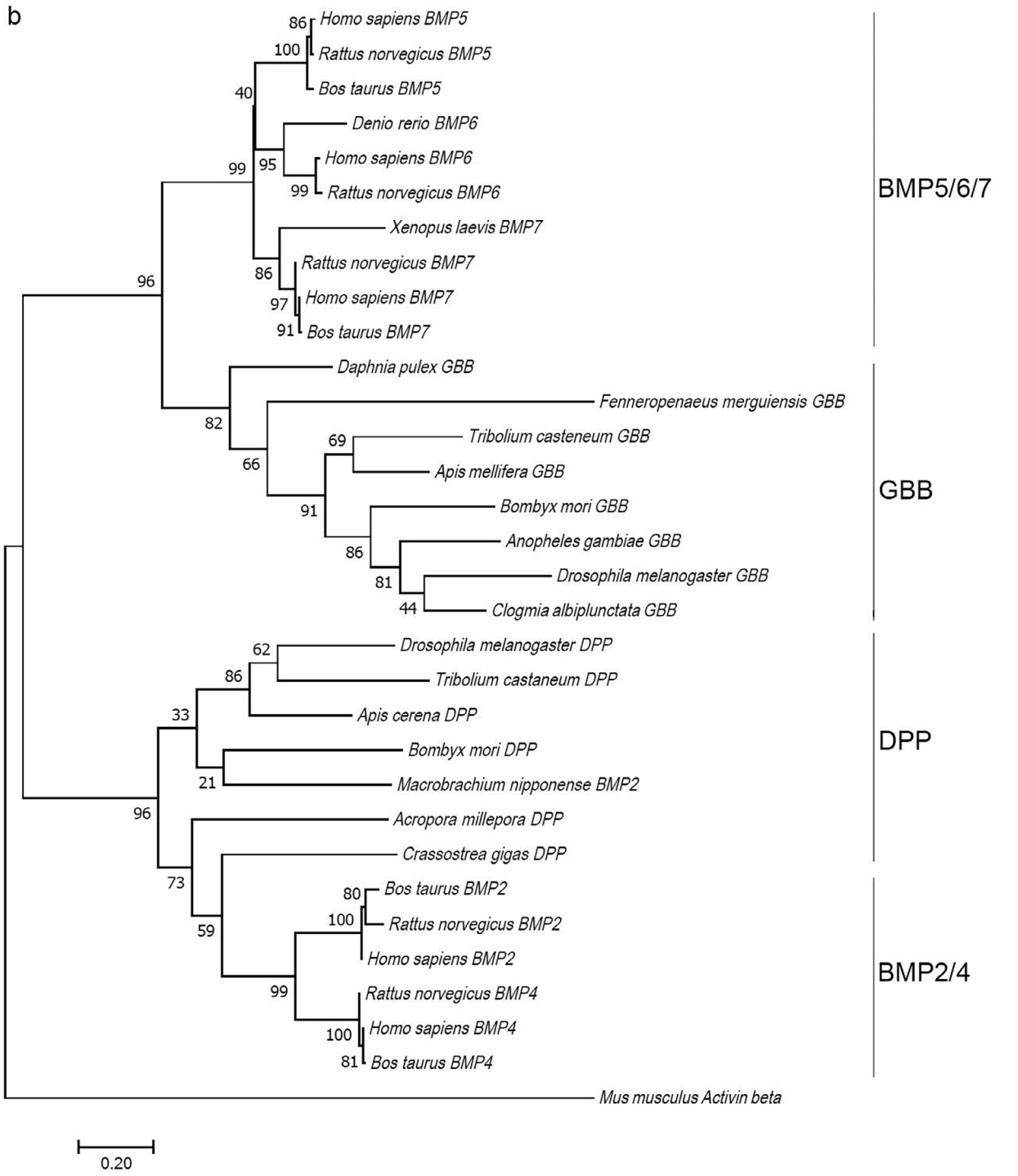

Fig. 2. (*continued*)

dimerization of His-TF-rgbb or His-TF (6X histidine-tagged trigger factor) under reducing and non-reducing conditions. The purified His-TF-rgbb peptide was also analyzed by western blot using an anti-6X-His antibody (Pierce) with a rabbit anti-mouse IgG conjugated with alkaline phosphatase as a secondary antibody. The colorimetric signal was detected by measuring the substrate produced in the presence of a NBT/BCIP solution. The protein concentration was measured using Lowry's method.

2.6. Effect of the His-TF-rgbb peptide on oocyte proliferation in ovarian explants

Ovaries were freshly isolated from four individual previtellogenic female shrimp and minced into small pieces. The ovarian pieces were washed with culture medium composed of 2X Leibovitz's L-15 (Gibco), 10% v/v fetal bovine serum, 0.1% w/v lactalbumin and 2X antibiotic and antimycotic (Gibco). Each ovarian piece was then cultured in 100 µl of culture medium in an individual well of a 96-well plate. Eighty microliters of various concentrations (50, 100 and 200 ng/ml) of His-TF-rgbb, His-TF or buffer was added to each well, and the ovarian explants were incubated at 28 °C for 2 hr. Subsequently, 20 µl of 5-

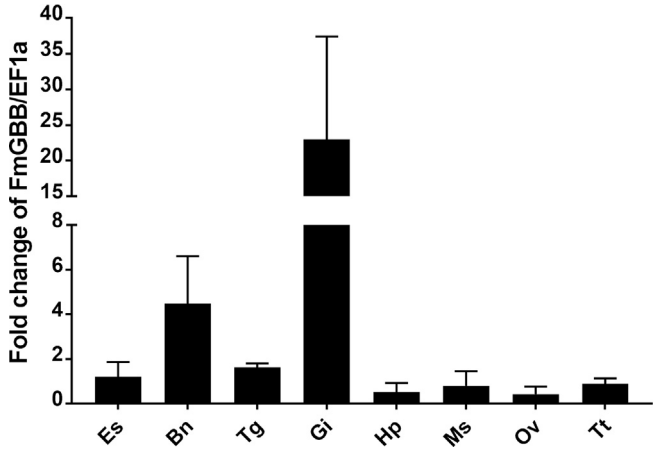

Fig. 3. Determination of *FmGBB* expression in shrimp tissues. The relative expression of *FmGBB* in shrimp tissues, including the eyestalks (Es), brain (Bn), thoracic ganglia (Tg), gills (Gi), hepatopancreas (Hp), ovary (Ov), and testis (Tt), was determined using qPCR. The bars and the error bars represent the means and SEMs, respectively.

