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Research Article

Characterization of a novel and testis-specific zinc finger protein during sexual development of Pacific white shrimp *Litopenaeus vannamei*[†]

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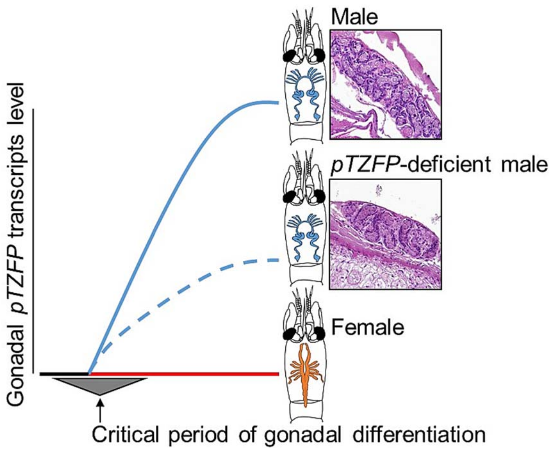
Abstract

Since females grow faster in penaeid shrimp, all-female aquaculture was proposed. Environmental conditions in the Pacific white shrimp were not found to affect genetic sex determination (ZZ/ZW system). The androgenic gland–secreting insulin-like androgenic gland hormone is a key controlling factor in crustacean male differentiation. However, functional sex reversal (neo-male) in penaeid shrimp has not yet been achieved by manipulating the insulin-like androgenic gland hormone–sexual switch. Therefore, understanding the molecular mechanisms of gonadal differentiation may help build appropriate tools to generate neo-male for all-female breeding. This study describes the potential role of the novel penaeid-specific testicular zinc finger protein (*pTZFP*) in the gonads of Pacific white shrimp. First, *pTZFP* transcripts show a male-bias expression pattern in undifferentiated gonads, which is then exclusively expressed in the testis and absent or slightly expressed in the ovary and other tissues. Besides, the knockdown of *pTZFP* in undifferentiated males results in smaller testes but no sex reversal. Immunohistochemical staining of proliferating cell nuclear antigen further confirmed that the smaller testes in *pTZFP*-deficient males are due to the lower proliferating activity of spermatogonia. These data reveal that *pTZFP* may be involved in testicular development but have fewer effects on gonadal differentiation. Moreover, testicular *pTZFP* transcription levels were not reduced with estradiol-17β (E2) administration or AG excision. Therefore, our data suggest that *pTZFP* may regulate testicular development through downstream genes regulating spermatogonia proliferation. Moreover, our data provide an appropriate molecular marker for identifying the sex of undifferentiated gonads.

Summary Sentence

pTZFP participates in testicular development by regulating spermatogonia proliferation, and its male-biased expression profile provides an appropriate molecular marker for identifying the critical window of gonadal sex differentiation in penaeid shrimp.

Graphical Abstract



Key words: crustacean sex determination, gonadal differentiation, 17β-estradiol (e2), androgenic gland, insulin-like androgenic gland (iag) hormone.

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Introduction

The Pacific white shrimp (*Litopenaeus vannamei*) is the most common crustacean species in global crustacean aquaculture. Generally, sex-dimorphic growth patterns are exhibited in most decapod species [1]. In penaeid shrimp, females are larger than males due to greater mass gain per molt cycle [2]. At the same time, females are less active in detecting feed and feeding [3]; therefore, all-female aquaculture was proposed as an advantageous culture strategy for penaeid shrimp.

Sex can be determined by genetic factors (genetic sex determination, GSD), environmental factors (environmental sex determination, ESD), or both (GSD with ESD) [4]. Sometimes, closely related species have different modes of sex determination [4]. Generally, most shrimp, prawn, and crayfish species bear the ZZ/ZW sex heritability system (females are heterogametic), while some crab and lobster species have the XX/XY system (males are heterogametic) [1]. Unlike the sex-determining gene Y-linked *SRY* in mammals and Z-linked (a dosage-dependent) DM domain–related transcription factor 1 (*DMRT1*) in birds being conserved, several Y-linked sex-determining genes have been identified in teleost fishes such as *dmrt1*/*dmy*, *transforming growth factor beta* (*tgfb*) family genes, *Sry-box transcription factors* (*sox*) gene, and *sexually dimorphic on the Y-chromosome* (*sdy*) [5]. In contrast, there are only a few reports of Z-linked and W-linked sex-determining genes such as Z-linked *dmrt1* in Chinese tongue sole (*Cynoglossus semilaevis*; [6]) and spotted scat (*Scatophagus argus*; [7]) and W-linked *Dm-w* in African clawed frog (*Xenopus laevis*; [8]). In decapod crustaceans, only one male sex-determining gene, *DMY*, has been identified in Eastern spiny lobster (*Sagmariasus verreauxi*; [9]) and gazami crab (*Portunus trituberculatus*; [10]) and is not found in other species yet. Furthermore, no environmental conditions affected sex determination in the Pacific white shrimp, including temperatures, photoperiod, density, and fasting [11].

In non-eutherian vertebrates, sex determination and gonadal differentiation are very sensitive to endogenous levels of estrogen [12]. In most fish, the induction of E2 administration and the blockage of aromatase (Cyp19a) activity results in feminization and masculinization, respectively [12]. Therefore, there is a great interest in generating an all-female population by vertebrate-type estrogen in crustaceans. Few reports support this suggestion that feminization follows the administration of E2 in prawn [13] and shrimp [14]. In contrast, there are no reports of the identification of high-affinity sex steroid–binding proteins (defined as receptors) in crustaceans [15]. Therefore, the estrogen receptor–independent signaling pathway of E2 that regulates sex determination and sex differentiation in crustaceans remains unclear.

In crustaceans, an androgenic gland (AG)–secreting insulin-like androgenic gland (IAG) hormone is a key controlling factor of sex differentiation [1] termed the IAG sexual switch. In the giant freshwater prawns (*Macrobrachium rosenbergii*), AG implantation [16] and AG cell transplantation [17] in juvenile females (approximately 10 days after metamorphosis to the post-larval stage, PL10) resulted in functional sex reversal of females to males. Furthermore, AG ablation [18] and *IAG*-deficiency [19] in juvenile males resulted in functional sex reversal of males to females in freshwater prawns. *IAG* knockout resulted in functional sex reversal of males to females in ridgetail white prawns (*Exopalaemon carinicauda*; [20]). However, functional sex reversal in Pacific white shrimp has not yet been achieved by AG manipulation, including AG implantation in females [21] and AG ablation in males [22]. Taken together, no sex reversal in andrectomized Pacific white shrimp demonstrates that AG/*IAG*-regulatory transdifferentiation might differ from previous reports in prawns. Therefore, even if the "IAG-switch" is a key player in decapod sex differentiation, the molecular and cellular mechanisms that regulate the sex determination and sex differentiation in penaeid shrimp remain unclear.

Recently, transcriptome analysis has been used to identify the sex-associated genes in decapod crustaceans, including prawns [23–26], penaeid shrimps [27–30], and crabs [31]. However, most transcriptome-identified genes are associated with gonad maturation and do not yet reveal their expression profiles during early gonadal differentiation. Moreover, in addition to the knockdown of AG-expressing *IAG*, functional sex reversal of males to females was only observed in the knockdown of testis-expressing *DMRT11E* [32] or masculinizer [33] in giant freshwater prawns. Therefore, we note that functional sex reversal in penaeid shrimp has not yet been achieved by artificial manipulation despite extensive studies around the world.

