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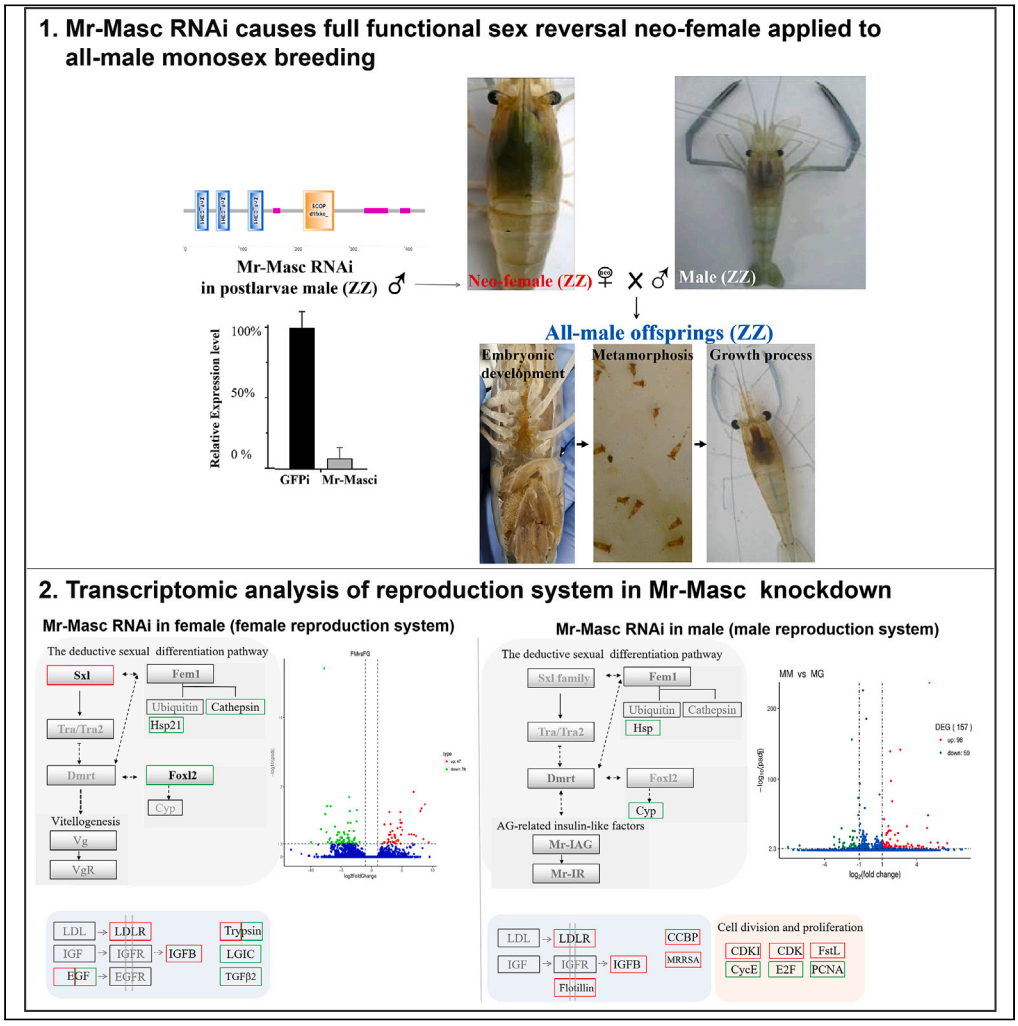

Jing-Yu Luo, Shuai-Qi Shen, Hai-Jing Xu, Jin-Shu Yang, Wen-Ming Ma

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Highlights

Identification a transcription factor masculinizer gene (Mr-Masc) inprawn

Mr-Masc silencing results in functional sex reversal females (neo-females)

Several significantly expressed transcripts and crucial signal pathways are focused

An innovative perspective on Masc proteins' evolution and physiological function

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The transcription factor masculinizer in sexual differentiation and achieved full functional sex reversal in prawn

Jing-Yu Luo,[1,3] Shuai-Qi Shen,[1,2,3] Hai-Jing Xu,[1] Jin-Shu Yang,[2] and Wen-Ming Ma[1,4,*]

SUMMARY

Some Zinc finger (ZnF) proteins are required for masculinization in silkworms. In the present study, a masculinizer gene (Mr-Masc) with multi-tissue expression is identified in the freshwater prawn *Macrobrachium rosenbergii*. The Mr-Masc is clustered into a separate branch with ZnF proteins from decapoda by phylogenetic tree analysis. Moreover, Mr-Masc silencing in male postlarvae prawn results in functional sex reversal females known as neo-females, which are applied to all-male monosex offspring breeding. This manipulation has been significant in sexually dimorphic cultured species. In addition, several significantly expressed transcripts are enriched and the effects of crucial signal pathways are focused through the comparative transcriptomic analysis in Mr-Masc gene knockdown. The significantly differentially expressed epidermal growth factor, upregulated low-density lipoprotein receptor, flotillin, and sex-lethal unigenes, downregulated heat shock proteins and forkhead box homologs are focused. The finding offers an innovative perspective on Masc proteins' evolution and physiological function.