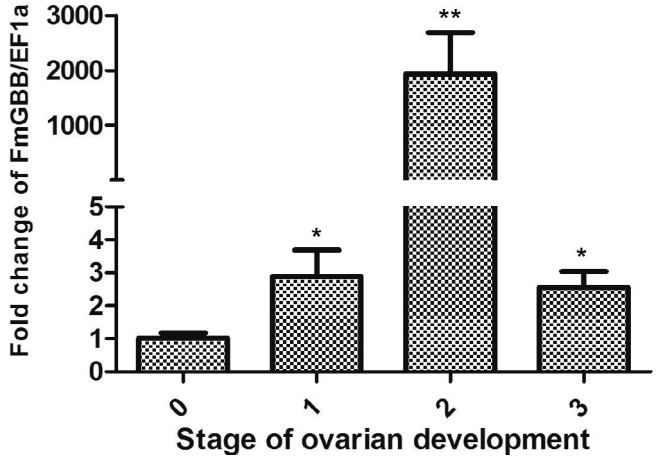

Fig. 4. Expression profile of *FmGBB* during ovary development. *FmGBB* expression in the ovary was determined using qPCR at different stages of ovarian development (n = 4–7 for each group), including the previtellogenic (0), early vitellogenic (1), late vitellogenic (2) and mature (3) stages. The bars and error bars represent the means and SEMs, respectively. Single and double asterisks denote significant differences between groups where $p < 0.05$ and $p < 0.01$, respectively, that were determined using one-way ANOVA and pair-wise comparison by Duncan's test.

ethynyl-2-deoxyuridine (EdU, Abcam) was added to a final concentration of 10 μM and the explants were incubated for another 22 h.

After incubation, the ovarian pieces were fixed in methanol at −20 °C overnight for EdU staining. Some of the ovarian tissues were processed and their total RNA was extracted, which was used to determine vitellogenin (*Vg*) expression using qPCR. For EdU staining, the fixed ovarian tissue was sonicated three times for 1 s. The obtained oocyte cells were rehydrated using a series of methanol dilutions in TBS. The oocytes were stained with 50 μl of staining solution (100 mM Tris (pH 8.0), 1 mM $CuSO_4$, 10 μM Alexa Fluor® 488 azide (Molecular Probe) and 100 mM L-ascorbic acid) for 20 min before washing with TBS-T (0.1% Triton X-100 in TBS) for 5 min, then incubated with TBS-T containing 10 μg/ml DAPI for 2–3 min. After the final washing, the oocyte cells were mounted on glass slides and maintained at 4 °C. Images were acquired using fluorescence microscopy (Olympus BX51) with CellSens Standard software (Olympus). Overlap of the EdU and DAPI signals was considered to be indicative of oocyte proliferation. The EdU index was calculated by dividing the number of EdU-positive oocyte cells by the total number of oocyte cells (n = 150–200).

2.7. Vitellogenin stimulation and oocyte proliferation in shrimp ovaries injected with His-TF-rgbb

Previtellogenic female shrimp (n = 3–4) weighing approximately 40 g were intramuscularly injected with 3 μg of His-TF-rgbb, His-TF or binding buffer. Three shrimp were used for each group. On the second day after injection, shrimp from each group were anesthetized on the ice and their ovaries were excised. The injection was performed during two independent experiments. In addition, shrimp were injected with the same amount of protein and the ovaries were isolated on days 7 and 9. Total RNA was extracted from the ovaries to determine *Vg* expression using qPCR as described above. The ovaries from shrimp injected with the protein for 2 days were cultured as ovarian explants and incubated with EdU for 20 h to determine oocyte proliferation.

2.8. Determination of the 20-hydroxyecdysone (20-HE) titer in hemolymph

Hemolymph was collected from previtellogenic female shrimp injected with 3 μg of His-TF-rgbb, His-TF or buffer on days 2, 5 and 7. Five shrimp in the intermolt stage of the molting cycle were used in each group. The hemolymph was extracted in an equal volume of cold methanol. After centrifugation, the supernatant was evaporated until it was completely dry. The hemolymph was then re-suspended in 100 μl of blocking solution (1% w/v BSA and 0.05% Tween-20 in PBS) prior to performing ELISA. The ovaries from shrimp after protein injection on day 7 were isolated to determine *Vg* expression using qPCR.

To determine 20-HE titers in hemolymph, a competitive ELISA was performed. 1.5 μg/ml of unconjugated goat anti-rabbit IgG in coating buffer (100 mM carbonate buffer, pH 9.6) was used to coat an ELISA plate, which was then incubated at 4 °C overnight. After washing with PBS-T (0.05% Tween-20 in PBS (pH 7.4)), each well was blocked with blocking solution for 2 h. The blocking solution was aspirated and 50 μl of 20-HE standard (Abcam) at concentrations of 0.1–100 ng/ml, 50 μl of hemolymph sample, 50 μl of 20-HE conjugated to HRP (Cosmo Bio; 1:200 dilution) or 50 μl of 1:1000 rabbit anti-20-HE antibody (Cosmo Bio) was added to each well. The experiments were duplicated for each sample or standard. The mixtures were incubated for 2 h, then washed with PBST three times before development with 100 μl of TMB solution (Abcam). After incubation for 45 min, the reaction was stopped by adding with 50 μl of 1 N H_2SO_4. The absorbance was measured at 620 nm. The detection limit of ELISA is 5 pg.

