Identification and Characterization of the Pyruvate Dehydrogenase E1 Gene in the Oriental River Prawn, *Macrobrachium nipponense*

Shubo Jin¹, Yuning Hu², Hongtuo Fu¹,²*, Sufei Jiang¹, Yiwei Xiong¹, Hui Qiao¹, Wenyi Zhang¹, Yongsheng Gong¹ and Yan Wu¹

¹ Key Laboratory of Freshwater Fisheries and Germplasm Resources Utilization, Ministry of Agriculture, Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences, Wuxi, China, ²Wuxi Fisheries College, Nanjing Agricultural University, Wuxi, China

Pyruvate dehydrogenase E1 (PDHE1) is thought to play essential roles in energy metabolism, and a previous study suggested that it also has potential regulatory roles in male sexual development in the oriental river prawn, *Macrobrachium nipponense*. In this study, we used rapid amplification of cDNA ends, quantitative polymerase chain reaction (qPCR), *in situ* hybridization, western blotting, RNA interference (RNAi), and histological analyses to assess the potential functions of Mn-PDHE1 in the sexual development of male *M. nipponense*. The full cDNA sequence of Mn-PDHE1 was 1,614 base pairs long, including a 1,077 base pair open reading frame that encodes 358 amino acids. qPCR analysis revealed the regulatory functions of PDHE1 in male sexual development in *M. nipponense* and in the metamorphosis process. *In situ* hybridization and western blot results indicated that PDHE1 was involved in testis development, and RNAi analysis showed that PDHE1 positively regulated the expression of insulin-like androgenic gland factor in *M. nipponense*. Compared with the cell types in the testes of control prawns, histological analysis showed that the number of sperm was dramatically lower after test subjects were injected with Mn-PDHE1 dsRNA, whereas the numbers of spermatogonia and spermatocytes were higher. Sperm constituted only 1% of cells at 14 days after injection in the RNAi group. This indicated that knockdown of the expression of PDHE1 delayed testis development. Thus, PDHE1 has positive effects on male sexual development in *M. nipponense*. This study highlights the functions of PDHE1 in *M. nipponense* and its essential roles in the regulation of testis development.

Keywords: *Macrobrachium nipponense*, PDHE1, qPCR analysis, RNAi, male sexual development
INTRODUCTION

The oriental river prawn, *Macrobrachium nipponense* (Crustacea; Decapoda; Palaemonidae), is widely distributed in China and other Asian countries, and had an annual aquaculture production of 205,010 tons in 2016 (1–4). Male prawns show better growth performance than their female counterparts, as they grow faster and reach larger size by harvest time (2). However, the rapid development of the testis during the reproductive season restricts the sustainable development of the testis during the reproductive season (5). Jin et al. (5) reported that the testis of *M. nipponense* aquaculture, Jin et al. (5) previously showed that the androgenic gland also has significant effects on male sexual differentiation, sexual maturity, and reproductive capability in crustacean species (8–11). Jin et al. (5) previously showed that the androgenic gland regulates testis development in *M. nipponense*.

The androgenic gland and its secreted hormones play important positive regulatory roles in the process of male sexual differentiation and development in crustacean species, and it is especially true for testis development (6, 7). The testis also has significant effects on male sexual differentiation, sexual maturity, and reproductive capability in crustacean species (8–11). Jin et al. (5) previously showed that the androgenic gland regulates testis development in *M. nipponense*.

Many environmental factors, including temperature, illumination, and the presence of chemical pollutants, can regulate sexual differentiation and development by affecting the expression profiles of sex-related genes (12). Jin et al. (13) previously described significant morphological differences in the testis and androgenic gland of *M. nipponense* between the reproductive and non-reproductive seasons. Using transcriptome profiling to analyse the testis and androgenic gland during the two seasons, it was found that glycolysis/gluconeogenesis and the tricarboxylic acid (TCA) cycle may play essential roles in promoting the process of male sexual differentiation and development in *M. nipponense* by providing ATP. Pyruvate dehydrogenase E1 (PDHE1) is an important gene that is enriched in both the glycolysis/ gluconeogenesis and TCA metabolic pathways (13). The PDH complex (PDHc) plays essential roles in the glycolysis pathway. Glucose is reduced to form pyruvate through glycolysis. PDHc can catalyse the oxidative decarboxylation of pyruvate to become acetyl-CoA, which is required by the TCA cycle, depending on the needs of cells (14, 15). PDH deficiency means that pyruvate cannot be converted into acetyl-CoA but instead is reduced to lactic acid, thus affecting the TCA cycle and resulting in decreased ATP production (16). An abnormal TCA cycle leads to metabolic disorders and tissue damage. Therefore, PDHc plays an important role in maintaining the normal metabolism of animals. PDHE1 is the key enzyme component of PDHc, as it catalyses the rate-limiting step of the oxidative decarboxylation of pyruvate (17, 18).

