Transcriptome Profiling Analysis of the Testis After Eyestalk Ablation for Selection of the Candidate Genes Involved in the Male Sexual Development in *Macrobrachium nipponense*

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The eyestalk of crustacean species secretes many hormones, affecting the process of reproduction, molting, metabolism of glucose, and other functions in crustaceans. In this study, important metabolic pathways and candidate genes involved in the male sexual development were identified through performing the transcriptome profiling analysis of the testis after the ablation of eyestalk from *Macrobrachium nipponense*. The histological observations revealed that the testis development became vigorous after eyestalk ablation, indicating that the hormones secreted by the eyestalk have negative effects on the testis development in *M. nipponense*. Transcriptome profiling analysis revealed that 1,039, 1,226, and 3,682 differentially expressed genes (DEGs) were identified between normal prawns (CG) vs single-side eyestalk ablation prawns (SS), SS vs double-side eyestalk ablation prawns (DS), and CG vs DS, respectively, indicating that the ablation of double-side eyestalk has more significant regulatory roles on male sexual development than that of single-side ablation, which was consistent with the histological observations. Lysosome, Apoptosis, Glycolysis/Gluconeogenesis, and Insulin signaling pathway were the main enriched metabolic pathways in all of these three comparisons, and the important genes from these metabolic pathways were also selected. The qPCR verifications of 10 DEGs from these metabolic pathways were the same as those of RNA-seq. The qPCR, *in situ* hybridization, and RNA interference analysis of Mn-NFκBα revealed that NFκBα has a positive regulatory effect on testis development. This study provided new insights on male sexual development in *M. nipponense*, promoting the studies on male sexual development in other crustaceans as well.

Keywords: *Macrobrachium nipponense*, eyestalk ablation, testis, male sexual development, NFκBα
**INTRODUCTION**

The oriental river prawn, *Macrobrachium nipponense* (Crustacea; Decapoda; and Palaemonidae), is widely distributed in China and other Asian countries (Cai and Shokita, 2006; Salman et al., 2006; Ma et al., 2011), which is an important commercial species with annual aquaculture production that reached 205,010 tons in 2016 (Ma et al., 2011), which is an important commercial species with other Asian countries (Cai and Shokita, 2006; Salman et al., 2006; Ma et al., 2011), which is an important commercial species with.

*Macrobrachium nipponense* aquaculture. But the rapid development of the testis in the reproductive season is another main problem that restricted the sustainable development of *M. nipponense*. Previous studies revealed that the testis of a newborn *M. nipponense* can reach sexual maturity within 40 days after hatching (Jin et al., 2016). Thus, inbreeding will happen between the newborn prawns. Inbreeding will lead to the decrease of the ability of resistance to adversity in their offspring, the small scale of market prawn, and the degradation of germplasm resources. Therefore, it is urgently needed to fully understand the male sexual determination and development mechanism, especially for the identification of the key metabolic pathways and genes involved in the mechanism of male sexual determination and development in *M. nipponense*, with the aims of establishing the technique to produce all male progeny on a commercial scale and regulating the process of testis development.

The eyestalk of crustacean species has many neurosecretory structures. The X-organ–SG complex (XO–SG) is located in the eyestalk in crustaceans, which was identified as a principal neuroendocrine gland (Hopkins, 2012). It stores and releases the crustacean hyperglycemic hormone (CHH) superfamily neurohormones, including CHH, gonad-inhibiting hormone (GIH), molt-inhibiting hormone (MIH), ion transport peptides, and mandibular organ-inhibiting hormone (MOIH), playing essential roles in reproduction (Treerattrakool et al., 2011, 2013; Revathi et al., 2013), molting (Pamuru et al., 2012; Salma et al., 2012; Shen et al., 2013), metabolism of glucose (Santos et al., 1997; Almeida et al., 2004), and other functions (Tiu and Chan, 2007; Sainz-Hernández et al., 2008; Diarte-Plata et al., 2012).

Knockdown of the expression of GH by RNA interference (RNAi) promotes the ovarian development in *M. nipponense* (Qiao et al., 2015). Knockdown of the expression of MIH by RNAi promotes the molting in *M. nipponense* (Qiao et al., 2018). CHH has been proven to promote testis development in *M. nipponense* (Jin et al., 2013b). Many previous studies have proven that the ablation of eyestalk from crustacean species has positive effects on the expression of insulin-like androgenic hormone (IAG; Sroyraya et al., 2010; Chung et al., 2011; Guo et al., 2019). IAG is an important gene playing essential roles in the male sexual differentiation and development in many crustacean species (Ventura et al., 2009, 2011). Knockdown of the expression of IAG resulted in sex reversal in *Macrobrachium rosenbergii*, and the all-male progeny were produced when the “reversal females” were mating with normal male *M. rosenbergii* (Ventura et al., 2012).