To understand the regulatory mechanism of gonadal differentiation in the Pacific white shrimp, we aimed to establish an early marker for gonadal differentiation through a comprehensive study. The different gonadal stages of both sexes were determined by histology. The gene expression profiles of some transcriptome-identified male-bias genes were analyzed by quantitative real-time PCR (qPCR) to identify genes involved in the mechanism of gonadal differentiation. A zinc finger protein (named by us in this study, penaeid-specific testicular zinc finger protein, pTZFP) showed male-bias expression during the early gonadal differentiation period (no morphological difference was found in the gonads of males and females at this period) and later exclusively expressed in the testis. The *pTZFP* function was further evaluated by knocking down gene expression (double-stranded RNA, dsRNA). Our data can provide valuable molecular markers to determine the sex during critical periods of gonadal differentiation in Pacific white shrimp, especially in genes involved in testicular differentiation.

Materials and methods

Animals

Juvenile Pacific white shrimp at 8–10 days post-larvae (PL8–10) were purchased from a commercial hatchery in Kaohsiung in southern Taiwan and transferred to the culture station of National Taiwan Ocean University, northern Taiwan. The experimental shrimp were acclimated to a seawater pond environment (32–35 parts per thousand, ppt; 26–30°C) in a 2.5-ton fiber-reinforced plastic (FRP) tank with natural lighting. They were fed commercial brine shrimp flakes mixed with black granule powders (Chuan-Kuan Inc., Kaohsiung, Taiwan) three times a day until body length reached 3 cm. Shrimp (>3 cm) were fed with commercial shrimp feeds twice daily. All procedures and investigations were approved by the National Taiwan Ocean University Institutional Animal Care and Use Committee and performed in accordance with standard guidelines.

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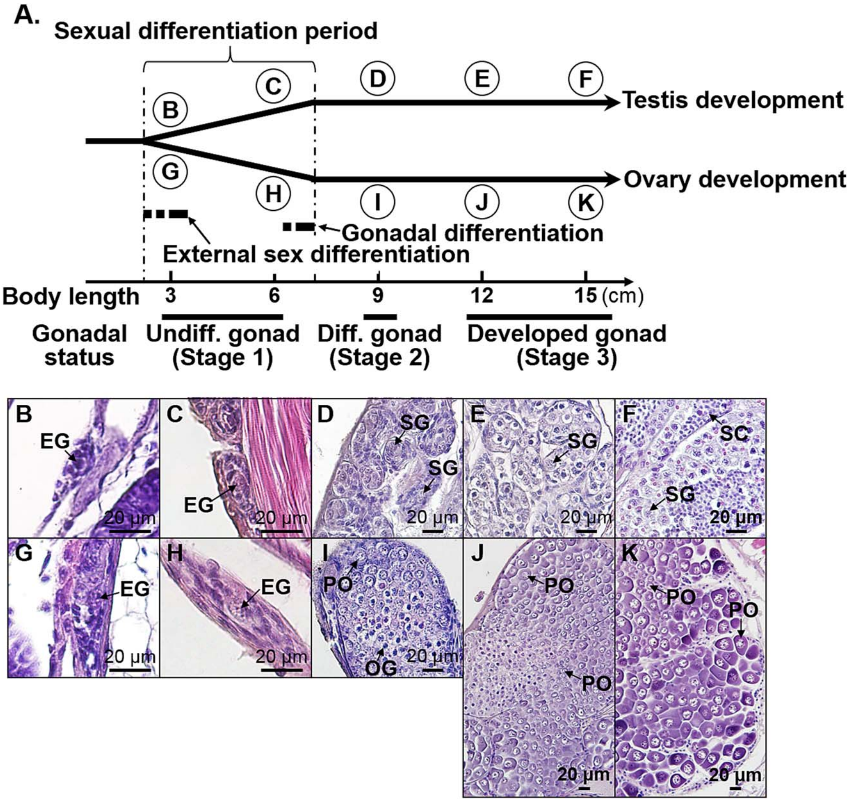

Figure 1. Body size-associated gonadal development in Pacific white shrimp. Schematic picture of the relationship between body size, external sex differentiation, and gonadal stage in male and female shrimp (A). Gonadal stages were shown in males as undifferentiated gonads with early germline cells (stages 1, B, and C); differentiated testis with spermatogonia (stages 2, D); and developed testis with spermatogonia (stage 3, E) and spermatocyte (stages 3, F). Gonadal stages were shown in females as undifferentiated gonads with early germline cells (stage 1; G and H); differentiated ovary with oogonia and primary oocytes (stage 2; I); and developed ovary (stage 3; J and K). Undiff., undifferentiated; diff., differentiated; EG, early germline cell; SG, spermatogonia; SC, spermatocyte; OG, oogonia; PO, primary oocyte.

Experimental design

Gene expression profiles during gonadal differentiation and gonadal development

The external character of sex differentiation was earlier than gonadal sex differentiation in Pacific white shrimp; it was recognized by the thelycum in females and gonopores in males about a body length of 3 cm [34]. Afterward, gonadal differentiation occurred when body length reached 7 cm [34]. The status of gonads is shown in Figure 1A. Shrimp were randomly collected from an undifferentiated gonad (3 cm) to a developed testis or ovary at 3 cm intervals until shrimp reached 15 cm (3, 6, 9, 12, and 15 cm with ±0.5 cm body length). Due to the small quantity of gonadal tissue in gonad undifferentiated shrimp (3 and 6 cm), gonads from individuals were collected with hearts and pooled (2–3 shrimp gonads in a sample) for RNA analysis. The genetic sex was determined by genetic sex markers, as in the previous report [35].

Effects of 17β-estradiol (E2) on gene expression

There is no report on identifying high-affinity E2-binding protein in crustaceans [15]. However, all females are inducted by E2 incubation in gonad-undifferentiated Pacific white shrimp [14]. E2 levels in ovarian extracts have been reported to reach 3.26 ng/g in Penaeidae shrimp [36]. To study the effects of E2 on *pTZFP* expression, we administrated an overdose of E2 (1.5 μg/g body weight; approximately 20 μg of E2 per shrimp) with three injections on days 0, 2, and 4 and then collected gonads on day 5) in immature males (12 and 15 cm; $n = 9$–10 in each group). E2 stock (6 mg/ml) was dissolved in ethanol and then diluted with phosphate buffered saline (PBS) to a working concentration (150 μg/ml). Males were determined by external sex organs. The control group was injected with PBS without E2.

Effects of insulin-like androgenic gland–expressing androgenic gland on pTZFP gene expression

To study the effects of IAG on *pTZFP* expression, we removed the fifth leg with terminal ampule (TA)-AG by surgery in immature males (TA-AG removal). The sham group had a similar operation for the fifth leg without TA-AG removal. We examined the short-term effects of TA-AG removal on *pTZFP* expression in testes after 3 days of TA-AG excision (13 ± 0.5 cm; $n = 7$ in each group).

Gene knockdown in juvenile shrimp

To examine the role of the target gene during gonadal differentiation, we performed a knockdown analysis in the undifferentiated gonad using dsRNA. We first examined the knockdown effects of dsRNA on the target gene transcripts in immature males (12 cm; $n = 7$–8 in each group). We administrated dsRNA against the target gene (5 and 10 μg/g body weight), two injections in 0 and 2 days, and then collected gonad in 3 days. We further examined the effects of dsRNA on the target gene during the initial gonadal differentiation and in later testis development. We administrated dsRNA against the target gene (5 μg/g body weight; 23 injections in 10 weeks, 3-day intervals) from undifferentiated gonads (about 6 cm) to a developed testis (reached 10 cm) ($n = 9$). The control

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group ($n = 8$) was injected with a dsRNA against the green fluorescence protein (*GFP*) gene.

