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Research article

Primordial germ cell migration in banana shrimp: *Fenneropenaeus merguiensis*

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Abstract

Importance of the work: For broodstock preparation and germ cell transplantation in *Fenneropenaeus merguiensis*, essential information on primordial germ cell (PGC) migration is lacking.

Objectives: To locate and track PGCs in *F. merguiensis* by detecting vasa-expressed cells during gonad development.

Materials & Methods: Immunohistochemistry of the vasa-like protein was performed to validate the vasa of the spermatogonia in the shrimp testis. Using whole-mount staining of the vasa-like protein, migrating PGCs were detected in the fertilized egg and the nauplius 4, zoea 3, mysis 3 and postlarva 1 stages.

Results: The vasa-like protein in the spermatogonia of the adult testis was specifically detected based on immunohistochemistry. Using whole-mount immunohistochemistry, the stained vasa-like protein was identified around the egg margin of fertilized eggs. In the nauplius 4 stage, vasa-like protein in PGCs was found in the ventral aspect of the cephalothorax region. In the zoea 3 and mysis 3 stages, vasa-like protein was detected in PGCs at the ventral aspect of the cephalothorax and migrated to the pereopod parts. Finally, in the postlarva 1 stage, the appearance of the vasa-like protein in PGCs was noted in the cephalothorax, pereopod and telson.

Main finding: Vasa-like protein was identified and indicated the migration of PGCs during gonad formation in *F. merguiensis* larvae. The obtained information should facilitate PGC tracking and provide valuable information for germ cell manipulation concerning gonad development in shrimp.

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Introduction

The Banana shrimp, *Fenneropenaeus merguiensis*, is a common species in the Gulf of Thailand and the Andaman Sea (Saetan et al., 2016). It is a commercial species and a consumer favorite that commands a reasonable price; however, broodstocks have declined because of overfishing to meet demand (Wonglapsuwan et al., 2009). Furthermore, the complicated life cycle of the species is a barrier to broodstock recovery since this shrimp requires an offshore coastal spawning ground linked to a mangrove-lined estuarine nursery (Noor-Hidayati et al., 2014).

In the early development of organisms, primordial germ cells (PGCs) are in a different location from the gonad and using amoeboid movements they migrate to the gonadal precursors where they further differentiate into sperm or eggs. Therefore, the PGCs have to reach the somatic part of the gonad for it to perform its crucial role in the propagation of the species, serving as a general model for long-range cell migration (Raz and Reichman-Fried, 2006; Yön and Akbulut, 2015). Generally, in the males of organisms, PGCs differentiate into sperm cells during the processes of spermatogenesis and spermiogenesis (Richardson et al., 2010). In fish, PGCs are large, with a low nucleocytoplasmic ratio due to their limited cell division (Duangkaew et al., 2019). In *Fenneropenaeus chinensis*, PGCs were found initially in the early embryonic stage (Feng et al., 2011). A distinct nuclear border and granular nuclear chromatin were noted as characteristics of PGCs (Feng et al., 2011; Duangkaew et al., 2019). In the giant freshwater prawn, *Macrobrachium rosenbergii*, PGCs were oval with a prominent nucleus comprising 4–6 nucleoli. The PGCs of *M. rosenbergii* varied between 6–14 µm in diameter as the gonad developed (Rungsin et al., 2012). Therefore, the PGCs in crustaceans and fish could be typified as large cells with distinct nuclear chromatin.

The *vasa* gene first identified in *Drosophila* spp. encodes an adenosine triphosphate -dependent RNA helicase belonging to the DEAD-box family, which is detected during development before zygotic gene activation and expression restricted to migratory cells, most likely to be primordial germ cells (Olsen et al., 1997). Since the *vasa* gene expression is localized to the PGCs during early development, its activity is required for cell lineage. Surprisingly, a *vasa* homolog is also expressed in the somatic cells of planarian that somatic cells were identified as neoblasts a totipotent cell type that functions in regeneration (Raz, 2000). Therefore the *vasa* function is essential for preserving totipotency which inhibits the expression of genes that would lead to somatic differentiation (Seydoux et al., 1996; Raz, 2000; Yön and Akbulut, 2015).

The *vasa* gene has been identified as a germ cell marker in most animals and its expression is restricted to migratory PGCs and gonial cells in reproductive organs (Olsen et al., 1997). The *vasa* gene has been used as a molecular marker for studies on germ cell development and reproductive processes in several animals, such as Chinese shrimp, *F. chinensis*, (Feng et al., 2011), fruit fly, *Drosophila melanogaster* (Renault, 2012), Pacific oyster, (*Crassostrea gigas*), (Fabioux et al., 2004), Japanese flounder, (*Paralichthys olivaceus*), (Wu et al., 2014) and the striped catfish, *Pangasianodon hypophthalmus*, (Duangkaew et al., 2019).