It is widely acknowledged that the testis plays essential regulatory roles in reproduction, sexual maturity, and sex differentiation. Previous male reproductive studies have been conducted at the molecular and cellular levels in *M. nipponense* (Qiu et al., 1995; Yang et al., 1999; Cao, 2006; Guo, 2007). A total of 52 candidate male reproduction-related genes were identified from the testis cDNA library of *M. nipponense* (Qiao et al., 2012). An integrated analysis of metabolomes and transcriptomes was also performed in the testis between the reproductive season and non-reproductive season, in order to select candidate male reproduction-related metabolites and genes, regulated by different water temperature and illumination time (Jin et al., 2020). Some candidate genes from these constructed testis transcriptomes have proven their functions in the mechanism of male sexual differentiation and development (Zhang et al., 2013a,b,c). These studies dramatically improved the studies on the mechanism of male sexual differentiation and development in *M. nipponense*. However, the effects of eyestalk on male sexual differentiation and development were still unclear.

In this study, we aimed to select the vital metabolic pathways and genes involved in the male sexual differentiation and development in *M. nipponense* through performing the transcriptome profiling analysis of the testis after the ablation of single-side and double-side eyestalks. The functions of nuclear factor kappa B inhibitor alpha (NfκBα) were further analyzed in-depth by using qPCR analysis, in situ hybridization, and RNAi. This study provided valuable evidences on the studies of male sexual differentiation and development in *M. nipponense*, as well as other crustacean species.

**MATERIALS AND METHODS**

**Ethics Statement**

We obtained the permission from the committee of Freshwater Fisheries Research Center and the Tai Lake Fishery Management Council during the experimental programs. MS222 anesthesia was used to sedate the prawns and shear the tissues.

**Sample Collection**

A total of 600 healthy male *M. nipponense* prawns were collected from a wild population in Tai Lake, Wuxi, China (120°13′44″E, 31°28′22″N) with body weights of 3.34–4.76 g. All the samples were randomly divided and transferred to three 500-L tanks and maintained in aerated freshwater for 3 days with dissolved oxygen of ≥6 mg/L prior to the tissue collection. The three groups were normal prawns (CG), single-side eyestalk ablation prawns (SS), and double-side eyestalk ablation prawns (DS). The death rate of DS prawns was 13.7 and 8.6% higher than that of CG prawns and SS prawns, respectively. The testis was collected from these three groups after 7 days of treatment and immediately preserved in liquid nitrogen until use for transcriptomic analysis.

**Transcriptomic Profiling Analysis**

The comparative transcriptome analysis of androgenic gland between the CG, SS, and DS was performed. In order to ensure the sufficient amount of RNA samples, the testis from at least 30
prawns were pooled to form one biological replicate, and three biological replicates were sequenced for all of these three groups. Thus, a total of nine libraries were generated for sequencing. The experimental process of transcriptome sequencing has been well described in the previously published studies (Jin et al., 2013a, 2017, 2020). Briefly, the total RNA from each pooled sample was extracted by using RNAsiso Plus Reagent (TaKaRa), following the manufacturer’s instructions. The concentration of total RNA was measured by a spectrophotometer (Eppendorf), and the integrity was measured by using a 2100 Bioanalyzer (Agilent Technologies, Inc.) with a minimum RNA integrity number (RIN) value of 7.0. A total of 4 µg of total RNA was used to construct the library, and Illumina HiSeq 2500 sequencing platform was used to perform the sequencing under the parameter of PE150.

Raw data of fastq format were firstly processed using Trimmomatic with default parameters (Bolger et al., 2014). The clean reads were assembled into expressed sequence tag clusters (contigs) and de novo assembled into transcripts by Trinity (version 2.4) with paired-end method with default parameters after removing the adaptor and low-quality sequences (Grabherr (version 2.4) with paired-end method with default parameters de novo (contigs) and Trimmomatic with default parameters (Bolger et al., 2014)). The sequencing platform was used to perform the sequencing under the parameter of PE150.

In situ Hybridization

The mRNA locations of Mn-NF1β0 in the testis, androgenic gland, and different reproductive cycles of ovary were analyzed by using in situ hybridization. The different reproductive cycles of ovary were collected, according to the previous study (Qiao et al., 2015). The testis and androgenic gland were collected in reproductive season. Primer5 software was used to design the anti-sense and sense probes of chromogenic in situ hybridization study and synthesized with DIG signal by Shanghai Sangon Biotech Company. The sequences of anti-sense and sense probes are listed in Table 1. The previous