Gonad histology

Gonads were fixed with Davison solution (300 ml of distilled water, 200 ml of 37% formaldehyde solution, 300 ml of 95% ethanol, and 100 ml of acetic acid glacial) for 24 h and repeated two times. The fixed tissues were dehydrated in 70% ethanol and stored at room temperature. Tissues were dehydrated in ethanol, replaced with xylene, and embedded in paraffin. Sections (5 μm thickness) were rehydrated and stained with hematoxylin and eosin (H&E).

RNA sequencing and transcriptome analysis

Gonads of immature males and females (12 ± 0.5 cm) were pooled (three gonads in a sample) for transcriptome analysis. Gonads were homogenized in TRIzol reagent (ThermoFisher Scientific), and total RNA was extracted following the manufacturer's protocol. RNA qualification, complementary DNA (cDNA) library construction, and Illumina (HiSeq 2000) sequencing were performed by Welgene Biotech, Inc. (Taipei City, Taiwan). Raw reads were input to CLC Genomics Workbench v8.5.1 (Qiagen) to remove adapters and low-quality reads, and then, de novo assembly was performed. The clean reads were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive database (BioProject ID PRJNA1092420). The expression level was evaluated by fragments per kilobase per million (FPKM). To determine the sex-specific genes in testes and ovaries, the low-abundance transcripts (FPKM < 0.213) were determined as background noise [37]. The testis-specific genes were identified as abundant transcript levels (FPKM > 10) in testes with no transcripts (FPKM < 0.213) in the ovary.

Cloning of Pacific white shrimp gonad–expressing genes *pTZFP*

Total RNA was extracted using the TRIzol reagent described in this study. Total RNA was reverse-transcribed to the first-strand cDNA using Superscript III (ThermoFisher Scientific) with the oligo $(dT)_{15}$ primer (Promega). To amplify the partial cDNA fragments of *pTZFP*, PCR primers were designed based on the nucleotide sequence of the genome and the transcriptome database of immature gonads (Table 1). Furthermore, the SMARTer RACE 5′/3′ Kit (Takara) was used to isolate the 3′ and 5′ ends of the cDNA and spliced a full-length sequence of *pTZFP* (GenBank accession no. PP356593). The confirmed cDNA sequence of *pTZFP* was used to design a specific qPCR primer for the RNA analysis (Table 1).

RNA analysis

The qPCR analysis was performed according to our previous study [38]. Gene quantification was conducted simultaneously by qPCR (CFX Connect Real-Time PCR Detection System; Bio-Rad Laboratories) with SYBR Green Master Mix (Bio-Rad Laboratories). qPCR primers for target genes are listed in Table 1. The qPCR specificity was confirmed by a single melting curve in standards (template-containing plasmid) and samples. No signal was detected in negative (non-template) controls. The reaction efficiency of different genes was evaluated using serial dilute standards. The values detected from different amounts of plasmid DNA contained the fragment of the target gene (10 times serial dilution) of the representative samples in parallel with the respective standard curve. The qPCR assay was conducted with duplicate repeats in each sample. Germline marker *VASA* (GenBank accession no. DQ095772) was used to determine the relative amount of gonads in tissue-mixed samples [39, 40]. Elongation factor 1 alpha (*EF1A*; GenBank accession no. GU136229) was used as an internal control standard to normalize the gene expression level. The relative gene expression levels (target gene/*EF1A*) were normalized according to the *EF1A* transcript levels. The highest value of the target genes was defined as 100%.

In situ hybridization

RNA probe synthesis was performed as described previously [41]. The cDNA fragment of *IAG* (GenBank accession no. XM_027374208; nucleotide 38-496) was used to synthesize the digoxigenin-11-UTP (DIG)–labeled RNA probe for in situ hybridization (ISH). The antisense RNA probe was used to detect the localization of *IAG*, and the sense probe was used as a reference for non-specific signals. The methodology for ISH staining was modified from our previous study [41]. Tissues were fixed with 4% paraformaldehyde in PBS at 4°C for 16 h, and then, fixed tissues were dehydrated in methanol and stored at −20°C. Sections (5 μm thickness) were rehydrated and processed for ISH. The sections were incubated overnight with DIG-labeled RNA probes (100 ng/ml) at 65°C. The gene expression was detected with alkaline phosphatase (AP)–conjugated sheep anti-DIG antibody (11093274910, Roche) and colorized by nitro-blue tetrazolium and 5-bromo-4-chloro-3′-indolyphosphate (NBT/BCIP) Detection System (11681451001, Roche).

Immunohistochemical and immunofluorescence staining

Immunohistochemical (IHC) and immunofluorescence (IF) staining was performed as described previously [38]. Tissue fixation (Davison solution) and section were performed as described in the present study. For IHC staining, the rehydrated gonadal sections (5 μm thickness) were treated with HistoVT One (Nacalai Tesque) to expose the antigens of the target protein. Gonadal sections were first incubated with 3% H_2O_2 for 10 min to block the endogenous peroxidase and then incubated with 2N HCl for 15 min to dissociate the chromatin-bound to nucleus protein. The sections were incubated with 5% non-fat milk powder for 30 min to block the non-specific binding. Mouse anti-proliferating cell nuclear antigen (PCNA) antibody (13-3900, ThermoFisher Scientific; 1:200 dilution with 1.5% non-fat milk powder) was incubated at 4°C overnight. This was followed by incubation with a biotinylated goat anti-mouse antibody (BA-9200, Vector; 1:1000 dilution with 1.5% non-fat milk powder) at room temperature for 1 h. The color formation was amplified with an ABC kit (Vector) and 3,3′-diaminobenzidine (DAB, Sigma-Aldrich). Sections were counterstained with hematoxylin. For IF staining, an Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (A-11029, ThermoFisher Scientific; 1:100 dilution with 1.5% non-fat milk powder) was used.

Double-strand RNA preparation

The fragment of the target gene and reference gene (green fluorescent protein) were amplified by specific primers with T7 promoter sequence (5'-TAATACGACTCACTATAGGG-target sequence-3′) on 5′-end (Table 1). Double-stranded RNA synthesis was performed using T7 RNA polymerase following the manufacturer's protocol (T7 RiboMAX Express RNAi System, Promega). This study used the fragment

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Table 1. Oligonucleotides for specific primers were used in this study

Gene name	Orientation	Sequence	Analysis
pTZFP	Sense	5’-GCTTGTGCTACCTGAAAATCCA-3’	Cloning and RT-PCR
pTZFP	Antisense	5’-CCGAACTAGTTTCAGGTGCTT-3’	Cloning and RT-PCR
pTZFP	Sense	5’-GCCTCATGAATCACTGACAGCGGAGGT-3’	3’-RACE
pTZFP	Antisense	5’-GGCCGAAATCCTGTGTGTATTCTGGCAT-3’	5’-RACE
pTZFP	Antisense	5’-CACATACTTTGCCTTCGCACCCTTA-3’	5’-RACE (nested PCR)
pTZFP	Sense	5’-CCACACAAGTTACGGTGAAGA-3’	qPCR
pTZFP	Antisense	5’-CTCTCCTGATACACCTGATCCT-3’	qPCR
VASA	Sense	5’-CACAGCGTGAACAAGCATTATC-3’	qPCR
VASA	Antisense	5’-ATTGACTACCACACCAATCCC-3’	qPCR
EF1A	Sense	5’-GGTGACTCCTGCATCGTAAA-3’	qPCR
EF1A	Antisense	5’-TTGATCACACCCACAGCTAC-3’	qPCR
IAG	Sense	5’-GCTACAACGTCACAGGGATT-3’	qPCR
IAG	Antisense	5’-GTATCGGCTGACCTTGACAC-3’	qPCR
IAG	Sense	5’-AATTAACCCTCACTAAAGGGCGTGCCTG-CTCAACTGTTA-3’	ISH
IAG	Antisense	5’-TAATACCACTCACTATAGGCCTCGCAAT-ACTCCAGGAT-3’	ISH
	Sense	5’-ACGTAAGCCAAGAGAGGGAA-3’	Genotyping
	Antisense	5’-TCAAGATGGCTGGCTTTGTTG-3’	Genotyping

(position −147 to 672, 819 bp) of *pTZFP* to synthesize dsRNA.