2.9. Determination of Vg expression in primary ovarian cells upon 20-HE treatment

Primary ovarian cells were prepared from five previtellogenic ovaries. The small ovary pieces were freshly isolated and washed with culture medium containing 2X antibiotic and antimycotic (Gibco) several times. The ovarian cells were then isolated by mincing the ovarian pieces with scissors in culture medium. 5×10^5 ovarian cells were seeded in a 24-well plate and incubated at 28 °C overnight. The ovarian cells were then treated with a different concentrations of 20-HE (50–400 ng/ml) or buffer and incubated for 3 h. Four samples were used for each group. Subsequently, the total RNA extracted from the ovarian cells was used to determine *Vg* expression using qPCR.

2.10. Statistical analysis

All data were analyzed to determine significant differences between groups using one-way ANOVA; pair-wise comparisons were conducted using Duncan's test (IBM SPSS Statistics 20).

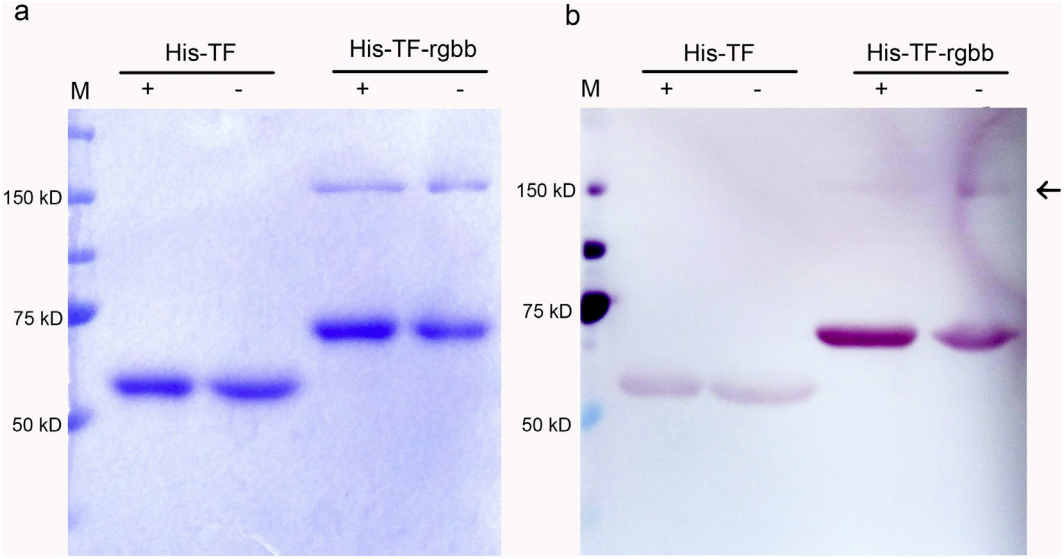

Fig. 5. Expression of the soluble His-TF-rgbb peptide in *E. coli*. The His-TF-rgbb or His-TF peptide purified using Ni^{+}-NTA resin was separated by SDS-PAGE and visualized using Coomassie staining (a). Western blot analysis was performed using anti-6X-His antibody, and the signal was detected using NBT/BCIP solution (b). The presence of homodimers in the protein sample was determined in the presence (+) or absence (−) of β-mercaptoethanol. The arrow indicates the expected size of the His-TF-rgbb homodimer. "M" represents a pre-stained protein marker.

3. Results

3.1. Molecular characteristics of GBB in F. merguiensis

In this study, a full-length cDNA of FmGBB was obtained from our ovary transcriptome data derived from *F. Merguiensis* (accession no. SRP075844; Saetan et al., 2016). The FmGBB transcript contained a 308-bp 5′UTR, a 1239 bp open reading frame encoding 413 amino acid residues, and a 577-bp 3′UTR (GenBank accession no. KX922856; Fig. 1). The deduced amino acid sequence of mature FmGBB is composed of a signal peptide that is 31 amino acid residues in length (as predicted by ProP 1.0), followed by 263 amino acid residues that comprise the prodomain that contains two furin cleavage sites (RXXR; underlined in Fig. 1 as predicted by ProP 1.0), and a C-terminus. The mature peptide contains a conserved cystine knot (CXGXC) peptide sequence that is shown in bold and underlined in Fig. 1.

The deduced amino acid sequence of mature FmGBB is similar to that of insect GBB and vertebrate BMP 5/6/7 (Fig. 2a). FmGBB also contains seven cysteine residues (indicated by arrows in Fig. 2a) that may form three intra-structural disulfide bonds via pairing of the 1st and 5th, 2nd and 6th, and 3rd and 7th cysteine residues (as determined by the DiANNA 1.1 web server). In addition, a phylogenetic tree revealed that FmGBB belongs to the GBB and BMP 5/6/7 subgroups but is dissimilar to decapentaplegic protein (DPP) and BMP 2/4 (Fig. 2b).

3.2. Expression analysis of FmGBB in shrimp tissues

The expression of *FmGBB* in shrimp tissues was determined using qPCR. The results showed that *FmGBB* was highly expressed in the gills and brain, whereas the *FmGBB* expression level was lower in other tissues, including the eyestalk, thoracic ganglia, hepatopancreas, muscle, ovary, and testis (Fig. 3).

3.3. Expression profile of FmGBB during ovarian development

To determine *FmGBB* expression in the ovary during different stages of ovarian maturation, qPCR was performed. The results indicated that *FmGBB* expression was at a low level in the previtellogenic stage, then increased by two-fold during the early vitellogenic stage. *FmGBB* expression rose dramatically in the late vitellogenic stage and declined in the mature stage of ovarian development (Fig. 4).