| Primer          | Sequence          |
|-----------------|-------------------|
| Slalin-F        | ATCAAAGGAAATGCTGCTACCCGT |
| Slalin-R        | TCAGGTAACATGCTCCACTGAAAG |
| Alpha-F         | CAAAGGTCTTTGCAAGGAAAA |
| Alpha-R         | TGGATTTCCCTGACCCCTAT |
| ASK1-F          | GAAATCTTCTCGAGACCACCTCTG |
| ASK1-R          | TCTTCAAGGAGAGGAGGCACCATC |
| Mn-NF1β0-F      | CATGTGAGAGCCGTATACAG |
| Mn-NF1β0-R      | CTTGCAAGGTGAGGATTCTT |
| TGF-F           | CATCTTTACAGGAGTGTGAG |
| TGF-R           | CTGCTTACAGATTTCTCCT |
| ADP-F           | CACACGGATTTACTCTTGGG |
| ADP-R           | CAAGCAAGATCCTCGAGG |
| PPT-F           | GCATCTGGAAGAGGATGAGTGG |
| PPT-R           | GAGAACGGAGGTGTGAGC |
| Hexokinase-F    | CAGACGATTCCTTTCTTGGAG |
| Hexokinase-R    | CAGATTTCCCTCCCTGAAAG |
| Alcohol-F       | TAAAACACATCCTCCAGGAAAG |
| Alcohol-R       | AGGTAGGATTTGGATCTATT |
| Acetyl-F        | CAAAGCTGAGAAAGAGTACAA |
| Acetyl-R        | GAGGAAACACACATCAGGAAAG |

The mRNA locations of Mn-NF1β0 in the testis, androgenic gland, and different reproductive cycles of ovary were analyzed by using in situ hybridization. The different reproductive cycles of ovary were collected, according to the previous study (Qiao et al., 2015). The testis and androgenic gland were collected in reproductive season. Primer5 software was used to design the anti-sense and sense probes of chromogenic in situ hybridization study and synthesized with DIG signal by Shanghai Sangon Biotech Company. The sequences of anti-sense and sense probes are listed in Table 1. The previous
studies have described well the detailed procedures (Jin et al., 2018; Li et al., 2018). Slides were examined under a light microscope for evaluation.

RNA Interference Analysis

RNA interference was performed to analyze the regulatory roles on Mn-NFκBα in *M. nipponense*. The specific RNAi primer with T7 promoter site was designed by using Snap Dragon tools¹ and is shown in Table 1. The Transcript Aid™ T7 High Yield Transcription kit (Fermentas Inc., United States) was used to synthesize the Mn-NFκBα dsRNA, followed by the procedures of the manufacturer. A total of 300 healthy mature male *M. nipponense* were collected with body weight of 3.17–4.96 g and divided into two groups. As described in previous studies (Jiang et al., 2014; Jin et al., 2018), the prawns from experimental group were injected with 4 µg/g of Mn-NFκBα dsRNA, while the prawns from the control group were injected with an equal volume of green fluorescent protein. The NFκBα mRNA expression was investigated in the androgenic gland by qPCR after the injection at 1, 7, and 14 days in order to detect the interference efficiency (N ≥ 5). The mRNA expressions of Mn-IAG were also measured in the androgenic gland templates from the same prawns in order to analyze the regulatory relationship between Mn-NFκBα and Mn-IAG.

RESULTS

Histological Observations of the Testis After Eyestalk Ablation

Histological observations were performed in order to analyze the morphological changes of the testis after the ablation of eyestalk in *M. nipponense* (Figure 1). The histological observations revealed that the greatest number of spermatogonia was observed in the CG prawns, followed by SS prawns and DS prawns. However, the dominant cells in the DS prawns were sperms, which were more than those in SS prawns and CG prawns. Spermatogonia were rarely observed in the DS prawns.

Transcriptome Analysis

The transcriptome generated 54,341 non-redundant transcripts with an average length of 1,311.61 bp. The non-redundant transcripts length ranged from 301 to 28,887 bp. The majority of the transcripts was 301–400 bp (23.62%) in length, followed by >2,000 bp (19.61%) and 401–500 bp (13.36%). The complete and duplicated BUSCOs of this assembled transcriptome reached 97.5%, indicating the completeness of this assembled transcriptome.

Histological Observation

The morphological changes of the testis between different days after RNAi treatment were observed by hematoxylin and cosin (H&E) staining. Five testicular samples were collected after 1, 7, and 14 days of RNAi treatment for H&E staining. The procedures have been described well in previous studies (ShangGuan et al., 1991; Ma et al., 2006). Olympus SZX16 microscope was used to observe the slides (Olympus Corporation, Tokyo, Japan). The various cell types were labeled based on morphological analysis (Jin et al., 2016).

Statistical Analysis

SPSS Statistics 23.0 was used to measure the statistical differences, estimated by one-way ANOVA followed by least significant difference and Duncan’s multiple range test. Quantitative data were expressed as mean ± SD. p < 0.05 indicates a significant difference.