Cell proliferation assay

The expression of the cell-proliferating marker, PCNA, was used to determine potential cell-proliferating activity. The effects of *pTZFP* knockdown (using *pTZFP* dsRNA) on cell proliferation were analyzed by IHC staining of PCNA. In both the control and the RNAi groups ($n = 4$ per group), testes were collected from the anterior, middle, and posterior parts for IHC staining with an anti-PCNA antibody. These sections were used to count the number of PCNA-positive spermatogonia and the total number of spermatogonia. The spermatogonia-proliferating activity was expressed as the percentage (%) of PCNA-positive spermatogonia out of the total number of spermatogonia. Additionally, gonadal activity was represented as the relative amount (per mm^2) of PCNA-positive spermatogonia and somatic cells, respectively. IHC staining was performed with triplicate sections for each tissue ($n = 4$ tissue samples in each group).

Data analysis

The data are presented as the mean ± standard deviation (SD). The values were analyzed by one-way analysis of variance (ANOVA), followed by a Tukey test, with $P < 0.05$ indicating a significant difference. The Student *t*-test was also conducted to determine significant differences ($P < 0.05$) between the two treatments.

Results

Size-associated gonadal status under tank culture conditions

Under tank culture conditions, shrimp grew slower than under normal pond culture conditions. Thus, gonadal status is defined by size rather than age. According to the histological results, undifferentiated gonads were found in shrimp with a body length smaller than 6 cm in males (Figure 1B and C) and females (Figure 1G and H). After reaching a size of 9 cm, all shrimp developed either differentiated testes (Figure 1D) or differentiated ovaries (Figure 1I). After reaching 12 cm, all shrimp had well-developed testes (Figure 1E and F) or ovaries (Figure 1J and K). Furthermore, male shrimp have entered spermatogenesis with spermatocytes after reaching a size of 15 cm (Figure 1F). Based on histological characteristics, three different stages were divided in the genetic (ZZ) males, including shrimp with undifferentiated gonads with early germ cells (Stage 1), shrimp with differentiated testes with spermatogonia (Stage 2), and shrimp with developed testes with germinal cysts (Stage 3) (Figure 1A). Three different stages were also divided in the genetic (ZW) females, including shrimp with undifferentiated gonads with early germ cells (Stage 1), shrimp with differentiated ovaries with oogonia (Stage 2), and shrimp with developed ovaries with primary oocytes (Stage 3) (Figure 1A). Combining these data, the developmental stage of gonads was associated with body length, but not with age, in Pacific white shrimp. Therefore, the gonadal condition of shrimp selected for qPCR analysis was based on their body size.

Determination of male-specific genes in the gonads

To identify the genes specifically expressed in the gonads of male Pacific white shrimp, we performed a transcriptome analysis of immature gonads of males and females. Through the transcriptome database, we found zinc finger domain-contained transcripts (contig53643, 585 nucleotides) that were abundantly and exclusively expressed in the testis of immature males (FPKM = 216), and no transcripts were found in the ovary of immature females (FPKM < 0.213). Zinc finger domain–contained transcripts are transcription factors that regulate downstream gene regulation. Thus, we focused on this transcript and performed further analysis. Furthermore, the sequence of this transcript was determined by cDNA cloning. We cloned a zinc finger domain–contained gene containing 5318 nucleotides (open reading frame from 621 to 4235 nt) and had an open reading frame encoding 1205 amino acid residues. The deduced amino acid sequence contained five C2H2-type zinc finger domains with two linkers (*TGFKP*) (Figure 2A). Our data demonstrated that three transcripts belonged to this zinc finger domain–contained gene including contig53463 (437–1022 nt), contig54003 (2042–2568 nt), and contig53560 (2517–3076 nt). According

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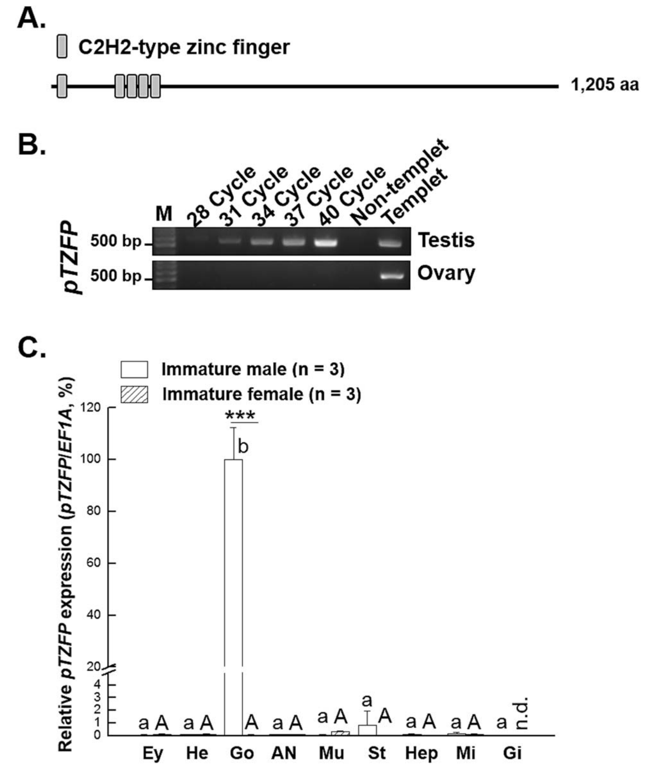

Figure 2. Domain structure of pTZFP and its mRNA expression pattern in various tissues of Pacific white shrimp. (A) The predicted structure of pTZFP in the Pacific white shrimp includes five C2H2-type zinc fingers in the N-terminal region. Reverse transcription (RT)-PCR (B) and qPCR (C) were used to analyze the expression levels of *pTZFP* mRNA in immature shrimps, assessing its sex-biased expression pattern and tissue distribution. Immature gonads from shrimp sizes of 15 and 12 cm were used for RT)-PCR and qPCR, respectively. Differences between the different tissues were normalized with *EF1A*, and the value of mRNA expression of the highest group was defined as 100%. The number of asterisks indicates the results of the Student *t*-test as follows: ***: $P < 0.001$. Small and capital letters indicate a one-way ANOVA and Tukey's multiple test ($P < 0.05$). M, DNA ladder; Ey, eyestalk; He, heart; Go, gonad; An, abdominal nerve; Mu, muscle; St, stomach; Hep, hepatopancreas; Mi, midgut; Gi, gills; N.d., nondetectable.

to the search results of amino acid pairwise alignment between these genes and the NCBI database sequences, an uncharacterized protein exhibited significant alignment in the penaeid shrimp, including *P. chinensis* (GenBank accession no. XP_047472780), *P. monodon* (GenBank accession no. XP_037776288), and *P. japonicas* (GenBank accession no. XP_042871911). Moreover, an incomplete sequence of this gene was found in *P. vannamei* (GenBank accession no. ROT77864). Despite them, the known genes in the GenBank database only showed sequence similarities to the zinc finger domain–contained area. Therefore, out of the penaeid shrimp, none of the known genes in the GenBank database exhibited strong sequence and structural similarities to this zinc finger domain–contained gene.

Noted PCR amplification of this gene was shown in immature testis, and no amplification was detected in immature females by gel electrophoresis (Figure 2B). The qPCR result confirmed transcriptomic data and showed that this gene was exclusively expressed in the immature testis with slight or absent transcripts in the immature ovary (Figure 2C).