The goal of this study was to verify the important functions of PDHE1 in the process of male sexual development in *M. nipponense* using rapid amplification of cDNA ends (RACE) cloning, quantitative polymerase chain reaction (qPCR), in situ hybridization, western blot, RNA interference (RNAi), and histological analyses. We focused on the potential regulatory roles of PDHE1 on male sexual development in *M. nipponense*. Results of this study provide essential data that can be used to establish a technique to regulate testis development in *M. nipponense*.

MATERIALS AND METHODS

Ethics Statement

All experiments involving *M. nipponense* were approved by the Institutional Animal Care and Use Ethics Committee of the Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences (Wuxi, China).

Sample Preparation

Healthy adult male and female *M. nipponense* with the body weight of 2.87–4.51 g for male prawns and the body weight of 2.37–3.45 g for female prawns were obtained from Tai Lake in Wuxi, China (120°13′44″E, 31°28′22″N). These specimens were transferred to a 500 L tank in the lab and maintained in aerated freshwater at room temperature (28°C) with a dissolved oxygen content ≥ 6 mg/L. The specimens were maintained under lab conditions for 72 h prior to tissue collection. The testis, ovary, hepatopancreas, muscle, eyestalk, gill, heart, and brain were collected from five different prawns (19). The ovarian sample was the mixed sample of the whole ovarian reproductive cycle, which included ovarian stage I (O I), O II, O III, O IV, O V. The five standard phases of the ovarian reproductive cycle have been well described by previous study (20). Additionally, one male prawn and one female prawn were mated and young were hatched in the lab to produce the full-sib population. Specimens for the different developmental stages were collected from this full-sib population during their maturation process (19). For males, the testis and androgenic gland were collected from five specimens during the reproductive season (at 28°C in summer) and from five specimens during the non-reproductive season (at 15°C in winter). Five prawns were also sampled from each of the five standard phases of the ovarian reproductive cycle. The samples were treated with phosphate buffered saline and immediately frozen in liquid nitrogen until used for RNA and protein extraction to prevent total RNA and protein degradation.

RACE

We followed previously described procedures to conduct RACE cloning (21, 22). The specific primers used for *Mn-PDHE1* RACE cloning were listed in Table 1, designed by the Primer-BLAST tool in NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 1). Table 2 lists sequences of PDHE1 from different species. The phylogenetic tree was constructed by MEGA X using the maximum-likelihood method with 1000 bootstrap replications.

qPCR Analysis

The relative mRNA expression levels of *Mn-PDHE1* in the different tissues were measured using qPCR, which was performed on the Bio-Rad iCycler iQ5 Real-Time PCR System.
TABLE 1 | Universal and specific primers used in this study.