3.4. Expression and purification of soluble recombinant 6X histidine-tagged trigger factor gbb (His-TF-rgbb)

The nucleotide sequence encoding mature FmGBB was cloned into the pCold®TF vector, which contains 6 histidine residues and the trigger factor (TF) at the N-terminus. A protein product of approximately 67 kDa, which represented soluble His-TF-rgbb, was successfully expressed in *E. coli*, while the His-TF peptide had a molecular weight of approximately 52 kDa (Fig. 5a). The identity of the purified His-TF-rgbb and His-TF peptides was confirmed by western blot analysis using an anti-6X-His antibody (Fig. 5b). SDS-PAGE was used to determine the degree of homo-dimerization of the His-TF-rgbb peptide in reducing and non-reducing conditions. The results showed that purified His-TF-rgbb existed mainly as a monomer (67 kDa) and that the expected homodimer was present in both recuding and non-reducing conditions (Fig. 5a and b). In contrast, the His-TF sample contained only monomers (Fig. 5a and b).

3.5. Effect of His-TF-rgbb on oocyte proliferation and vitellogenin mRNA levels in ovarian explants

Ovarian explant cultures were used to demonstrate the effects of the His-TF-rgbb peptide on oocyte proliferation and *Vg* mRNA expression. The complete merging of the EdU and DAPI signals that was observed in the nuclei of some oocyte cells (Fig. 6a) was considered to signify oocyte proliferation. The calculated EdU indices indicated that oocyte proliferation in ovarian explants treated with 200 ng/ml His-TF-rgbb was increased approximately three-fold compared to those treated with buffer or TF alone (Fig. 6b). However, compared to buffer-treated ovarian explants, *Vg* mRNA levels were decreased approximately two-fold in ovarian explants treated with either His-TF-rgbb or His-TF alone (Fig. 6c).

3.6. Effects of His-TF-rgbb injection on vitellogenesis in shrimp

The FmGBB peptide was demonstrated to affect oocyte proliferation and *Vg* expression in ovarian tissue in previtellogenic female shrimp. Previtellogenic female shrimp were injected with His-TF-rgbb, His-TF

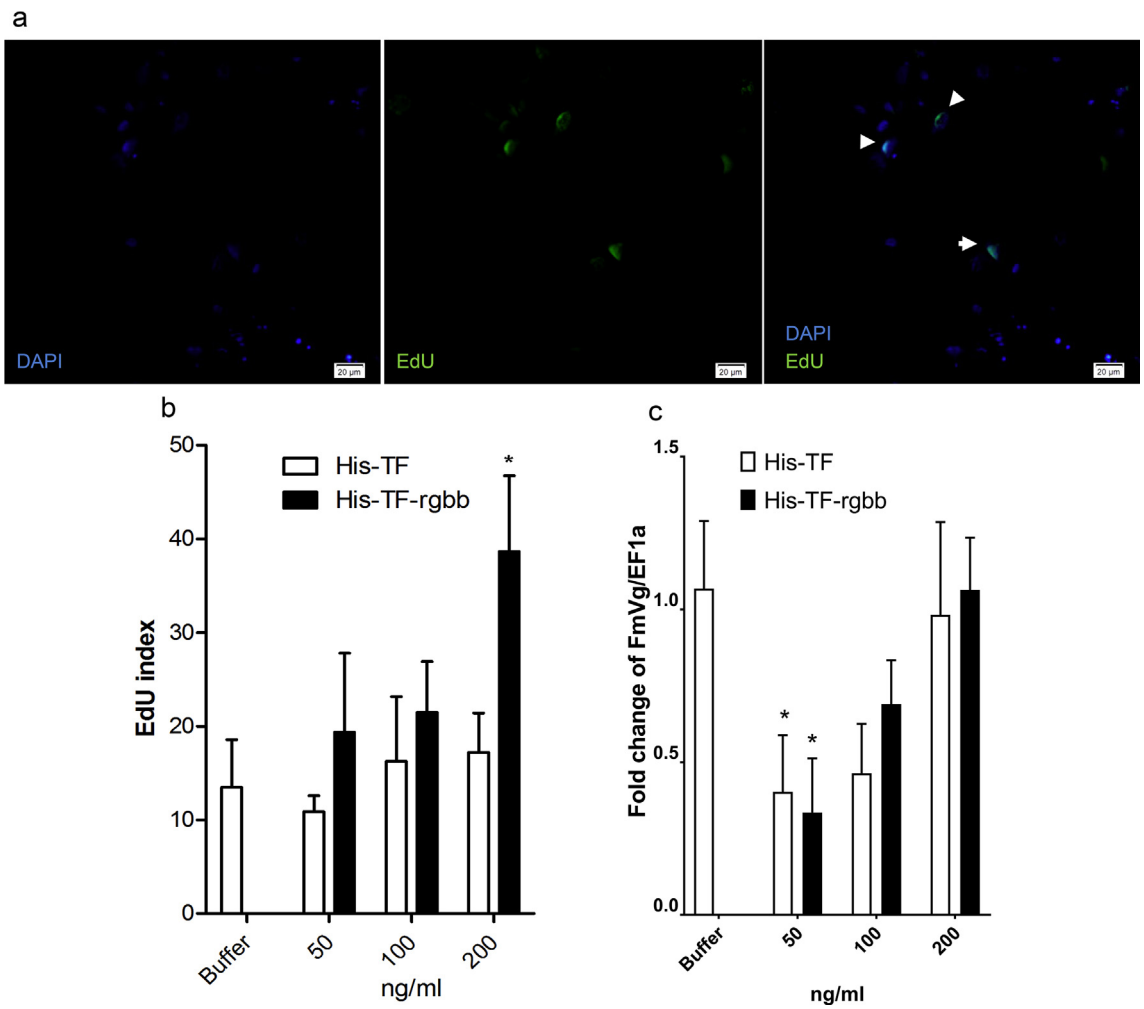

Fig. 6. Effects of His-TF-rgbb treatment on oocyte proliferation and *Vg* expression in ovarian explants. Previtellogenic ovaries from 4 individual shrimp were cultured and treated with different concentrations of His-TF-rgbb, His-TF or buffer for 24 h. The EdU (green) and DAPI (blue) signals were detected in the fixed oocyte cells under a fluorescence microscope (a). The merged signals, shown by arrowheads, were determined to be indicative of proliferation and used to calculate the EdU index. The effect of treatment on oocyte proliferation was determined using EdU index analysis of 150–200 oocyte cells in each group (b). The relative expression of *Vg* mRNA was determined using qPCR (c). In Fig. b and c, the bars and error bars represent the means and SEMs, respectively. An asterisk denotes the presence of a significant difference between groups where $p < 0.05$, as determined by randomized complete block design using one-way ANOVA and pair-wise comparison by Duncan's test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