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¹http://www.flyrnai.org/cgibin/RNAifind_primers.pl

## Table 2: Primers used for NFκB analysis.

| Primer name       | Nucleotide sequence (5′ → 3′) | Purpose                      |
|-------------------|------------------------------|------------------------------|
| NFκBα-RTF         | CATGGTACGGCA ATACGGAAG     | FWD primer for NFκBα expression |
| NFκBα-RTR         | CGTCAAGTGGTTGACAGGATTTCTT  | RVS primer for NFκBα expression |
| EIF-F             | CATGATGTACCTGGTGAGAAAC     | FWD primer for EIF expression |
| EIF-R             | CTGTCAGCAAGAAGTCCTGATTATA  | RVS primer for EIF expression |
| NFκBα anti-sense | TTCGCGAAAGAGGGAGAGGAGGAATCAGCGATCCCT | Probe for NFκBα ISH analysis |
| NFκBα sense      | AGGGATCGCTGATTCCTCCTCTTGGC | Probe for NFκBα ISH analysis |
| NFκBα RNAi-F      | TAATACGACTCACCTATAGGGGCTCGT | FWD primer for RNAi analysis |
| NFκBα RNAi-R      | TAATACGACTCACCTATAGGGGCTCGT | RVS primer for RNAi analysis |

## Figure 1

The morphological differences of the testis after the ablation of eyestalk. SG, spermatogonia; SC, spermatocytes; S, sperms; and CT, collected tissue. Scale bars = 20 µm.
unigenes were annotated in the Nr database, while the other unannotated unigenes represent novel genes, but the functions need further investigations.

The assembled unigenes were then annotated in the GO, COG, and KEGG databases. GO and COG analyses provide a structured vocabulary to describe the transcripts. A total of 12,109 unigenes matched the known proteins in GO database, composed of 60 functional groups (Figure 2). The number of unigenes in each functional group ranged from 1 to 10,057. Cell, Cell part, Cellular process, and Binding represent the main functional groups, in which the number of unigenes was > 8,000. A total of 2,509 unigenes were assigned to the matched proteins in COG database, including 22 functional categories (Figure 3). The unigenes in each functional category ranged from 1 to 811. The main functional category includes General function prediction only, Signal transduction mechanisms, and Posttranslational modification, protein turnover, and chaperones, in which the number of unigenes was more than 200.

Kyoto Encyclopedia of Genes and Genomes analysis plays essential roles in releasing the regulatory relationship between
the unigenes, assembled in this transcriptome. A total of 4,971 unigenes were matched with the known proteins in the KEGG database involved in the 338 metabolic pathways. The metabolic pathways, in which the number of unigenes was more than 200, included Alzheimer disease, Pathways in cancer, and Huntington disease.

Identification of Differentially Expressed Genes

The DEGs were identified using the criterion of >2.0 as up-regulatory genes and <0.5 as down-regulatory genes, and p-value < 0.05. A total of 1,039 DEGs were identified between CG and SS, including 617 up-regulated genes and 422 down-regulated genes. Eighty-seven metabolic pathways were identified, and the number of DEGs in each metabolic pathway ranged from 1 to 8. A total of 3,682 DEGs were found between CG and DS, including 1,978 up-regulatory genes and 1,704 down-regulatory genes. A total of 285 metabolic pathways were identified, and the number of DEGs in each metabolic pathway ranged from 1 to 56. KEGG analysis revealed that Lysosome, Apoptosis, Insulin signaling pathway, and Glycolysis/Gluconeogenesis were the main enriched metabolic pathways in all of these three comparisons.

Ten important DEGs were identified from these metabolic pathways, which were differentially expressed in at least two comparisons (Table 3). Sialin-like, alpha-L-fucosidase, and acetyl-CoA carboxylase (ACC) were selected from the metabolic pathway of Lysosome. Apoptosis signal-regulating kinase 1 (ASK1), NFkBa, and TGF-beta-activated kinase 1 (TGF) were selected from the metabolic pathway of Apoptosis. Alcohol dehydrogenase class-P (ADP), Palmitoyl-protein thioesterase 1 (PPT1), and Hexokinase (HXK) were selected from the metabolic pathway of Glycolysis/Gluconeogenesis.

Table 3: Important DEGs through transcriptome profiling analysis.

| Name                             | Accession number     | p value | CG vs SS | CG vs DS | SS vs DS | Metabolic pathways                                      |
|----------------------------------|----------------------|---------|----------|----------|----------|---------------------------------------------------------|
| Sialin-like                      | XP_018006493.1       | 0.0367  | 0.32     | 0.39     |          | Lysosome                                               |
| Alpha-L-fucosidase               | KFM70007.1           | 0.0105  | 2.01     | 2.23     |          | Lysosome; other glycan degradation                      |
| Apoptosis signal-regulating kinase 1 (ASK1) | AKI88007.1       | 0.0145  |          | 2.21     | 2.13     | Apoptosis; platinum drug resistance; tight junction    |
| NF-kappa B inhibitor alpha (NFkBa) | AET34918.1          | 0.0161  | 0.46     | 0.28     | 0.43     | Apoptosis; Pathways in cancer; shigellosis              |
| TGF-beta-activated kinase 1 (TGF) | AKV88638.1           | 0.0338  | 2.31     | 2.17     |          | Apoptosis; NF-kappa B signaling pathway; Shigellosis; MAPK signaling pathway |
| Alcohol dehydrogenase class-P (ADP) | XP_019577393.1       | 0.0293  | 2.43     | 2.16     |          | Glycolysis/Gluconeogenesis; fatty acid degradation; tyrosine metabolism |
| Palmitoyl-protein thioesterase 1 (PPT1) | XP_015686279.1       | 0.0082  |          | 0.27     | 0.31     | Glycolysis/Gluconeogenesis; shigellosis                 |
| Hexokinase                       | ABO21409.1           | 2.58E-06 | 3.23     | 3.32     |          | Glycolysis/Gluconeogenesis; fatty acid degradation; drug metabolism—cytochrome P450 |
| Alcohol dehydrogenase class II (Alcohol) | CCQ25768.1          | 0.0275  |          | 0.45     | 0.48     | Insulin signaling pathway; pyruvate metabolism; AMPK signaling pathway |
| Acetyl-CoA carboxylase (Acetyl)   | ALK82309.1           | 8.70E-05 | 3.23     | 3.13     |          | Lysosome; fatty acid elongation                        |