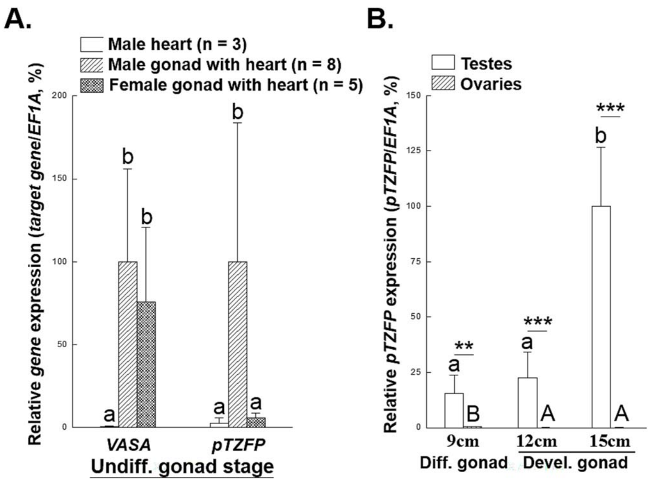

Figure 3. Gonadal *pTZFP* mRNA expression profiles during gonadal differentiation and later development. Detection of *pTZFP* mRNA expression in undifferentiated (A) and differentiated (B) gonads by qPCR. For undifferentiated gonads, both sexes were collected with heart tissues from shrimp at 6 cm ($n = 8$ males and 5 females), using male heart tissues ($n = 3$) as a reference. The germline marker *VASA* was used to calibrate the relative proportions of the heart and gonad in these mixed samples. Differentiated gonads were collected from shrimp at 9 cm ($n = 8$ per group) and developed gonads at 12 and 15 cm ($n = 8$ per group). Differences between groups were normalized using *EF1A*, and the highest mRNA expression value was set as 100%. The number of asterisks indicates the results of the Student *t*-test as follows: **: $P < 0.01$, ***: $P < 0.001$. Small and capital letters indicate a one-way ANOVA and Tukey's multiple test ($P < 0.05$). undiff., undifferentiated; diff., differentiated; devel., developmented.

Moreover, tissue distribution of this gene in immature shrimp showed that this zinc finger domain–contained gene was specifically expressed in the testis, with only slight or no expression in the other tissues (Figure 2C). Based on these results, we designated this zinc finger domain–contained gene as a novel penaeid-specific gene and tentatively named it penaeid-specific testicular zinc finger–contained protein (*pTZFP*).

Expression profiles of *pTZFP* transcripts

Different gonad stages were used to analyze the gene transcript levels in both sexes. The germline marker *VASA* was used in tissue-mixed samples to determine the relative number of gonads in the collected samples. qPCR results showed that *VASA* transcripts had no change in the gonads (with hearts) of males and females (Figure 3A). Low expression of *VASA* was found in the hearts compared to the gonads with hearts (Figure 3A). qPCR results showed that *pTZFP* transcripts had high expression levels in undifferentiated male gonads compared to undifferentiated female gonads (Figure 3A). Similarly, *pTZFP* also showed a high level of expression during testis development, with slight expression during ovary development (Figure 3B). Thus, *pTZFP* transcripts not only showed male-bias expression in undifferentiated gonads but also exclusively expressed in testes.

Estrogen did not suppress *pTZFP* expression

For individuals treated with vertebrate-type estrogen (E2), two sizes of shrimp were injected with E2 (1.5 μg/g) for

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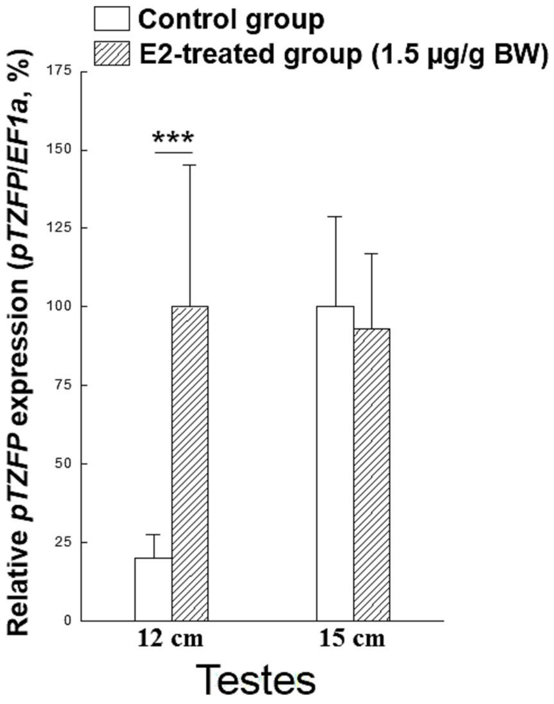

Figure 4. Effects of E2 on *pTZFP* mRNA expression in developed testes. Detection of *pTZFP* mRNA expression in E2-treated shrimp by qPCR. Shrimp were injected with E2 (1.5 μg/g body weight) on days 0, 2, and 4, and testes were collected on day 5 for RNA analysis. The control group received PBS injections without E2. Sample sizes were 12 cm shrimp ($n = 10$ for control, $n = 9$ for E2-treated) and 15 cm shrimp ($n = 6$ for control, $n = 8$ for E2-treated). Differences between groups were normalized using *EF1A*, and the highest mRNA expression value was set as 100%. The number of asterisks indicates the results of the Student *t*-test as follows: ***: $P < 0.001$.

5 days. The shrimp size includes 12 cm (only spermatogonia) and 15 cm (entering spermatogenesis with spermatocytes). qPCR results showed that E2 treatments significantly increased *pTZFP* transcript levels in immature testes of 12 cm shrimp (Figure 4). In contrast, E2 treatments did not affect *pTZFP* expression in immature testes of 15 cm shrimp (Figure 4). These results reveal that *pTZFP* expression in the testes is not suppressed by E2. Instead, *pTZFP* expression is further enhanced by E2 in the testes of 12 cm shrimp. These data suggest a differential regulatory effect of E2 on *pTZFP* messenger RNA (mRNA) expression depending on the developmental stage of the testes.

The effects of terminal ampoule-androgenic gland excision in the testicular development

To determine the distribution of *IAG*-expressing AG in males of Pacific white shrimp, we analyzed the *IAG* transcription levels in different parts of the vas deferens, including ascending middle vas deferens, descending middle vas deferens, distal vas deferens, and terminal ampoule (Figure 5A). qPCR results showed that *IAG* transcripts were mainly expressed in the terminal ampoule and non-detectable in other regions (Figure 5B). The H&E result showed the location of AG on the terminal ampoule (Figure 5C). ISH results showed that *IAG* signals were only observed in the surrounding region of the terminal ampoule by antisense probe (Figure 5D). No signal was observed by sense probe (Figure 5E). Therefore, *IAG*-expressing AG was attached to the terminal ampoule. To examine the role of *IAG*-secretion in regulating the *pTZFP* transcription level, the terminal ampoule-AG was removed by surgery. After 3 days of TA-AG excision, qPCR results showed that testicular *pTZFP* transcript levels did not change in andrectomized shrimp compared to a sham group (Figure 5F). Thus, these results revealed that *pTZFP* expression in the testes was not influenced by *IAG*-expressing AG.