or buffer for 2, 7 or 9 days. The results showed that oocyte proliferation was increased in the His-TF-rgbb-injected shrimp compared to the buffer- and His-TF-injected shrimp 2 days after injection (Fig. 7a). Interestingly, ovarian *Vg* mRNA expression levels were higher in the His-TF-rgbb-injected shrimp than in buffer-injected shrimp, whereas *Vg* was expressed at a lower level in His-TF-injected shrimp 2, 7 and 9 days after injection (Fig. 7b–d).

3.7. Effects of His-TF-rgbb injection on the 20-HE titer in shrimp hemolymph

The effects of FmGBB peptide on the production of 20-HE in previtellogenic female shrimp was investigated. On days 2, 5 and 7 after injection of His-TF-rgbb, His-TF or buffer, the level of 20-HE in hemolymph was determined using a competitive ELISA assay. The results indicated that the amount of 20-HE in hemolymph significantly decreased in shrimp injected with His-TF-rgbb compared with shrimp injected with buffer or His-TF peptide on days 2, 5 and 7 after injection (Fig. 8a). The *Vg* mRNA level increased in His-TF-rgbb-injected shrimp on day 7 after injection (Fig. 8b). Shrimp in the molting stage were observed on day 7 after injection. The results showed that most shrimp injected with His-TF-rgbb were in the premolt stage of the molting cycle ($D_{1\text{-}2}$), while shrimp injected with His-TF and buffer were in the late premolt stage ($D_{3\text{-}4}$) (Table 2).

3.8. Effects of 20-HE treatment on Vg expression in primary ovarian cell cultures

After treatment of primary ovarian cell cultures with 20-HE for 3 h, *Vg* expression was determined. The results indicated that the *Vg* mRNA level significantly increased in ovarian cells treated with 50 ng/ml 20-HE compared to those treated with buffer (Fig. 9). Higher concentrations of 20-HE (100–400 ng/ml) did not affect *Vg* expression.

4. Discussion

Ovarian development in shrimp is controlled by several gonad-stimulating factors, such as peptide hormones, neurotransmitters, and steroids (Nagaraju, 2011). Several growth factors that are potentially involved in gonad development in vertebrates are members of the TGF-β superfamily, which includes TGF-β, BMP, and GDF (Knight and Glister, 2006). In this study, FmGBB, an ortholog of BMP 5/6/7, was identified based on *F. merguiensis* transcriptome data and found to be differentially-expressed during the previtellogenic and mature stages of

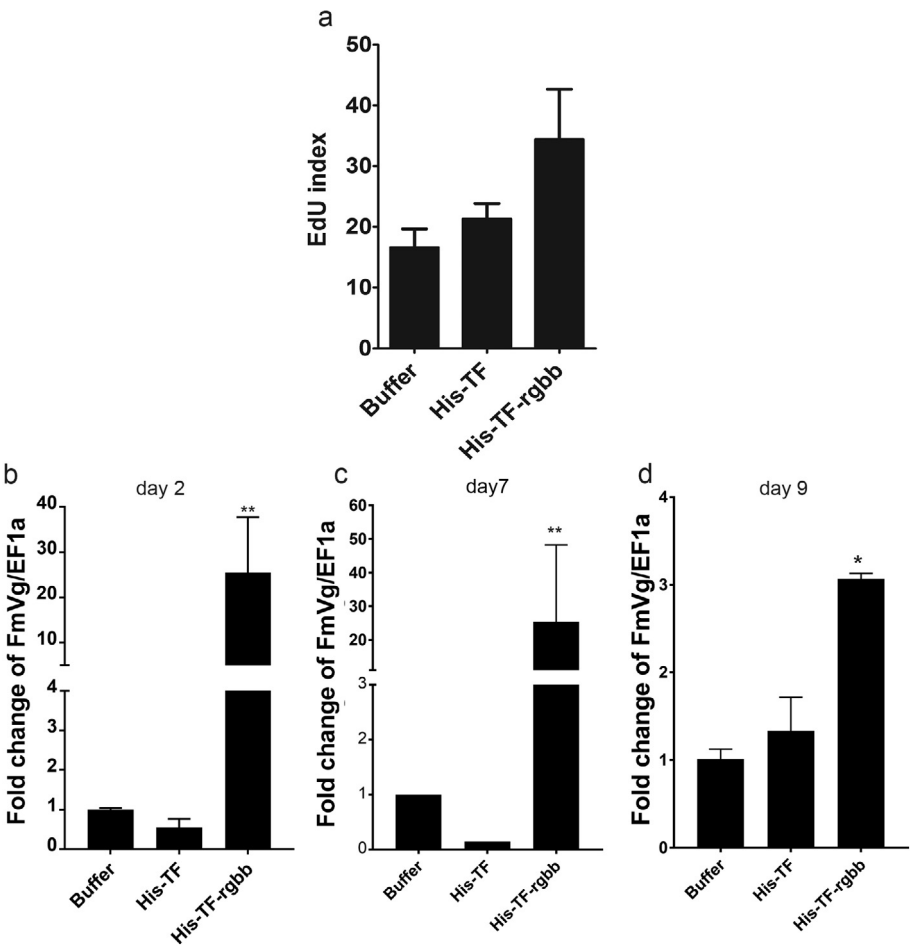

Fig. 7. Effects of His-TF-rgbb injection on oocyte proliferation and *Vg* expression in previtellogenic shrimp. Previtellogenic shrimp (n = 3–4) were injected with 3 μg of His-TF-rgbb, His-TF or buffer. The ovaries were excised from shrimp on day 2 after injection, and oocyte proliferation was determined based on the EdU index (a). The relative expression levels of *Vg* in ovaries from shrimp on day 2 (b), day 7 (c) and day 9 (d) after injection were determined using qPCR. The bars and error bars represent the means and SEMs, respectively. Single and double asterisks denote significant differences between groups where $p < 0.05$ and $p < 0.01$, respectively, using one-way ANOVA and pair-wise comparison by Duncan's test.