qPCR Verification of Important Differentially Expressed Genes

The expressions of 10 important DEGs were verified by qPCR, which showed the same expression pattern with that of RNA-Seq (Figure 4). The expressions of NFkBa and PPT1 were gradually increased from the control group to double-side ablation and showed a significant difference between each group (p < 0.05). The lowest expression of sialin-like was observed in the control group and showed a significant difference with that of single-side ablation and double-side ablation (p < 0.05), while the highest expressions of alpha-L-fucosidase, TGF, HXK, and ACC were observed in the control group and showed a significant difference with that of single-side ablation and double-side ablation (p < 0.05). The highest expression of Alcohol dehydrogenase class II was observed in the double-side ablation and showed a significant difference with that of the control group and single-side ablation (p < 0.05), which the lowest expressions of ASK1 and ADP were observed in double-side ablation and showed a significant difference with that of the control group and single-side ablation (p < 0.05).

qPCR Analysis of Mn-NFkBaα

The physiology functions of a gene can be reflected by the qPCR analysis. According to the qPCR analysis in different tissues,
the highest expression of Mn-NFκBα was observed in the testis, which was significantly higher than the other tested tissues and showed a significant difference \( (p < 0.05; \text{Figure 5A}) \). The lowest expression was observed in the brain. The expression in the testis was 6.58-fold higher than that of the brain.

The mRNA expression of Mn-NFκBα was also measured during the post-larval developmental stages. The PCR analysis revealed that the expression of Mn-NFκBα was gradually increased in time with specimen development (Figure 5B). The gonad can be distinguished for the first time by the naked eye at
PL25. The expression of Mn-NF_{k}\beta was higher at both PL25^{\text{♂}} and PL25^{\text{♀}} and showed a significant difference with that of other developmental stages (p < 0.05). However, the expression at PL25^{\text{♂}} was higher than that of PL25^{\text{♀}} (p < 0.05). The lowest expression was observed in PL5, and the expressions in PL25^{\text{♂}} and PL25^{\text{♀}} were 11.83- and 9.15-fold higher than those of PL5, respectively.

**In situ** Hybridization of Mn-NF_{k}\beta

The cell type was labeled, based on the previous study (Figure 6). According to the **in situ** hybridization analysis, signals of Mn-NF_{k}\beta were observed in spermatogonia and spermatocytes, whereas no signal was observed in sperms. Strong mRNA signals in the androgenic gland were only observed in the ejaculatory bulb surrounding the androgenic gland cells, while no signals were directly found in all stages of androgenic gland cells (Figure 6). Clear signals were rarely observed in O I and O V, while signals were observed in the nucleus, yolk granule, yolk granule, and cytoplasmic membrane in O II, O III, and O IV.

**The RNA Interference Analysis of Mn-NF_{k}\beta**

The potential functions of Mn-NF_{k}\beta on male sexual development in *M. nipponense* were analyzed by using RNAi. The expression levels of Mn-NF_{k}\beta were measured in the testis after the treatment of Mn-NF_{k}\beta dsRNA. According to the qPCR analysis, the expression of Mn-NF_{k}\beta remained stable in the control group after the injection of GFP and showed no significant difference (p > 0.05). However, the expression of Mn-NF_{k}\beta significantly decreased at days 7 and 14 after the injection of Mn-NF_{k}\beta dsRNA. The decrease reached 95 and 85% at days 7 and 14, respectively, compared with that in the control group (Figure 7A).

The expressions of Mn-IAG were also measured in the androgenic gland from the same prawns (Figure 7B). According to the qPCR analysis, the expression of Mn-IAG at day 1 in the control group was slightly higher than that of day 7 and day 14, while it generally remained stable. In the RNAi group, the expressions of Mn-IAG were significantly decreased at day 7 and day 14 after the injection of Mn-NF_{k}\beta dsRNA. The expression decreased about 61 and 54% at days 7 and 14, respectively, compared with that in the control group.