| Primer name | Nucleotide Sequence (5’→3’) | Purpose |
|-------------|-----------------------------|---------|
| PDHE1-3GSP1 | CTGAGATCTGCTGACTGAAATAG     | FWD first primer for PDHE1 3’ RACE |
| PDHE1-3GSP2 | CCAAGTATTGCTGTAACGGQG     | FWD second primer for PDHE1 3’ RACE |
| PDHE1 -5GSP1 | TCAAGGAAACACCGTCTATCCC   | RVS first primer for PDHE1 5’ RACE |
| PDHE1 -5GSP2 | TACTCTTGGTTGCGAAACT       | RVS second primer for PDHE1 5’ RACE |
| 3’ RACE OUT | TACCGCTGTCACAGAATCTAGTTT  | RVS first primer for 3’ RACE |
| 5’ RACE OUT | CAGCAGTACTGAGCATCGATGATGGTACATAGG | RVS second primer for 3’ RACE |
| 5’ RACE IN | CGCAGTACCGCAAGCGGAGG     | FWD first primer for 5’ RACE |
| PDHE1 -RTF | TGACCTTAACCGCAAGGGG     | FWD second primer for 5’ RACE |
| PDHE1 -RTR | TACCAGGAGAGATTGAGAGC   | FWD primer for PDHE1 expression |
| IAG-RTF | CGCCTCTCCACCTCCATACGCC | RVS primer for PDHE1 expression |
| IAG-RTR | CTCCTCTCCACCTCCATACGCC | FWD primer for IAG expression |
| EIF-F | CATGGAATGAATCGGCCAACAC | RVS primer for EIF expression |
| EIF-R | CTGTCGAAGAACAGTGCTCCTATTA  | FWD primer for EIF expression |
| PDHE1 anti-sense Probe | GGTGACCTCTGCTATCGTCTTTGTTGCGAAGAAAACTACGG | Probe for PDHE1 ISH analysis |
| PDHE1 sense Probe | CGCTGGTTCCTCCACAAGAGGAGATACGCAAGGTCAAC | Probe for PDHE1 ISH analysis |
| PDHE1 RNAi-F | TAATACGACTACATATAAGGGGTGGTCTCTAATGACATGGAGAGG | FWD primer for RNAi analysis |
| PDHE1 RNAi-R | TAATACGACTACATATAAGGGGTGGTGATGAGGCCGAAGGAGG | RVS primer for RNAi analysis |

(Bio-Rad, Hercules, CA, USA) and used to carry out the SYBR Green RT-qPCR assay (21, 22). Table 1 lists the primers used for qPCR analysis. The expression levels between different tissues were measured by using 2^(-ΔΔCT) method (23).

In Situ Hybridization

In situ hybridization (22, 24) was performed to analyse the mRNA locations of Mn-PDHE1. Primer5 software was used to design the anti-sense and sense probes for chromogenic in-situ hybridization, with the digoxigenin signal based on the cDNA sequence of Mn-PDHE1 (Table 1). The primers for in situ hybridization analysis were synthesized by Shanghai Sangon Biotech Company (Shanghai, China). Slides were examined under light microscope for evaluation the cell types.

Western Blot Analysis

Western blot analysis was conducted on the 20 mg tissues samples following the previous study (25). The total protein concentrations were quantified by using 2-(diazobenzaldehyde)-(6-cyano-1,3,5-triazine)-3-sulfonic acid (Biorad, Hercules, CA, USA) and used to carry out the Western Blot analysis. A total of 50 mg protein from each sample was separated on a 10% sodium dodecyl-polyacrylamide gel. The gel was then transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA).

TABLE 2 | Species used for the construction of PDHE1 phylogenetic tree.

| Species | Accession number |
|---------|-----------------|
| Macrobrachium nipponense | MW366892 |
| Penaeus vannamei | ROT67345.1 |
| Blattella germanica | PSN53167.1 |
| Zootermopsis nevadensis | XP_021917776.1 |
| Brunneria bonealis | QAB-H74097.1 |
| Orthodera ornata | QAB-H74097.1 |
| Nictolea phytophila | QAB-H74097.1 |
| Thermobia domestica | QAB-H74097.1 |
| Atta cephalotes | XP_012055795.1 |
| Atta colombica | XP_018049360.1 |
| Bemisia tabaci | XP_018097986.1 |
| Aedes albopictus | Aedes albopictus XP |

RNAi Analysis

RNAi was performed to analyse the potential regulatory roles of Mn-PDHE1 in M. nipponense. For this analysis, 300 healthy mature male M. nipponense were collected (body weight 3.34–4.47 g). These prawns were randomly divided into the RNAi group and the control group, each containing 150 male prawns. Each group was further divided into three replicates of 50 prawns, which were maintained in 500 L tanks at 28°C with dissolved oxygen content ≥ 6 mg/L. The procedures for designing specific RNAi primer and synthesizing the dsRNA have been well described in previous studies (24, 27). The concentration of Mn-PDHE1 dsRNA was measured using a spectrophotometer and diluted to 4 μg/μL. The prawns in the RNAi group were injected with 4 μg/g of Mn-PDHE1 dsRNA (24, 27) and those in the control group were injected with an equal volume of green fluorescent protein, based on their body weights. qPCR was used to investigate the PDHE1 mRNA expression in the testis after injection (N=5). To assess the regulatory relationship between Mn-PDHE1 and insulin-like androgenic gland hormone (Mn-IAG), the IAG mRNA expression levels were also measured in the same Mn-PDHE1 dsRNA-treated prawns on the same days (N=10).