Aflalo et al. (2007) characterized the *vasa* gene and considered it as a maternal factor contributing to embryos in *Litopenaeus vannamei*. In *F. chinensis*, the *vasa* transcript was detected in mature oocytes, fertilized eggs and throughout multiple embryonic stages. This gene is still expressed in the PGCs of the nauplius 4, zoea 3, mysis 3 and postlarva 1 stages (Türkmen, 2005; Feng et al., 2011).

To date, germ cell development and the reproductive processes in *F. merguiensis* are not known; however, they are essential for breeding technology, such as germ cell transplantation and artificial insemination (Herrid and McFarlane, 2013). The current study aimed to identify a vasa-like protein in migrating PGCs during the gonadal development of *F. merguiensis*. The study should provide basic information on gonad formation and help to clarify the optimal stage for spermatogonia transplantation during the cultivation of *F. merguiensis* and potentially of other shrimp species.

Materials and Methods

Animals

Banana shrimps were collected from a farm in Nakhon Sri Thammarat province, Thailand and reared in a cement tank containing aerated seawater. The mature shrimps were fed three times daily with shrimp pellets and the larvae were fed four times daily with artemia. To start the experiment, the fertilized eggs, the nauplius 4, zoea 3, mysis 3, postlarva 1 stages, and the adult testis (length 12 cm) were collected, fixed in 4% paraformaldehyde for 24 hr at 4 °C, transferred to phosphate buffered saline (PBS) and kept at 4 °C until used.

Antibody

The nucleotide sequence of the *vasa* gene of *F. merguiensis* (GenBank accession no. MZ173499.1) was obtained from gonad transcriptome (Saetan et al., 2016); it contained the same conserved domains with *Drosophila* spp. (Lasko, 2013). In shrimp species, the glycine-rich domain is one of the conserved domains of *vasa* among *F. merguiensis*, *M. rosenbergii* (GenBank accession no. DQ339110.1) and *P. monodon* (GenBank accession no. HQ385221.1). The glycine-rich region containing the translated GRSRGGGRGGGRGGC amino acids was synthesized and used for anti-vasa production by the Genscript Company (NJ, USA).

Immunohistochemistry and whole-mount staining of vasa-like protein

The fixed testis (n = 3) were processed using the paraffin technique (Fischer et al., 2008) and the tissue was cut into 5 µm thick slices to be mounted on glass slides. Endogenous peroxidase was removed by submerging samples in a 3% H_2O_2 and methanol solution for 30 min in darkness at room temperature. Then, samples were placed in PBS for 2 min. The tissue slides were permeabilized with 1% glycine in 0.3% TritonX-100 diluted in PBS (0.3% $PBST_{x\text{-}100}$) for 30 min at room temperature. The tissue sections were mildly digested further with 0.5 µg/mL of proteinase K for 20 min at 37 °C before soaking the blocking solution (10% FBS in 0.3% $PBST_{x\text{-}100}$), for 2 hr at room temperature. Then, the tissues then incubated by covering the slides in anti-vasa antibody at 1:200 dilution for 16–18 hr at 4 °C. After washing three times with $PBST_{x\text{-}100}$ for 10 min each time, they were incubated in horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody at a dilution of 1:500 (Merck, Germany) for 1 hr at room temperature. Then, the tissue slides were washed three times with 0.3% $PBST_{x\text{-}100}$ for 10 min each time.

Whole-mount samples of fixed fertilized eggs and the nauplius 4, zoea 3, mysis 3, and postlarva 1 stages (n = 3 for each stage) were immersed for 2 d in 0.5% TritonX-100 diluted in PBS and then soaked in Dent's solution (80% methanol and 20% dimethyl sulfoxide) at –20 °C for 6 hr. The samples were washed five times in PBS for 30 min each time before being incubated in anti-vasa solution (1:1,000 dilution) for 4 d. Following washing as described above, the samples were incubated in the HRP-conjugated goat anti-rabbit IgG antibody at a dilution of 1:2,000 for 2 d, and subsequently washed three times with $PBST_{x\text{-}100}$ for 10 min each time. All the washing and incubation steps were performed at 4°C. The tissue slides and whole samples were stained with 0.05% 3,3′-diaminobenzidine solution (Sigma, USA) containing 0.015% H_2O_2 prepared in PBS. After a brown color had developed, the reaction was stopped in distilled water. After that, the testis sections were stained using hematoxylin. The sections were stopped in tap water and were finally permounted while the whole-mount immunohistochemistry samples were skipped for this step. Finally, photographs were taken under a BX–53 light microscope equipped with a DP–72 digital camera (Olympus, Tokyo, Japan) (Joyner and Wall, 2008; Graham and Vermeron, 2022).