ovarian development in *F. merguiensis* (data not shown). The primary structure of FmGBB is similar to that of homologs in other species. FmGBB contains an N-terminal signal peptide, a pro-domain and a mature peptide at a C-terminal domain (Fig. 1), which is similar to the general structure of growth factors in the TGF-β superfamily (Roch and Sherwood, 2014). The mature FmGBB peptide sequence was found to be highly similar to that of other arthropods as well as that of vertebrate BMP 5/6/7 (Fig. 2a); whereas, its sequence differs significantly from that of insect DPP and vertebrate BMP 2/4 (Fig. 2b), which suggests that FmGBB belongs to the GBB and BMP 5/6/7 subgroups. The conserved sequence corresponding to the cystine knot hormone was found in the amino acid sequence of mature FmGBB, and three intramolecular disulfide bonds were predicted to form among the six cysteine residues within the mature FmGBB sequence (Fig. 2a), similar to that observed in members of the TGF-β superfamily (Roch and Sherwood, 2014), suggesting that the tertiary structure of FmGBB is most likely a cystine knot structure. The remaining unpaired cysteine residue in mature FmGBB may contribute to the formation of homodimers. However, we found that the His-TF-rgbb peptide mainly existed in a monomer and less in a homodimer (Fig. 5), indicating that the formation of the homodimer of the His-TF-rgbb may bind with other covalent bonds not a disulfide bond.

The *FmGBB* transcript was highly expressed in gills and brain, whereas its expression was lower in other tissues (Fig. 3), similar to the reported expression of BMP 7 in the mud crab *Scylla paramamosain*, which is present at a higher level in the brain and a lower level in various other tissues, such as the hepatopancreas and gonads (Shu et al., 2016). The expression of *GBB* had also been observed in the central nervous tissues and muscles of *Drosophila melanogaster* embryos (McCabe et al., 2003). We also determined the expression profile of *FmGBB* in the ovary during its development. The expression of *FmGBB* was dramatically increased in the early and late vitellogenic stages of ovarian development (Fig. 4), thus suggesting its possible role in vitellogenin synthesis and oocyte growth. Likewise, BMP-2, -6, -15 and other TGF-β proteins have been found to be expressed in oocyte and follicle cells during preovulatory follicle maturation in pigs (Paradis et al., 2009), and the expression of BMP-4 and -7 have been observed in theta cells in rat ovaries (Shimasaki et al., 1999).

Here, the effects of purified His-TF-rgbb peptide on oocyte proliferation and *Vg* expression were determined both *in vitro* and *in vivo*. The results indicated that the His-TF-rgbb peptide could induce oocyte proliferation in ovarian explant cultures as well as in shrimp injected with His-TF-rgbb (Figs. 6b and 7a), suggesting its role in the stimulation of oocyte proliferation in the shrimp ovary. Similarly, treatment with BMP-5 was shown to influence granulosa cell proliferation in rat ovaries (Pierre et al., 2005). Follicle cell proliferation was shown to be stimulated by BMP-2, -4, 6 and -7 in sheep (Juengel et al., 2006). In ovarian explant cultures, treatment with either His-TF-rgbb or His-TF alone led to reduced *Vg* expression (Fig. 6c) most likely due to the negative effects of various materials in FBS on the active substances. FBS and BSA were previously demonstrated to interact with active compounds, such as epigallocatechin gallate, which resulted in a decrease in intracellular oxygen species (Zhang et al., 2016). Therefore, the reduction in *Vg* expression upon His-TF or His-TF-rgbb treatment may result from the FBS supplementation. The other assumption is that GBB did not directly activate on the ovary for the *Vg* expression. Interestingly, *Vg* expression was dramatically increased in shrimp injected with His-TF-rgbb, while His-TF alone led to decreased *Vg* expression on days 2, 7 and 9 after injection (Fig. 7b–d). The differences in the effects of His-TF-rgbb on *Vg* expression *in vitro* and *in vivo* may suggest that the vitellogenesis-