**Histological Observations of the Testis After RNA Interference**

According to the histological observations, the number of sperms was more than that of spermatogonia and spermatocytes in the control groups. Compared with that of the control group at day 7 and day 14, the number of sperms in the RNAi group was significantly decreased. In the RNAi group, the number of sperms was gradually decreased in time with Mn-NF_{k}\beta dsRNA treatment, and sperms were rarely found at day 14 after Mn-NF_{k}\beta dsRNA treatment (Figure 8).

**DISCUSSION**

The eyestalk of crustaceans secreted many neurosecretory structures and mediated the reproduction, molting, and metabolism of glucose in crustaceans (Jin et al., 2013b; Qiao et al., 2015, 2018). In this study, we aimed to analyze the regulatory effects on male sexual development through performing the transcriptome profiling analysis of the testis after eyestalk ablation. The histological observations of the testis after eyestalk ablation from *M. nipponense* indicated that the number of sperms in the DS prawns was significantly more than that of SS prawns and CG prawns, and spermatogonia were rarely observed in the DS prawns. This indicated that the hormones secreted by the eyestalk have negative regulatory effects on the testis development. This is the same as the results of a previous study that the hormones secreted by eyestalk inhibit the expression of IAG in *M. nipponense* (Li et al., 2015), and IAG promoted the male sexual characteristic development in many crustacean species (Ventura et al., 2009, 2011).

A total of 54,341 transcripts were generated in this study, providing valuable evidences on the studies of male sexual development. According to the GO and COG analyses, the genes related to the male sexual development were predicted to be mainly found in the functional groups of Cell, Cell part, Cellular process, and Binding in the GO assignment, and in the functional groups of General function prediction.
FIGURE 6 | In situ hybridization analysis of Mn-NFαBα gene in the testis and androgenic gland from reproductive season, and different reproductive cycle of ovary of *M. nipponense*. SG, spermatogonia; SC, spermatocytes; S, sperms; CT, collected tissue; I, Stage I of androgenic gland cell; II, Stage II of androgenic gland cell; III, Stage III of androgenic gland cell; EB, ejaculatory bulb; OG, oogonium; OC, oocyte; CM, cytoplasmic membrane; N, nucleus; Y, yolk granule; and FC, follicle membrane. Scale bars = 20 µm.
respectively, indicating that the ablation of double-side eyestalk vs SS, SS vs DS, and CG vs DS was 1,039, 1,226, and 3,682, (Jin et al., 2017, 2020). The number of DEGs between CG classification, which were consistent with the previous studies modification, protein turnover, and chaperones in the COG only, Signal transduction mechanisms, and Posttranslational responses to exogenous stimuli and indigenous stimulation. ASK1 plays an important role in the response to reactive oxygen species, endoplasmic stress-responsive MAPK kinase kinase. ASK1 is one of the markers of hepatocellular carcinoma (Fukushima et al., 1985). ACC is the enzyme playing essential roles in the synthesis of malonyl-coenzyme A (malonyl-CoA; Winder and Hardie, 1996). Malonyl-CoA is a key metabolite in the regulation of energy homeostasis. Malonyl-CoA was an inhibitor of fatty acid oxidation in skeletal muscle mitochondria, decreased in rat skeletal muscle during exercise or in response to electrical stimulation (Abu-Elheiga et al., 2001). ASK1 is one of the stress-responsive MAPK kinase kinase. ASK1 plays an important role in the response to reactive oxygen species, endoplasmic reticulum stress, and pro-inflammatory cytokines (Kawarazaki et al., 2014). It is involved in the pathogenesis of various diseases, including cancer, neurodegenerative diseases, infections, diabetes, and cardiovascular diseases (Liu et al., 2000). Nuclear factor kappa B (NFκB) plays essential roles in activating immune responses to exogenous stimuli and indigenous stimulation. NFκB proteins translocate into the nucleus to perform its functions during the stress condition (Thanos and Maniatis, 1995). NFκB expression was found in almost all cell types and has more regulatory effects on male sexual development than the single-side ablation in M. nipponense, which was consistent with histological observations of the testis after eyestalk ablation. KEGG analysis revealed that Lysosome, Apoptosis, Insulin signaling pathway, and Glycolysis/Gluconeogenesis were the main enriched metabolic pathways in all of these three comparisons, predicting that these mainly enriched metabolic pathways and vital DEGs from these metabolic pathways may play essential roles in male sexual development in M. nipponense. qPCR verification of these DEGs showed the same expression pattern with that of RNA-Seq, indicating the accuracy of the RNA-Seq.