Histological Observations

Morphological differences between the testis of prawns in the RNAi group and the testis of samples in the control group were observed using haematoxylin and eosin (HE) staining after Mn-PDHE1 dsRNA injection (N = 5). We followed the methods described in previous studies (28, 29). The prepared testis samples were observed using an Olympus ZX16 microscope (Olympus Corporation, Tokyo, Japan).

Statistical Analysis

SPSS Statistics 23.0 (IBM, Armonk, NY, USA) was used to conduct all statistical analyses. Statistical differences were identified by one-way analysis of variance followed by least significant difference and Duncan’s multiple range tests.
The statistical difference between the control group and the RNAi group on the same day was assessed using the paired t-test. Quantitative data were expressed as mean ± standard deviation. A \( p \)-value < 0.05 was considered to be statistically significant.

RESULTS

Genome and cDNA Sequence Analysis

The full cDNA sequence of Mn-PDHE1 was 1,614 base pairs (bp) long, with a 5’ untranslated region (UTRs) and a 3’ UTR of 264 bp and 273 bp, respectively (Figure 1). Mn-PDHE1 included a 1,077 bp open reading frame that encoded 358 amino acids. The sequence was submitted to NCBI with accession number MW366892. The molecular weight of the Mn-PDHE1 protein was 38,539 kDa, and the theoretical isoelectric point of Mn-PDHE1 protein was 6.56.

The BLASTP similarity analysis in NCBI showed that Mn-PDHE1 had the highest sequence similarity with that of Penaeus vannamei (90.72%). The similarities with other PDHE1 sequences from insect species were also > 70%, including Blattella germanica (81.66%), Zootermopsis nevadensis (81.47%), Brunneria borealis (77.31%), and Nicoletia phytophila (79.30%) (Figure 2). The amino acid sequences listed in Table 2 were used to construct the phylogenetic tree, which showed two main clusters. The amino acid sequence of Mn-PDHE1 clustered with that of P. vannamei, whereas the amino acid sequences of PDHE1 from the insect species clustered together as another group. Thus, Mn-PDHE1 has the closest evolutionary relationship with that of P. vannamei and a dramatically distant evolutionary relationship with those from insect species (Figure 3).

Expression of Mn-PDHE1 in Different Tissues and Developmental Stages

Among the tissues tested, the Mn-PDHE1 mRNA expression level was highest in the testis, followed by the heart and ovary, which showed significant difference with other tested tissues \( (p < 0.05) \). The lowest expression was observed in muscle, and the expression levels in the testis and ovary were 20.37-fold and 13.18-fold higher than that in muscle, respectively (Figure 4A).

![FIGURE 1](image-url)  | Nucleotide and deduced amino acid sequence of Mn-PDHE1. The nucleotide sequence is displayed in the 5'-3' directions and numbered at the left. The deduced amino acid sequence is shown in a single capital letter amino acid code. 3’ UTR and 5’ UTR are shown with lowercase letters. Codons are numbered at the left with the methionine (ATG) initiation codon, an asterisk denotes the termination codon (TGA).
Juvenile prawns can be visually distinguished as male or female for the first time at post-larval developmental stage 25 (PL25) by observing the presence of external gonadal features. Overall, the expression levels of Mn-PDHE1 were higher in the larval developmental stages compared with the post-larval developmental stages. During larval and post-larval development, the highest expression level of Mn-PDHE1 was observed in larval developmental stage 15 (L15), whereas the lowest expression level was observed in PL25 females (PL25♀). The expression at L15 was 2.87-fold higher than that of PL25♀. During post-larval development, expression peaked at PL5 and then gradually decreased to PL15. The expression level in PL25 males (PL25♂) was 1.64-fold higher and significantly different than that of PL25♀ (p < 0.05) (Figure 4B).
Expression of Mn-PDHE1 in Different Ovarian Developmental Stages

The expression pattern of Mn-PDHE1 was analysed in different ovarian developmental cycle of *M. nipponense*. The expression of Mn-PDHE1 reached the bottom at O III during the ovarian developmental cycle, and showed significant difference with other tested stages (*p* < 0.05). The highest expression level was observed in O V, followed by O I, which is 2.13-folder and 1.98-folder higher than that of O III, respectively (Figure 5).