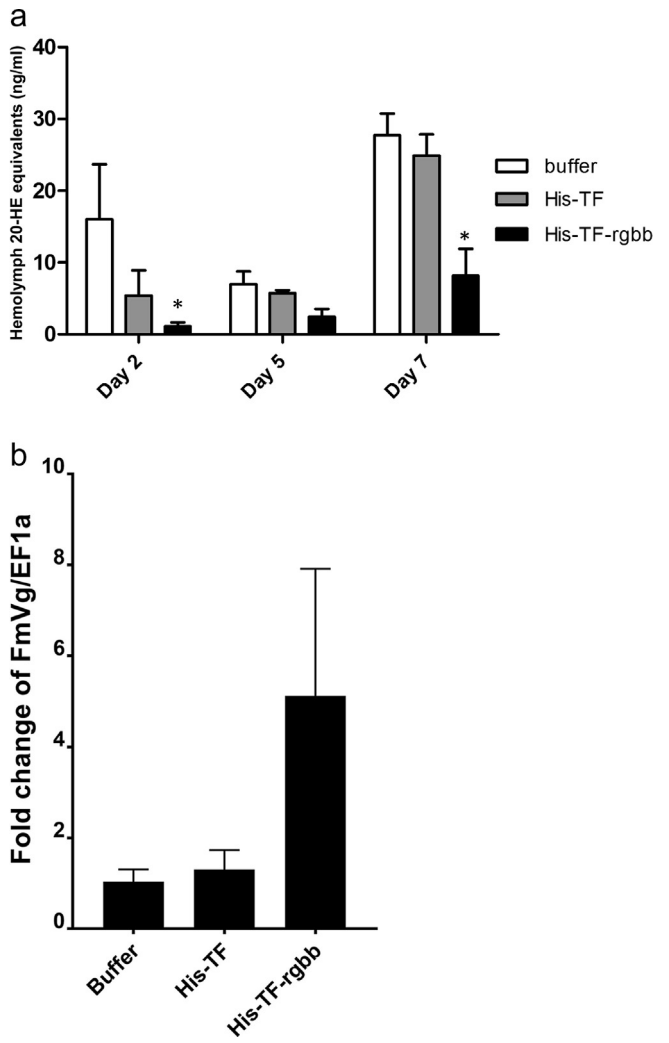

Fig. 8. Effects of His-TF-rgbb injection on 20-HE production in hemolymph. Hemolymph from previtellogenic female shrimp (n = 4) injected with 3 μg of His-TF-rgbb peptide, His-TF peptide or buffer was collected to determine the 20-HE levels on day 2, 5 and 7 (a). On day 7 after the injection, *Vg* mRNA expression in the ovaries was determined using qPCR (b). The bars and error bars in Fig. a and b represent the means and SEMs, respectively. Asterisks indicate significant differences between the groups ($p < 0.05$), as determined using one-way ANOVA and pair-wise comparison by Duncan's test.

Table 2
Molting stage of shrimp after injection with recombinant FmGBB.

Treatment	Molting stage on day 7 after injection (N = 4)
Buffer	$D_{3\text{-}4}$ and post-molt
His-TF	$D_{3\text{-}4}$
His-TF-rgbb	C and $D_{1\text{-}2}$

stimulating activity of FmGBB is mediated by FmGBB-inducible factors in shrimp or activated by the interaction of FmGBB with other molecules. In general, members of the TGF superfamily can form either homodimers or heterodimers with other members of the family. For example, the GBB can form a heterodimer with DPP, which was shown to stimulate wing development in *D. melanogaster* (Shimmi et al., 2005). Other TGF-β superfamily members can heterodimerize, including BMP 4/7 (Yuan et al., 2013), BMP 2/6 (Valera et al., 2010) and BMP 2/5, BMP 2/6 and BMP 2/7 (Israel et al., 1996). BMP heterodimers have been demonstrated to be more effective than homodimers in the induction of certain effects. For instance, the BMP 2/6 heterodimer can

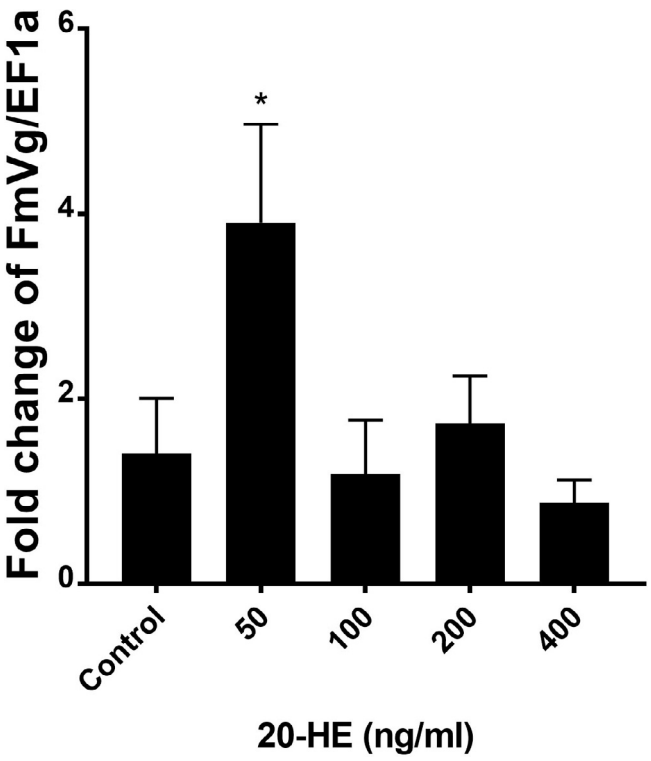

Fig. 9. Determination of *Vg* expression in primary ovarian cells upon 20-HE treatment. Different concentration of 20-HE were used to treat primary ovarian cells (n = 4) for 3 h. *Vg* mRNA expression in the ovaries was determined using qPCR. T bars and error bars represent the means and SEMs, respectively. Asterisks indicate the presence of significant differences between groups ($p < 0.05$), as determined using one-way ANOVA and pair-wise comparison by Duncan's test.

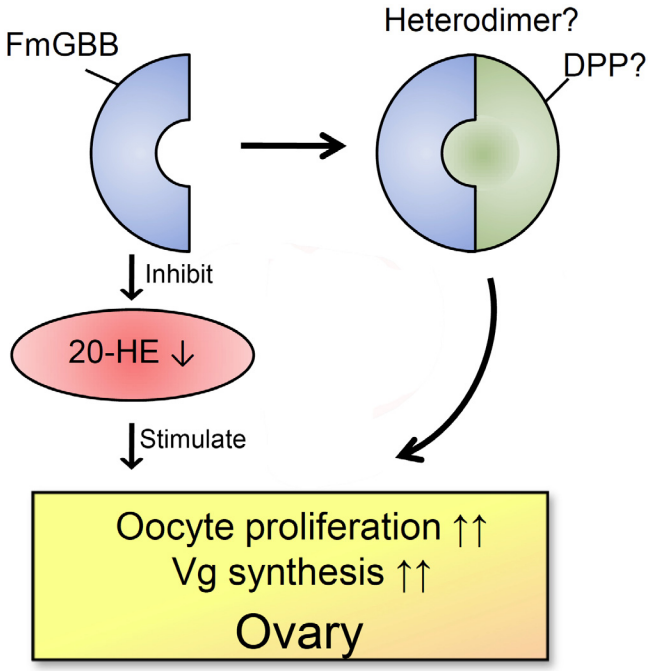

Fig. 10. Schematic diagram of the proposed mechanisms underlying the effects of FmGBB on oocyte proliferation and *Vg* regulation.