Ethics

This study was approved and conducted according to the Institutional Animal Care and Use Committee (IACUC) of Prince of Songkla University (Approval no. MHESI 6800.11/558).

Results

Vasa-like protein was detected as a brown color in the spermatogonia of the adult testis (Fig. 1A). No reactivity in spermatogonia was detected in the negative control section (Fig. 1B), while the section incubated with pre-immunize serum was a very pale brown color with connective tissue (Fig. 1C) which was clearly discriminated from the positive cell. In the fertilized egg, the vasa-like protein appeared as dark brown dots around the egg margin (Fig. 2A). In the nauplius 4 stage, this protein was concentrated in PGCs located in the ventral aspect of the cephalothorax region (Fig. 2B). In the zoea 3 stage, the vasa-like protein was found in PGCs migrating to the ventral aspect of the cephalothorax and pereopod regions (Figs. 2C–2D). The negative control without anti-vasa did not develop a brown color, as presented in the insets of Figs. 2A–2C. In the mysis 3 stage, the vasa-like protein signal remained in the cephalothorax and pereopod regions (Figs. 3A–3B). Finally, the signal of vasa-like protein by PGCs in the postlarva 1 stage was noted in the cephalothorax, pereopod (Figs. 3D–3E) and telson (Fig. 3F). The negative control without anti-vasa did not develop a brown color and is shown in Figs. 3C and 3G.

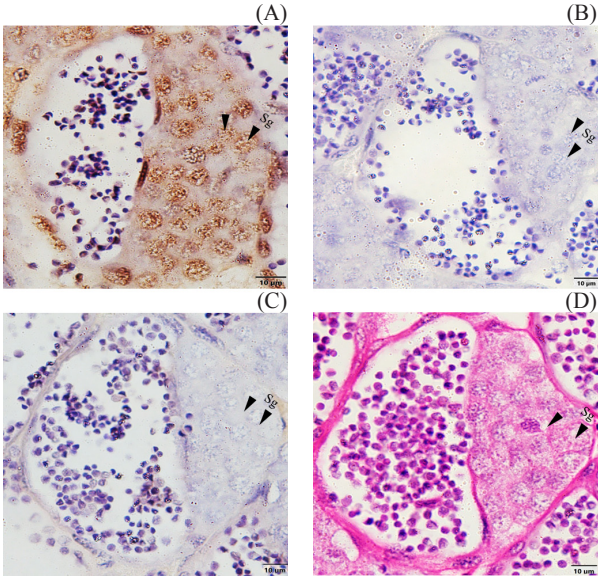

Fig. 1 Immunohistochemistry demonstrating presence of vasa-like protein in spermatogonia of the shrimp testis: (A) testis section of *Fenneropenaeus merguiensis* incubated with anti-vasa antibody showing positive vasa-like protein in spermatogonia (arrowheads); (B) control section without anti-vasa antibody showing no reactivity; (C) pre-immunized serum incubated section showing no reactivity and connective tissue showing as very pale brown color; (D) testis section of *F.merguiensis* stained with hematoxylin and eosin, scale bars are 10 µm and experiment was performed in triplicate.

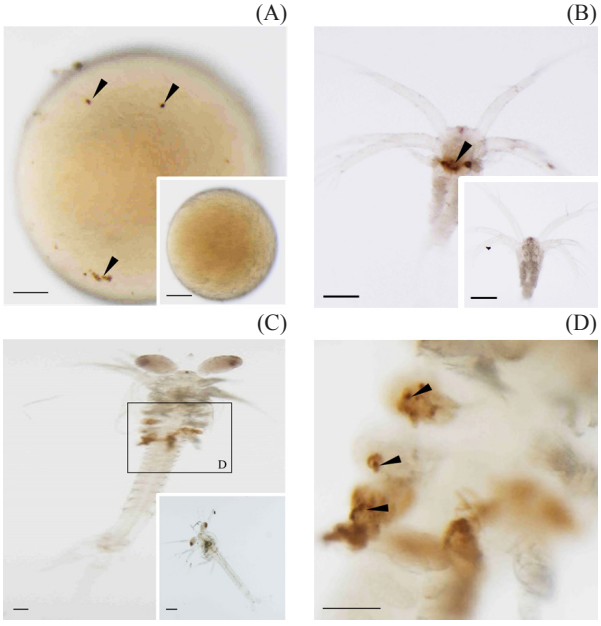

Fig. 2 Whole-mount immunohistochemistry showing vasa-like protein in various developmental stages of *Fenneropenaeus merguiensis*: (A) the fertilized egg; (B) nauplius 4 stage; (C) zoea 3 stage; (D) zoea 3 stage with magnification, where negative control without anti-vasa did not develop a brown color in any tested stage, as shown in the inserts for A–C, scale bars represent 2 µm for C and C-insert, 8 µm for A, A-insert, B and B-insert and 13.5 µm for D and experiment performed in triplicate using three samples of each stage.