Western Blot Analysis

The western blot analysis showed that the molecular mass of Mn-PDHE1 was approximately 50 kDa, which was slightly larger than that of the predicted molecular weight. Jin et al. (13) previously reported that mRNA expression of Mn-PDHE1 in the testis was higher during the reproductive season than during the non-reproductive season. Clear protein bands were visible in the testis samples from the reproductive season, but the bands were blurry in the samples from the non-reproductive season. However, clear bands were observed in the androgenic gland for both of the two seasons (Figure 6A).

During ovarian development, clear bands were only observed for stages OI and OV, indicating that Mn-PDHE1 was transcribed during these stages. Mn-PDHE1 protein expression was more up-regulated in the OV stage than in the OI stage. No bands were observed in stages OII, OIII, and OIV, indicating that Mn-PDHE1 protein was not transcribed during these periods of development (Figure 6B).

In Situ Hybridization Analysis

HE staining showed that the androgenic gland included androgenic gland cells and the ejaculatory bulb. The cells in the testis included spermatogonia, spermatocytes, sperm, and collecting tissues. Strong mRNA signals for Mn-PDHE1 in the androgenic gland were only observed in the ejaculatory bulb surrounding the androgenic gland cells (Figure 7). mRNA signals for Mn-PDHE1 were only observed in spermatogonia in the testis (Figure 7).

Oogonia and follicle cells, which are derived from ovarian epithelial cells, were observed in stage OI. The follicular cavity formed in stage OII, oocyte volume gradually increased in stage OIII, and yolk granules accumulated in the oocytes in stage OIV. mRNA signals for Mn-PDHE1 were observed in all of the cell types and organelles from stages OI to OIV, including the nucleus, oogonia, oocytes, cytoplasmic membrane, yolk granules, follicle cells, and follicle membrane (Figure 7).

RNAi Analysis

The relative mRNA expression levels of Mn-PDEH1 were measured in the testis on days 1, 7, and 14 after the Mn-PDHE1 dsRNA treatment in both the RNAi and control groups. Mn-PDHE1 mRNA expression remained at a stable level in the control group and did not differ significantly over time (*p* > 0.05). In contrast, Mn-PDHE1 mRNA expression in the RNAi group gradually decreased from day 1 to 7, reached the lowest level on day 7, and increased slightly by day 14. The Mn-PDHE1 mRNA expression level was > 95% and 85% lower on days 7 and 14 in the RNAi group, respectively, compared to the control group on the same day (*p* < 0.01) (Figure 8A).

Knockdown of the expression of Mn-PDHE1 also had a positive regulatory effect on the expression of Mn-IAG. In the RNAi group, mRNA expression of Mn-IAG was > 49% and 31% lower on days 7 and 14, respectively, compared to the control group on the same day (*p* < 0.01) (Figure 8B).

HE staining showed that sperm (> 60%) were the dominant cells in the testis in the control group, and spermatogonia and spermatocytes were rare. The morphology of the cells did not differ significantly over time. Compared with the control group,
the number of sperm in the RNAi group was dramatically lower on day 7. On day 14, sperm accounted for only about 1% of the cells in this group, whereas the numbers of spermatogonia and spermatocytes had increased (Figure 9).

**DISCUSSION**

The PDHc is involved in the glycolysis pathway, catalysing the oxidative decarboxylation of pyruvate to become acetyl-CoA, which is required by the TCA cycle to generate ATP (14, 15). PDHE1 is the key enzyme component of the PDHc, as it promotes ATP generation in the glycolysis/gluconeogenesis and TCA metabolic pathways (16, 17). Jin et al. (13) previously predicted that PDHE1 plays important roles in generating ATP for male sexual development in *M. nipponense*. In this study, we analysed the functions of PDHE1 in *M. nipponense*, focusing especially on the regulatory effects of Mn-PDHE1 on Mn-IAG expression and on testis development. Our results may be useful for developing a technique to regulate testis development in this species. To date, the actual functions of PDHE1 have not been reported for any crustacean species.

The BLASTP analysis showed 90.72% similarity between Mn-PDHE1 and PDHE1 of *P. vannamei*, and Mn-PDHE1 also shared over 70% sequence identity with PDHE1 from other insect species. To the best of our knowledge, the only PDHE1 sequence available for a crustacean species prior to our study was that for *P. vannamei*. The neighbour-joining analysis showed that Mn-PDEH1 and the PDHE1 from *P. vannamei* clustered together as a group, and those from the insect species clustered together as another group. Thus, Mn-PDEH1 has the closest evolutionary relationship with the crustacean species and a much longer evolutionary relationship with insect species, which is consistent with evolutionary analysis of other genes in *M. nipponense* (22, 30, 31). More PDHE1 sequences from other species are needed, especially for crustacean species, in order to better analyse the evolutionary relationship of Mn-PDHE1.