Effects of *pTZFP* silencing on male gonads

To evaluate the knockdown efficiency of *pTZFP* transcripts, a short-term dsRNA treatment (3 days) was used in male shrimp (12 cm). qPCR results showed that the transcript levels of *pTZFP* were significantly reduced (up to 51%) in the RNAi (*pTZFP* dsRNA-treated) group compared to the control (*GFP* dsRNA-treated) group (Figure 6A). To further examine the potential role of *pTZFP* in gonadal differentiation, a long-term dsRNA treatment (10 weeks) was used in undifferentiated gonads (6 cm shrimp) of genetic (ZZ) males (6 cm). After 10 weeks of treatment, shrimp size reached 8.6–10.8 cm, and there was no difference in body length between both groups (Figure 6B). However, the body weight was significantly decreased in the RNAi group compared to the control group (Figure 6C). Histological results showed that differentiated testes with spermatogonia were observed in both the control group (Figure 6D and E) and the RNAi group (Figure 6F and G). Furthermore, histological results showed that the thickness of testes and the density of germinal cysts were significantly reduced in the RNAi group compared to the control group (Figure 6H and I). To understand the reduction in testicular size in *pTZFP*-deficient male shrimp, we further examined cell-proliferating activity using PCNA as a marker through IHC and IF staining (Figure 7A–E). IHC results showed that PCNA-positive signals were observed in both spermatogonia and somatic cells in both the control group (Figure 7A and B) and the RNAi group (Figure 7D and E). To confirm the presence of PCNA-positive spermatogonia, given the slight IHC signal in spermatogonia, we performed IF staining of PCNA to further characterize PCNA-positive spermatogonia. Similar to the IHC results, IF staining showed that some spermatogonia had PCNA signals in the nucleus (Figure 7C). IHC results indicate that the control group had a higher percentage and number of PCNA-positive spermatogonia compared to the RNAi group (Figure 7F and G). Additionally, IHC results showed no change in the number of PCNA-positive somatic cells in the RNAi group compared to the control group (Figure 7H). These data demonstrate that *pTZFP* deficiency leads to reduced proliferative activity in germline cells, with no difference observed in somatic cells. Overall, these findings reveal that *pTZFP* had no effect on testis differentiation but plays a role in regulating spermatogonia proliferation.

Discussion

In this study, we found a novel gene, *pTZFP*, in the gonadal transcriptome database of the Pacific white shrimp. In alignment with the NCBI database, only penaeid shrimp had high similarity sequences. This penaeid-specific pTZFP contains five C2H2-type zinc finger domains and may be a transcription factor in gene regulatory pathways. We analyzed the mRNA expression profile of *pTZFP* in various tissues of both sexes; high *pTZFP* expression was only observed in the

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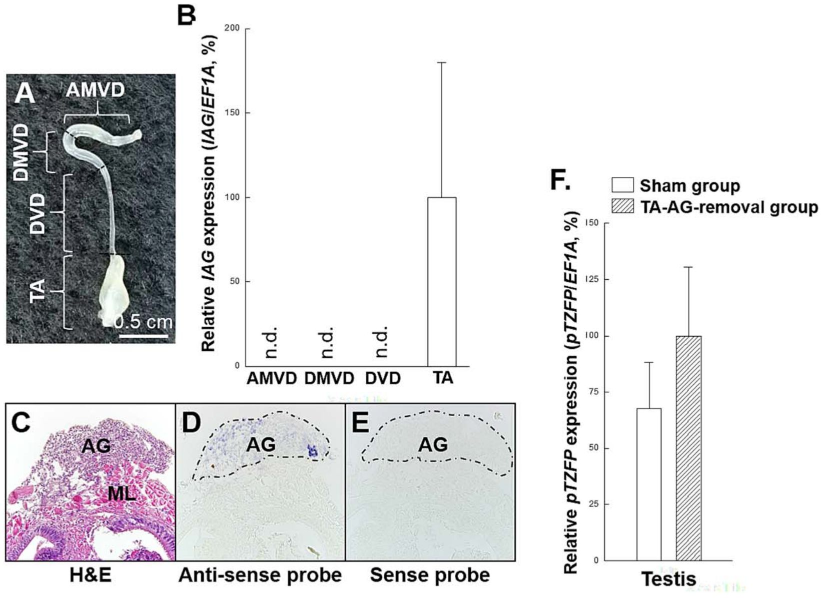

Figure 5. Distribution of *IAG*-expressing androgen gland and terminal ampule-androgen gland excision on *pTZFP* mRNA expression in developed testes. (A) The vas deferens were morphologically divided into four regions: ascending middle vas deferens (AMVD), descending middle vas deferens (DMVD), distal vas deferens (DVD), and terminal ampoule (TA). (B) Detection of IAG mRNA expression in different regions of vas deferens by RT-PCR ($n = 4$). (C) The AG was attached to the surface of the terminal ampoule, as shown by histology. *IAG* mRNA was detected by ISH using an antisense probe (D) and a sense probe (E). Detection of *pTZFP* mRNA expression in terminal ampoule-androgen gland-removal shrimp by qPCR (F). The fifth leg with terminal ampoule-androgen gland complex was surgically removed, and testes were collected 3 days post-surgery for RNA analysis ($n = 7$). The sham group was removed from the fifth leg ($n = 7$). Differences between groups were normalized using *EF1A*, and the highest mRNA expression value was set as 100%. A dashed line represents the AG region. AMVD, ascending middle vas deferens; DMVD, descending middle vas deferens; DVD, distal vas deferens; TA, terminal ampoule; AG, androgenic gland; ML, muscle layer.

testis, with slight or absent expression in other tissues. We further analyzed the expression profile of *pTZFP* at different stages during gonadal development. Our data showed that *pTZFP* had male-bias expression in undifferentiated gonads and then dominantly in differentiated testes and slightly in differentiated ovaries. Furthermore, the transcription level of testis-specific *pTZFP* was not reduced by short-term E2 treatment. Moreover, *pTZFP* expression was not changed by terminal ampoule-androgenic gland excision. In further functional analysis, the knockdown of *pTZFP* at undifferentiated gonads in males resulted in smaller testes and no sex reversal. These data suggest that *pTZFP* is mostly involved in testicular development and has little or no effects on gonadal differentiation. In addition, *pTZFP* could serve as a valuable molecular marker to determine the male gonads during a critical period of gonadal differentiation in Pacific white shrimp.

Potential role for the novel *pTZFP* in gonads of Pacific white shrimp

The deduced amino acid sequence of pTZFP contained five C2H2-type zinc finger domains with two linkers in the N-terminal region. Generally, this protein structure is a transcription factor mediating the downstream pathway, such as glioma-associated oncogene (*GLI*) [42]. In addition to binding DNA as a transcription factor, some C2H2 zinc finger proteins can bind to RNA or protein, and more zinc fingers have specific affinity for different ligands [43]. The amino acid sequence of pTZFP only exhibited similarity to the uncharacterized protein of penaeid shrimp and did not exhibit similarity to other crustaceans. These results suggest that *pTZFP*/pTZFP is a novel gene/transcription factor specific to penaeid shrimp. Because a *pTZFP*-like gene was not observed in the genomic and transcriptomic databases of other decapods, pTZFP might participate in some penaeid shrimp–specific regulatory pathways. To determine the downstream regulatory cascade of *pTZFP* in future studies, the chromatin immunoprecipitation method is planned to discover and characterize the candidate genes acting downstream of pTZFP.