Lysosomes are organelles playing essential roles in decomposing proteins, nucleic acids, polysaccharides, and other biological macromolecules. Lysosomes contain many hydrolases, functioning in the decomposition of the substances that enter into cells from the outside or the digestion of the local cytoplasm or organelles in the cells. The lysosomes will rupture, and the hydrolases will be released to digest the whole cells when cells are aged (Duve and Wattiaux, 1966; Luzio et al., 2007). Apoptosis refers to the programmed cell death, carefully controlled by genes to maintain the stability of internal environment. Apoptosis is an active process, which is different from cell necrosis. The process of apoptosis involves the activation, expression, and regulation of a series of genes, in order to better adapt to the living environment. Apoptosis plays important roles in the mechanism of physiology and pathology, in order to respond various stimuli, including ischemia, hypoxia, exposure to certain drugs and chemicals, immune reactions, infectious agents, high temperature, radiation, and various disease states (Thompson, 1995; Johnstone et al., 2002). According to the histological observations, the testis development was vigorous after eyestalk ablation. Thus, Lysosomes and Apoptosis were needed to digest the aged cells and to adapt to stress, in order to maintain the normal testis development. Alpha-L-fucosidase was a lysosomal enzyme, presented in all mammalian cells. Its activity has been proven to be deficient in the human autosomal recessive disease fucosidosis. It is also proposed as a marker of hepatocellular carcinoma (Fukushima et al., 1985). ACC is the enzyme playing essential roles in the synthesis of malonyl-coenzyme A (malonyl-CoA; Winder and Hardie, 1996). Malonyl-CoA is a key metabolite in the regulation of energy homeostasis. Malonyl-CoA was an inhibitor of fatty acid oxidation in skeletal muscle mitochondria, decreased in rat skeletal muscle during exercise or in response to electrical stimulation (Abu-Elheiga et al., 2001). ASK1 is one of the stress-responsive MAPK kinase kinase. ASK1 plays an important role in the response to reactive oxygen species, endoplasmic reticulum stress, and pro-inflammatory cytokines (Kawarazaki et al., 2014). It is involved in the pathogenesis of various diseases, including cancer, neurodegenerative diseases, infections, diabetes, and cardiovascular diseases (Liu et al., 2000). Nuclear factor kappa B (NFκB) plays essential roles in activating immune responses to exogenous stimuli and indigenous stimulation. NFκB proteins translocate into the nucleus to perform its functions during the stress condition (Thanos and Maniatis, 1995). NFκB expression was found in almost all cell types and
tissues (Oeckinghaus and Ghosh, 2009). Furthermore, NFκB is involved in the mechanism of many processes, including immune and inflammatory responses, stress responses, and regulation of cell proliferation and apoptosis (Oeckinghaus and Ghosh, 2009).

Glycolysis/gluconeogenesis promotes the conversion of glucose (C₆H₁₂O₆) into pyruvate (CH₃COOCO⁻ + H⁺), releasing free energy to form the high-energy molecule ATP and reduced nicotinamide adenine dinucleotide (Labert, 1995). The main precursors of Glycolysis/gluconeogenesis include lactic acid, pyruvic acid, amino acid, and glyceral. In mammals, the process of Glycolysis/gluconeogenesis mainly occurred in the liver. The Glycolysis/gluconeogenesis ability of the kidney is only 10% of that of the liver in a normal condition. However, the ability of Glycolysis/gluconeogenesis in the kidney will significantly enhance at a starved condition for a long time. HXK proteins play important roles in catalyzing hexose phosphorylation and sugar sensing and signaling. Sucrose must be cleaved in hexoses (glucose and fructose) during the sucrose metabolism (Siemens et al., 2011). The hexoses are phosphorylated by HXKs or fructokinases. The phosphorylated hexoses are then involved in metabolic processes (David-Schwartz et al., 2013). PPT1 plays essential roles in catalyzing the hydrolysis of lipid thioesters on S-acylated proteins, which has a lysosomal localization and function in non-neuronal cells (Lu et al., 1996; Verkruyse and Hofmann, 1996; Lu et al., 2002). Individuals lacking functional PPT1 present with progressive psychomotor decline within the first year of life, followed by loss of vision and profound seizures, before entering a persistent vegetative state that invariably ends in premature death (Santavuori et al., 1973; Hofmann et al., 2002).