The physiological functions of PDHE1 in *M. nipponense* can be reflected by the qPCR results of expression in various adult tissues and developmental stages. For example, qPCR analysis of PDHE1 in the roundworm *Ascaris suum* revealed that the Type I sequence was highly expressed in adult muscle, whereas the Type II sequence was abundant in the third-stage larvae as well as in adult muscle (32). In *Streptococcus mutans*, Korithoski et al. (33) found that PDHE1α expression dramatically increased during adaptation to acidic growth conditions. They also reported that PDHE1α expression increased in conditions favouring heterofermentative growth, decreased in the presence of excess glucose, and increased during the stationary phase compared with the mid-log phase of growth. In adult mice, PDHE1α showed testis-specific expression as well as somatic forms (34). They detected expression of PDHE1α in spermatogonia, Leydig cells, and Sertoli cells at a low level in somatic form. Our in situ hybridization analyses showed that PDHE1α was abundant in spermatocytes. The highest expression of Mn-PDHE1 was detected in the testis, which indicated that the activities of the TCA cycle were sufficient to generate ATP and promote male sexual development in *M. nipponense*. Mn-PDHE1 was more...
FIGURE 5 | Expression characterization of Mn-PDHE1 in different reproductive cycles of ovary. The amount of Mn-PDHE1 mRNA was normalized to the EIF transcript level. Data are shown as mean ±SD (standard deviation) of tissues from three separate individuals. Capital letters indicate expression difference between different samples.

FIGURE 6 | (A) Western-blot analysis of Mn-PDHE1 in the testis and androgenic gland from the reproductive season and non-reproductive season. Line 1: Testis from the non-reproductive season; Line 2: Testis from the reproductive season; Line 3: Androgenic gland from reproductive season; Line 4: Androgenic gland from the non-reproductive season. (B) Western-blot analysis of Mn-PDHE1 in the different ovarian developmental stages. Line 1: Ovarian developmental stage I; Line 2: Ovarian developmental stage II; Line 3: Ovarian developmental stage III; Line 4: Ovarian developmental stage IV; Line 5: Ovarian developmental stage V.
FIGURE 7 | Location of PDHE1 gene was detected in testis, androgenic gland and ovary of *M. nipponense* by using in situ hybridization. ST, seminiferous tubule; SG, Spermatogonia; SC, spermatocyte; S, sperm; CT, collecting tissue; E, wall epithelium; EM, eosinophilic matrix; VD, vas deferens; EB, ejaculatory bulb; OG, oogonium; OC, oocyte; CM, cytoplasmic membrane; N, nucleus; Y, yolk granule; FC, follicle membrane. Scale bars = 20 μm.
highly expressed during the larval developmental stages than during the post-larval developmental stages of juvenile *M. nipponense*, and the highest expression was observed in L15, indicating that PDHE1 promotes the metamorphosis process in *M. nipponense* (35, 36). During post-larval development, the highest expression level of *Mn-PDHE1* occurred in PL5, and it gradually decreased to PL15. Previous histological studies showed that both the testis and ovary could be differentiated at PL5, and they matured at PL19 and PL22, respectively (5). Thus, the peak expression in PL5 indicated that PDHE1 plays essential roles in promoting gonad differentiation. The gender of *M. nipponense* can be distinguished for the first time by the naked eye at PL25, and the expression of PDHE1 was 2-fold higher in PL25♂ than that in PL25♀. This result was consistent with results of the qPCR analysis of different tissues, which showed that PDHE1 plays more essential roles in male sexual development in *M. nipponense*.