The male-bias transcription of *pTZFP* was observed in undifferentiated gonads, and then *pTZFP* transcripts were predominantly expressed in testes with absent or slight expression in ovaries and other tissues. In *pTZFP*-deficient males (by RNAi), males had small testes compared to control males. Furthermore, no sex reversal was found in *pTZFP*-deficient males. Therefore, our data demonstrates that *pTZFP* is involved in testicular development and has less effects on gonadal differentiation. In prawns, functional sex reversal of males to females is caused by the knockdown of AG-secreting hormone *IAG* [19, 20] or testicular transcription factors *DMET11E* [32] and masculinizer [33]. However, no report documented functional sex reversal in penaeid shrimp by AG manipulation [21, 22] or knocking down terminal ampoule (with androgen gland)-expressing genes such as *insulin-like peptide* [44] and *insulin-like receptor* [45]. Furthermore, our data showed that AG excision (with terminal ampoule) of immature males had no reproductive defect. Therefore, these data suggest that penaeid shrimp have a regulatory pathway different from the IAG-switch pathway in prawns and some crustaceans.

Our data showed that the knockdown of *pTZFP* at undifferentiated gonads in males resulted in a smaller testis. IHC results of PCNA staining revealed that the proliferating activity of spermatogonia was decreased in *pTZFP*-deficient testes (Figure 7). A similar result (smaller testis) was also shown

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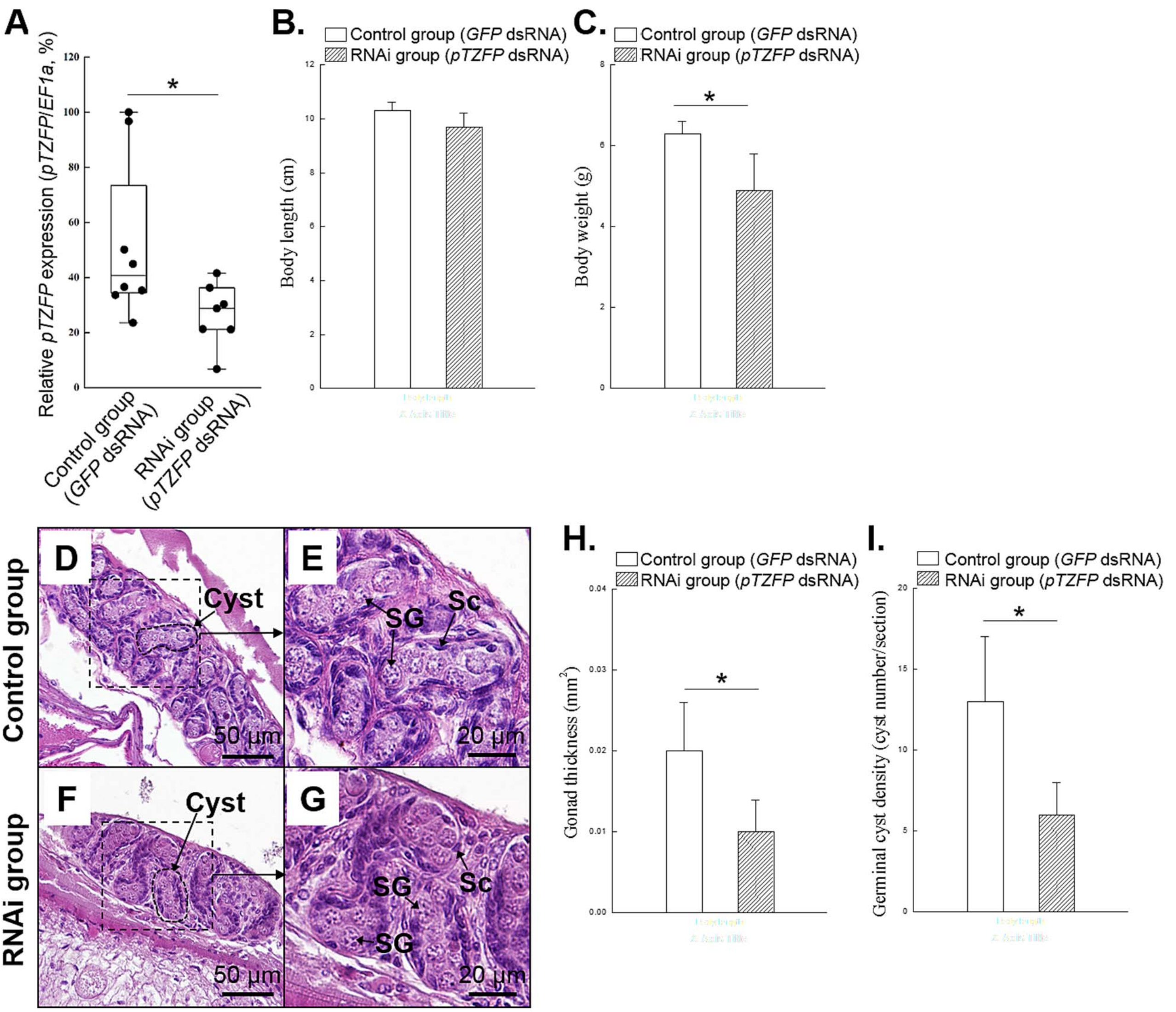

Figure 6. Silencing of *pTZFP* mRNA expression in the male gonads of Pacific white shrimp. Short-term (A) and long-term (B–I) dsRNA administration was used to examine the knockdown efficiency of *pTZFP* dsRNA and the effects of *pTZFP* deficiency, respectively. The experimental shrimp were divided into a control (*GFP* dsRNA) and an RNAi group (*pTZFP* dsRNA). (A) The *pTZFP* mRNA expression levels were determined 3 days after dsRNA treatment by qPCR ($n = 8$ and 7 in the control and RNAi group, respectively). The body length (B), body weight (C), gonad histology (D–G), testicular size (H), and germinal cyst distribution (I) were analyzed 10 weeks after dsRNA treatment ($n = 4$ in each group). The mRNA expression differences between groups were normalized using *EF1A*, and the highest mRNA expression value was set as 100%. The number of asterisks indicates the results of the Student *t*-test as follows: *: $P < 0.05$. SG, spermatogonia; Sc, somatic cell. A dashed line represents the germinal cyst region.

in the knockdown of gonad-specific argonaute 4, which led to smaller testes in black tiger shrimp (*Penaeus monodon*) [46]. Furthermore, the knockdown of gonad-specific *PIWI* led to reproductive defects with fewer sperms in the spermatophore sac [47]. Similar to these gonad-specific genes in penaeid shrimp, our data suggest that *pTZFP* may play an important role in controlling spermatogenesis by regulating spermatogonia proliferation. To determine the reproductive defects of *pTZFP*-deficient male shrimp in future studies, transcriptome analysis and ISH (IHC) should be used to discover the downstream regulatory cascade and characterize the gene/protein localization. In this study, we tried to detect *pTZFP* mRNA and pTZFP localization with ISH and IHC, respectively. Unfortunately, the ISH results showed that strong background signals were presented in the sense probe of *pTZFP*. In addition to performing ISH, we generated a C-terminal region peptide (DQVYQERIYTPSSY)-immunized antiserum for pTZFP, and it failed to recognize the pTZFP protein.