induce the differentiation of human embryonic stem cells more efficiently than either BMP 2 or BMP 6 alone (Valera et al., 2010). Furthermore, BMP 4 and BMP 7 were demonstrated to play anti-apoptotic

roles in granulosa cells by interacting with different signaling pathways (Shimizu et al., 2012). Recently, the up-regulation of juvenile hormone (JH) production by a GBB/DPP heterodimer was demonstrated in the insect *Clogmia albiplunctata* (Ishimaru et al., 2016). JH is structurally similar to methyl farnesoate (MF), which is involved in vitellogenesis in several crustaceans (Buchi et al., 2016; Laufer et al., 1987, 1998; Mak et al., 2005; Rodriguez et al., 2002; Tiu et al., 2006). In addition, BMP 6 and 7 can trigger the expression of FSH receptor in granulosa cells, suggesting their role in early gonad development (Ocon-Grove et al., 2012; Shi et al., 2010; Shi et al., 2009). The action of BMPs has been shown to be synergistic with that of FSH in the stimulation of estrogen production, but has also been shown to inhibit progesterone production in vertebrates (Miyoshi et al., 2007; Pierre et al., 2005; Shimasaki et al., 1999).

The effects of the FmGBB peptide on ecdysteroid production, which is involved in vitellogenesis, was investigated in this study. Interestingly, the results indicated that the level of hemolymph 20-HE was lower in shrimp injected with His-TF-rgbb than in shrimp injected with His-TF or buffer (Fig. 8a). Additionally, the progression of the molting cycle in shrimp injected with His-TF-rgbb ($D_{1\text{-}2}$) was slower than that in shrimp injected with His-TF or buffer ($D_{3\text{-}4}$), which suggests an inhibitory effect of FmGBB on 20-HE production that resulted in delay of the molting cycle. During ecdysteroid biosynthesis, cholesterol is converted into ecdysone via the activity of ecdysteroidogenic enzymes, particularly those of the Halloween gene family (Mykles, 2011). The secreted ecdysone is transported to the tissues, such as the ovaries, midgut and epidermis, where it is converted into 20-HE by CYP314A1 (Mykles, 2011; Petryk et al., 2003). The reduction of 20-HE titers in hemolymph by FmGBB is clear, although it is unclear whether FmGBB negatively regulates ecdysteroidogenic enzymes in the Y-organs or the peripheral tissues. Furthermore, *Vg* expression was increased in the His-TF-rgbb-injected shrimp (Fig. 8b). It has been reported that the stimulation of gonad development occurs within the intermolt stages and is complete before progression to the premolt stage in several crustaceans, including *L. vannamei*, *Macrobrachium rosenbergii* and *Emerita asiatica* (Gunamalai and Subramoniam, 2002; Kang et al., 2014; Okumura and Aida, 2000; Raviv et al., 2006; Subramoniam, 2000). The 20-HE level in hemolymph was observed to be at a low level (10–50 ng/ml) in the postmolt and early premolt stages, then was dramatically increased (220–230 ng/ml) in the premolt stage in *P. monodon* (Kuo and Lin, 1996). Both the expression of *Vg* mRNA and Vg levels in hemolymph were shown to be increased in female *L. vannamei* during the intermolt stage (Kang et al., 2014), and a high level of hemolymph Vg was found in freshwater prawn at the premolt stage ($D_{0\text{-}2}$) (Okumura and Aida, 2000). In addition, a low level of 20-HE was shown to be essential for the normal progression of oogenesis in *D. Melanogaster*, while a higher level (approximately 200 nM) induced apoptosis (Terashima et al., 2005), indicating that low levels of 20-HE in the intermolt and early premolt stages has the potential to stimulate *Vg* production. Similarly, *Vg* expression was stimulated by a low concentration of 20-HE (50 ng/ml; equivalent to ~100 nM) in primary ovarian cells (Fig. 9). These findings may suggest that FmGBB plays an inhibitory role during the molting cycle by suppressing 20-HE to levels that stimulate *Vg* expression. In crustaceans, 20-HE also induces vitellogenin expression both *in vitro* and *in vivo*. *Vg* expression was observed in ovarian explants derived from *Metapenaeus ensis* after treatment with 67–6700 nM 20-HE for 3 h (Tiu et al., 2006). Based on our results, a hypothesized model of the effects of FmGBB on *Vg* stimulation via the inhibiting 20-HE is depicted in Fig. 10. Additionally, FmGBB can directly trigger oocyte proliferation. On the other hand, it is unclear whether His-TF-rgbb monomers can form heterodimers with other growth factors, particularly DPP, and subsequently trigger *Vg* expression. FmGBB also negatively controls 20-HE production to trigger ovarian development. However, the mechanisms underlying these processes need to be further elucidated in the future. Finally, our study showed that FmGBB is a factor that could potentially be utilized to stimulate gonad development and, thereby, eliminate the use of the traditional eyestalk ablation technique in shrimp aquaculture.

5. Conclusions

Our study investigated roles of FmGBB in vitellogenesis in female *F. merguiensis*. The FmGBB was shown to be widely expressed in various shrimp tissues. The expression of *FmGBB* mRNA was increased during ovarian development. The His-TF-rgbb peptide induced oocyte proliferation both *in vitro* and *in vivo*. Injection of His-TF-rgbb peptide induced *Vg* expression in the ovary and suppressed 20-HE levels in hemolymph.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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