Western blot analysis revealed that the molecular weight of *Mn-PDHE1* protein was about 50 kDa, which was slightly larger than that of predicted molecular weight. This result indicates that the *Mn-PDHE1* protein was modified after transcription. Jin et al. (13) previously reported that *Mn-PDHE1* mRNA was more highly expressed in the testis and androgenic gland during the reproductive season than during the non-reproductive season. Our western blot results showed that *Mn-PDHE1* proteins were transcribed in the testis and androgenic gland during the reproductive season and non-reproductive season, while the protein expression in the testis from non-reproductive season may be quite low. Together these findings indicate that PDHE1 promotes testis development during the reproductive season. qPCR analysis of the ovarian reproductive cycle revealed that the high expression levels of PDHE1 were observed at the OI and OV stages. Western-blot analysis of the ovarian reproductive cycle indicated that the *Mn-PDHE1* protein was only transcribed in the OI and OV stages, and expression in stage OV was up-regulated relative to that in stage OI, which is consistent with that of qPCR analysis. Oogonia and follicle cells, which were derived from ovarian epithelial cells, were observed in stage OI. In stage OV, the mature oocytes were excreted (24).
FIGURE 9 | The histological observations of testis between RNAi and control group. SG, Spermatogonia; SC, spermatocyte; S, sperm; CT, collecting tissue. Scale bars = 20 μm.
**In situ** hybridization and immunostaining analysis showed that PDHE1 was abundant in spermatocytes in adult mice (34). We only observed strong mRNA signals of Mn-PDHE1 in spermatogonia in the testis in *M. nipponense*, indicating that PDHE1 plays essential roles in activating the testis developmental process. In the androgenic gland, strong mRNA signals were only observed in the ejaculatory bulb surrounding the androgenic gland cells. This finding indicated that PDHE1 was not directly secreted by the androgenic gland cells in *M. nipponense* but that it plays essential roles in maintaining the normal structure and function of the androgenic gland (22, 30, 31). In the ovary, PDHE1 was widely observed in all cell types, indicating that it was involved in the whole ovarian developmental process (24).

RNAi is an efficient method to analyse gene functions (37–39), and it has been widely used in studies of *M. nipponense* (24, 40). PDHE1 was shown to be involved in the glycolysis/gluconeogenesis and TCA metabolic pathways, promoting the ATP generation, which has been proven to play essential roles in testis development in adult mice (34). Jin et al. (13) predicted that PDHE1 might be involved in male sexual development in *M. nipponense*. Our qPCR analysis revealed that injection of Mn-PDHE1 dsRNA efficiently inhibited the mRNA expression of Mn-PDHE1 on days 7 and 14 after treatment. To analyse the regulatory roles of PDHE1 on IAG, the expression level of Mn-IAG was also measured in the same prawns. The expression of Mn-IAG was also significantly decreased on days 7 and 14 after treatment with Mn-PDHE1 dsRNA, indicating that PDHE1 positively regulates the expression of IAG in *M. nipponense*. Previous studies reported that IAG, which is secreted by the androgenic gland, is involved in male sexual differentiation and development in many crustacean species (41–43). Knockdown of the expression of IAG resulted in sex reversal in *Macrobrachium rosenbergii* (44). The positive regulatory relationship between PDHE1 and IAG highlights the important functions of PDHE1 in male sexual development in *M. nipponense*, as stipulated by Jin et al. (13). Morphologically, the number of sperm was significantly lower beginning on day 7 in the Mn-PDHE1 dsRNA treatment group compared to the control group. On day 14, sperm accounted for only 1% of the cells in the RNAi group, which indicated that Mn-PDHE1 positively regulates testis development in *M. nipponense*.

In conclusion, our results indicate that PDHE1 is involved in the metamorphosis and gonad development of *M. nipponense*, and it is especially important for male sexual development. Mn-PDHE1 was shown to positively regulate the expression of IAG and testis development in *M. nipponense*. This study provides essential data needed to establish a technique to regulate testis development in *M. nipponense*.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

**ETHICS STATEMENT**

The animal study was reviewed and approved by Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences.

**AUTHOR CONTRIBUTIONS**

SJin designed and wrote the manuscript. YH performed the RNAi analysis. HF supervised the experiment. SJia and YX provided the experimental prawns. HQ performed the qPCR analysis. WZ performed the western-blot analysis. YG performed the in situ hybridization analysis. YW performed the histological observations. All authors contributed to the article and approved the submitted version.

**FUNDING**

This research was supported by grants from the National Key R&D Program of China (2018YFD0900201); Central Public-interest Scientific Institution Basin Research Fund CAFS (2021JBFM02; 2020TD36); Jiangsu Agricultural Industry Technology System (JATS[2020]461); the China Agriculture Research System-48 (CARS-48); the New cultivar breeding Major Project of Jiangsu province (PZCZZ201745).

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