The role of vertebrate-type sex steroids in gonadal development of Pacific white shrimp

Our data demonstrate that *pTZFP* is involved in the testicular development of Pacific white shrimp. However, E2 (vertebrate-type female sex steroid) administration did not cause a decrease in *pTZFP* mRNA expression. In immature male shrimp (12 cm), the expression level of *pTZFP* was significantly increased in E2-treated males compared to the control group (Figure 4). These data suggest that testicular *pTZFP* expression was not directly suppressed with E2 in Pacific white shrimp. E2 levels in hemolymph and hepatopancreas correlate with the mature stage of the penaeid shrimp ovaries [48]. Furthermore, hepatopancreas

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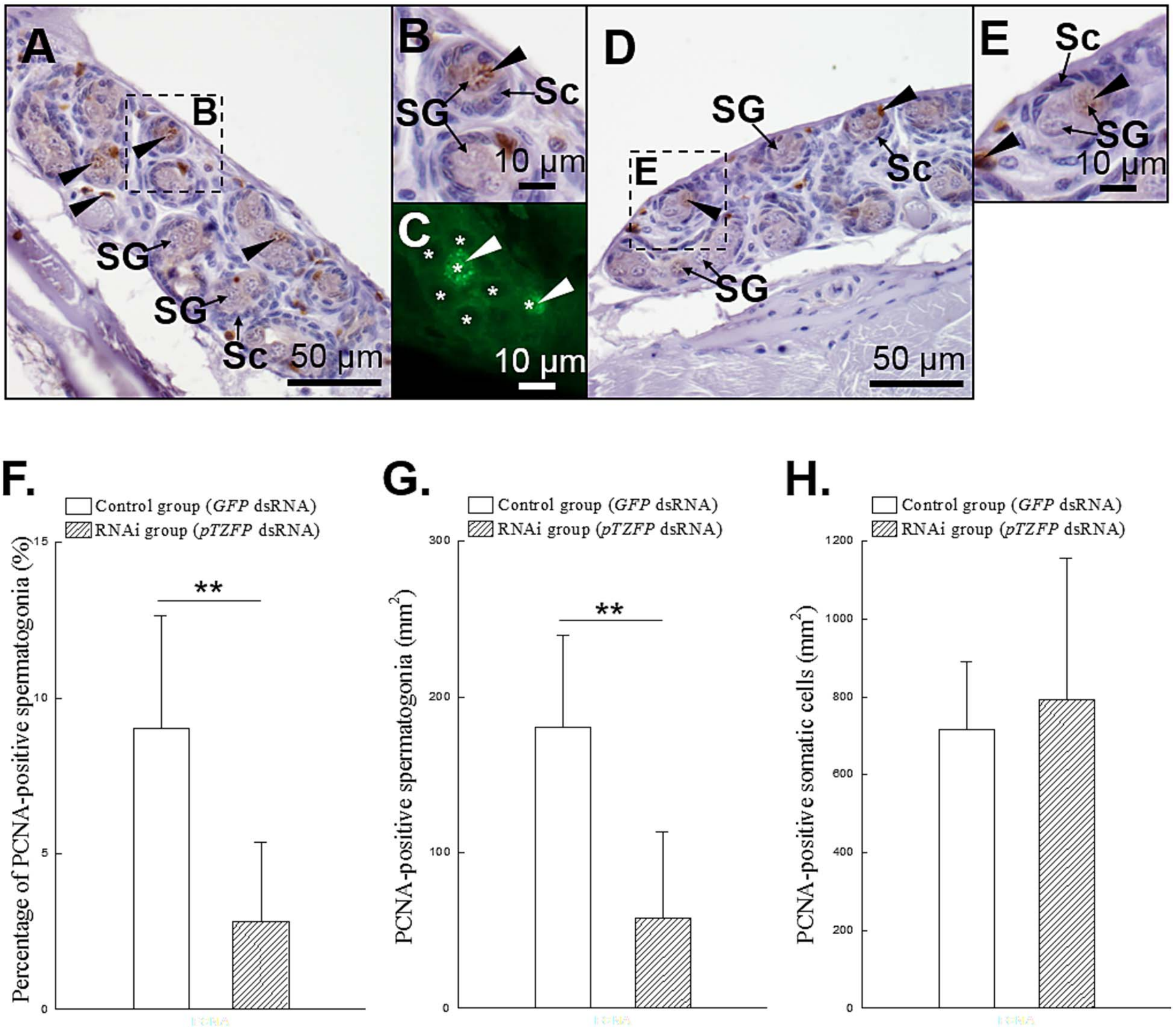

Figure 7. Lower spermatogonia-proliferating activity in the *pTZFP*-deficient male shrimp. The experimental shrimp were divided into two groups: a control group (treated with *GFP* dsRNA) and an RNAi group (treated with *pTZFP* dsRNA). The long-term effects (10 weeks) of *pTZFP* knockdown were analyzed through IHC staining in control (A and B) and RNAi group (D and E) ($n = 4$ in each group). IF staining was employed to characterize the distribution of PCNA signals (C). Based on the IHC staining result, gonadal activity was evaluated by a relative percentage of PCNA-positive cells in both the control and RNAi groups (F). Additionally, the gonadal activity was evaluated by the density of PCNA-positive cells in the control and RNAi groups, specifically in PCNA-positive spermatogonia (G) and somatic cells (H). SG, spermatogonia; Sc, somatic cell. Black and white arrowheads indicate PCNA-positive signals in IHC and IF staining, respectively. In the IF result (C), asterisks denote spermatogonia. The number of asterisks indicates the results of the Student *t*-test as follows: **: $P < 0.01$.

vitellogenin (*VG*) mRNA expression and ovarian development (vitellogenesis) are stimulated by exogenous E2 in penaeid shrimp [49, 50]. In oriental river prawn, sex-differentiated males underwent sex reversal into functional females following the administration of E2 (200 mg/kg feed) [13]. In Pacific white shrimp, all females are inducted by E2 (0.5–1.5 mg/L) incubation in gonad-undifferentiated shrimp [14]. While these studies have employed an overdose of E2 for feminization in shrimp sex manipulation experiments, exceeding endogenous E2 levels, the data support suggestions of a role for E2 in crustacean female reproduction.

In contrast, based on our literature reviews, there are no reports of the identification of high-affinity sex-steroid binding proteins (defined as receptors) in crustaceans [15]. Furthermore, no sex steroid receptor genes were found in the genome of arthropods such as water fleas (*Daphnia pulex*; [51]), fruit flies (*Drosophila melanogaster*; [52]), and nematodes (*Caenorhabditis elegans*; [52]). According to the findings presented above, sex steroid receptors have most likely never been present in the arthropod lineage [53]. Therefore, a possible reason why extremely high doses (non-physiological level) of vertebrate-type sex steroids might affect crustaceans is due to general steroidogenic effects such as enhanced energy usage and alternative energy allocation [15].

Conclusion

Pacific white shrimp is the most popular species in crustacean aquaculture in the world. All-female aquaculture of this species can accelerate the growth rate and productive yield. However, functional sex reversal (neo-male) in penaeid

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shrimp has not yet been achieved by artificial manipulation. Vertebrate-type female sex steroid (E2) in extremely higher than physiological doses has been used to produce an all-female population of Pacific white shrimp [14]. However, high-affinity estrogen-recognized protein (estrogen receptor) has never been found in crustaceans [53]. Therefore, the molecular mechanism by which E2-induced feminization required further elucidation [15]. In addition, based on our literature reviews, there is no report on the transcriptome analysis of the gonadal differentiation period in penaeid shrimp. In this study, our data provide an appropriate molecular marker, male-bias *pTZFP* of undifferentiated gonads. We can now have the ability to develop a transcriptome analysis of the critical window of gonadal differentiation using *pTZFP* as a marker of testicular differentiation. This opens the future opportunity to examine transcriptomic profiles of the critical windows of gonadal differentiation in penaeid shrimps using *pTZFP* as a marker of male gonad to understand the precise molecular mechanism of gonadal sex differentiation.

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Conflict of interest: The authors have declared that no conflict of interest exists.

Author contributions

C-SW performed the RNAi experiment and E2 administration. H-SC performed the terminal ampoule-androgen gland excision experiment and ISH. H-SC and P-WT conducted the transcriptome analysis. C-SW, W-TC, H-SC, C-CH, and H-WL collected the samples and conducted the histology and RNA analysis. AS and C-FC gave the comments. G-CW designed and conducted experiments, analyzed the data, and participated in compiling the main manuscript. All authors reviewed and approved the manuscript.

Data availability

The data underlying this article are available in the article and its online supplementary material.

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