The functions of NFκBα were further analyzed by qPCR, in situ hybridization, RNAi, and histological observations, because NFκBα was the most up-regulated gene in the double-side eyestalk ablation prawns. The previous studies and histological observation revealed that the hormones secreted by eyestalk have negative effects on testis development (Sroyraya et al., 2010; Chung et al., 2011; Guo et al., 2019). The histological observations after the ablation of eyestalk in M. nipponense revealed that the testis development became vigorous after eyestalk ablation, and RNA-Seq analysis predicted that Apoptosis played essential roles in the maintaining the normal testis development after eyestalk ablation through digesting the aged cells. NFκBα is enriched in the metabolic pathway of Apoptosis, which has essential DEGs, differentially expressed in all of these three comparisons. Thus, the significant up-regulation of NFκBα expression after eyestalk ablation indicated that NFκBα was predicted as a strong candidate gene for the mechanism of male sexual development in M. nipponense. The previous studies reported that the activation and repression of the NFκBα expression were involved in the pathogenesis of inflammatory diseases, such as adult respiratory distress syndrome (ARDS) and breast cancers (Blackwell and Christman, 1997; Biswas et al., 2001). NFκBα was proven to be involved in the immune system of Macrobrachium rosenbergii, because the expressions of NFκBα were up-regulated after the feed of scutellaria polysaccharide and soybean antigen protein (Yang et al., 2019). However, to the best of our knowledge, no study has reported the potential functions of NFκBα in the mechanism of male sexual development in any species. The qPCR analysis in different mature tissues revealed that the highest expression of Mn-NFκBα was observed in the testis, which was significantly higher than the other tested tissues and showed a significant difference with other tested tissues, indicating that Mn-NFκBα may have potential functions during the testis development in M. nipponense. qPCR was also used to measure the Mn-NFκBα expression in post-larval developmental stages of M. nipponense. The results revealed that the Mn-NFκBα expression was gradually increased with the specimen development, and PL25♂ showed higher expression than that of PL25♀. The sensitive period of gonad differentiation and development of M. nipponense has been proven to be from PL7 to PL22 (Jin et al., 2016). Thus, Mn-NFκBα was predicted to play essential roles in male sexual development in M. nipponense, combined with the qPCR analysis in different mature tissues and post-larval developmental stages. In situ hybridization revealed that signals were observed in spermatogonia and spermatocytes, indicating that Mn-NFκBα played essential roles in the testis development in M. nipponense. No signal was directly observed in the androgenic gland cells, while strong signals were observed in the ejaculatory bulb surrounding the androgenic gland cells, indicating that Mn-NFκBα has potential functions in maintaining the normal functions and structures of androgenic gland in M. nipponense (Jin et al., 2018, 2019). In different ovarian developmental stages, no signal was observed in O I and O V, while signals were observed in the nucleus, yolk granule, yolk granule, and cytoplasmic membrane in O II, O III, and O IV, indicating that Mn-NFκBα promotes yolk accumulation in M. nipponense (Li et al., 2018). RNAi analysis revealed that the ds-RNA of Mn-NFκBα can efficiently knockdown the expression of Mn-NFκBα in M. nipponense. In addition, the expression of Mn-IAG was also decreased with the decrease of Mn-NFκBα, indicating that Mn-NFκBα has a positive regulatory relationship with Mn-IAG. Thus, Mn-NFκBα was involved in the male sexual development in M. nipponense, based on the importance of IAG in the male sexual development in crustacean species (Ventura et al., 2009, 2011, 2012). Histological observations after the treatment of Mn-NFκBα dsRNA revealed that the number of sperms was decreased with the time of Mn-NFκBα dsRNA treatment, indicating that Mn-NFκBα has positive effects on testis development in M. nipponense.

In conclusion, histological observations revealed that eyestalk has negative effects on male sexual development in M. nipponense. A total of 1,039, 1,226, and 3,682 DEGs were identified between CG vs SS, SS vs DS, and CG vs DS, respectively, indicating that the ablation of double-side eyestalk has more regulatory roles on male sexual development in M. nipponense. Lysosome, Apoptosis, Glycolysis/Gluconeogenesis, and Insulin signaling pathway were the main enriched metabolic pathways in all of these three comparisons, and 10 important genes from these metabolic pathways were also selected. The functional analysis of NFκBα by qPCR, RNAi, and histological observations revealed that NFκBα has a positive regulatory effect on testis development in M. nipponense. This study identified the important functions of NFκBα in male sexual development...
in *M. nipponense*, providing new insights for the construction of the technique to regulate the testis development. Crisper9 techniques will be further used to knock out the gene expression of NF\(_k\)B in *M. nipponense* and to identify whether NF\(_k\)B is also an important gene in the mechanism of sex determination in *M. nipponense*, resulting in the sea reversal.

**DATA AVAILABILITY STATEMENT**

The data presented in the study are deposited in the NCBI repository, accession numbers: SRX9832767–SRX9832775.

**ETHICS STATEMENT**

The animal study was reviewed and approved by the committee of Freshwater Fisheries Research Center and the Tai Lake Fishery Management Council. Written informed consent was obtained from the owners for the participation of their animals in this study.

**AUTHOR CONTRIBUTIONS**

ShJ designed and wrote the manuscript. HF supervised the study. YH performed the eyestalk ablation and transcriptome profiling.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.675928/full#supplementary-material

**Supplementary Table 1 | Summary of BLASTx results for unigenes of testis *M. nipponense* transcriptome.**

**AUTHOR CONTRIBUTIONS**

ShJ designed and wrote the manuscript. HF supervised the study. YH performed the eyestalk ablation and transcriptome profiling analysis. YF and YG revised the manuscript. HQ performed the qPCR analysis. WZ performed the *in situ* hybridization analysis. YX performed the RNAi analysis. YW performed the histological observations. All authors contributed to the article and approved the submitted version.
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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