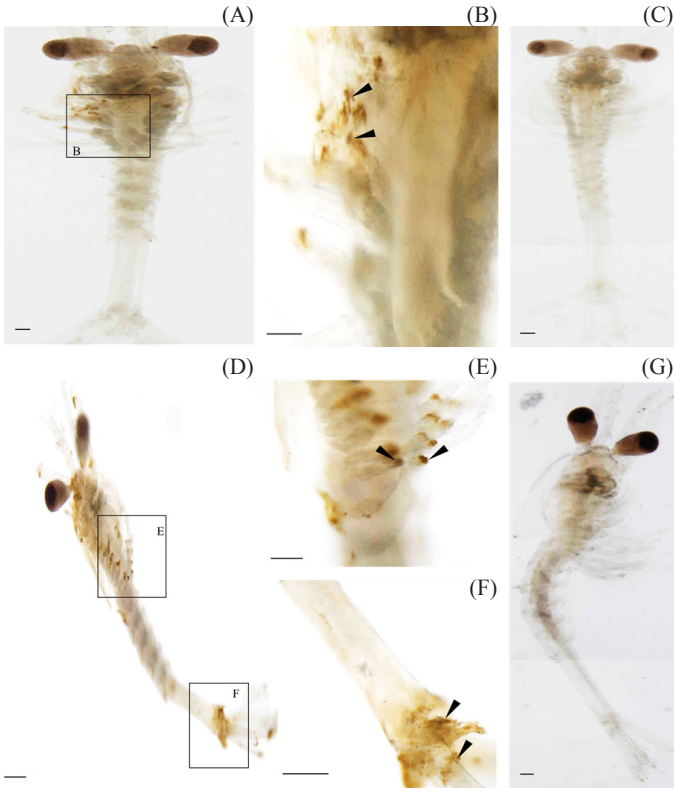

Fig. 3 Whole-mount immunohistochemistry showing vasa-like protein in various developmental stages of *Fenneropenaeus merguiensis*: (A) the mysis 3 stage; (B) with magnification; and (D) the postlarva 1 stage; (E, F) with magnifications; (C, G) the negative control without anti-vasa did not develop a brown color in any tested stage, scale bars represent 2 µm for A, C, D and G, 8 µm B, E and F. The experiment was performed in triplicate using three samples of each stage.

Discussion

The *vasa* gene has been previously characterized and used as a germ cell marker in crustaceans (Feng et al., 2011). The current study has demonstrated for the first time the distribution of vasa-like protein in the fertilized eggs and larvae of *F. merguiensis*. In *L. vannamei*, the *vasa* gene has been cloned and its transcript was detected in the oogonia of the shrimp ovary (Aflalo et al., 2007). The amino acid sequence homology was used to design the peptide for generating the antibody used in the current study and the selected region is a conserved region among the *vasa* of *F. merguiensis*, *P. monodon*, *M. rosenbergii* and *Drosophila* spp. However, the selected region was not found in the *vasa* of *L. vannamei* and therefore may be used to differentiate the germ cell of *F. merguiensis* from the germ cell of *L. vannamei*. Then, the produced anti-vasa was used to track the vasa-producing cells.

The developed antibody successfully depicted the spermatogonia of the testis, which resembled the vasa protein specificity.

The current whole-mount study successfully demonstrated the presence of PGCs in various juvenile stages. In addition, the vasa-like protein detected in the current study corresponded with the vasa transcript found in the eggs, embryo and PGCs at the cephalothorax of the nauplius 4 stage, in pereopods of the zoea 3 and mysis 3 stages and near the hepatopancreas of the postlarva 1 stage of *F. chinensis* (Feng et al., 2011). The pattern of migration was initiationed from the egg to the ventral aspect of the cephalothorax (nauplius), followed by from the cephalothorax to the pereopod (zoea 3 and mysis 3 stages) and then to the telson (postlarva 1 stage). The early vasa-like protein detected in fertilized eggs and embryos was possibly transmitted vertically by the maternal route (Feng et al., 2011). In addition, the current findings suggested that PGC migration followed a similar pattern in these two shrimp species and also suggested that any manipulation concerning gonad development could be done at any stage from the egg until the postlarva 1 stage (the last stage in the current study). In mammals, PGCs are found near the yolk sac outside the embryonic region, from where they migrate to the hindgut and separate from the gut epithelium to enter the dorsal mesentery to form a gonadal ridge. Therefore, PGC migration was proposed in three steps: separation, migration and colonization (Soto-Suazo and Zorn, 2005). The current study showed only PGC migration in separation and in part of the migration step; thus, further study is required until the PGCs have colonized the genital ridge to confirm the route of PGC migration.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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