INTRODUCTION

The important economic aquaculture freshwater prawn *Macrobrachium rosenbergii* displays remarkable sexual dimorphism, which means the males grow faster than females and show larger body sizes in adults.[1,2] Thus, considerable research efforts have been devoted to unraveling the molecular mechanism of sex determination and sexual differentiation of *M. rosenbergii* to achieve monosex production.[1,3,4] Sex determination and sexual differentiation are complex processes with various mechanisms in different species. Several signaling molecules essential for sex determination and sexual differentiation have been known to be sex-specific characteristics, such as the male-specific androgenic gland (AG) and insulin-like AG-related factors (IAGs) in crustaceans,[5,6] the doublesex (Dsx) genes in insects,[7] and the masculinizer (Masc) gene and feminizer (Fem) piRNA in *Bombyx mori*.[8]

Firstly, sexual differentiation is primarily monitored by a male-specific AG regulating the male sexual differentiation and maintaining the male characterization in crustaceans.[9,10] Several IAGs and insulin-like receptors (IRs) have been identified among decapods over the past decades. For instance, both the insulin-like androgenic gland hormone (Mr-IAG)[11–13] and its confirmed insulin-like receptor (Mr-IR)[14,15] have participated in male sexual differentiation whereas either of their silencing caused a full and functional sex reversal species, leading to the production of all-male monosex populations in *M. rosenbergii*. Hence, to date, insulin-like signal containing IAGs and IRs is primarily considered to regulate male sexual differentiation in crustaceans.

Secondly, all of the arthropod Dsx,[16–18] nematode mab-3,[19] and vertebrate dmrt1[20] are members of doublesex and mab-3-related transcription factor (Dmrt) gene family and involved in sex determination and/or sexual differentiation in bilaterian animals.[21,22] Meanwhile, the Dmrt is one kind of well-conserved Zinc finger (ZnF) proteins.[21,22] ZnF proteins play significant roles in gene regulation and are known as transcription factors. Different kinds of ZnF motifs exhibit a wide variety of biological functions.[23] MroDmrt11E is a member of Dmrt gene family and participated in male sexual differentiation in *M. rosenbergii*.[24,25] *In vivo* knockdown of MroDmrt11E in male prawn induced a complete and functional sex reversal and achieved the production of an all-male monosex population.[24] Moreover, Dmrt gene family probably participated in the

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transcriptional activation of Mr-IAG and possibly played great roles in the switch of Mr-IAG-related regulatory signaling of male differentiation.[26]

Thirdly, the W chromosome is known to possess a dominant role in female determination for the WZ sex-determination system in insects. For example, a ping-pong mechanism of sex determination has been well-studied in a model species of lepidopteran, *B. mori*.[7] In brief, a masculinizer gene (Masc) was located on the Z chromosome and encoded a CCCH-tandem ZnF protein that controlled both masculinization and dosage compensation.[27] In females, Masc mRNA was cleaved by the PIWI protein complexed with the W chromosome-derived female-specific PIWI interacting RNA (Fem piRNA), which resulted in the female variants of Bmdsx (doublesex ortholog of *B. mori*), inhibited masculinization and led to feminization.[7] Meanwhile, in male embryos, which lack Fem piRNA, Masc regulated the male-specific cleavage of Bmdsx with the two main functional ZnF domains.[7]

Moreover, Masc gene played a vital role in sex determination in other insects besides *B. mori*. For instance, a Masc homolog (TvMasc) in silkworm moth *Trilocha variants* was characterized and played a similar role in the sex-determination cascade in Bombycidae.[28] A Masc ortholog from the crambid *Ostrinia furnacalis* (OfMasc) was required for masculinization.[29] In a Noctuidae pest species, disruption of masculinizer gene (AiMasc) in the black cutworm *Agrotis ipsilon* caused abnormalities in the external genitalia and resulted in male-specific sterility.[30] Interestingly, a masculinizer gene (Ar-Masc) was also identified in brine shrimp *Artemia franciscana* and participated in the process of sex determination.[31] *In vivo* knockdown of Ar-Masc altered the ratio of males to females in sexual offspring and caused a high female-male ratio of progeny.[31] These data have led to the conclusion that Mascs participate in sexual regulation. However, no data on Masc variants of crustaceans involved in sexual differentiation has been published except for that of the brine shrimp. Thus, some questions need to be asked are whether a similar Masc homolog exists in prawns and what role would it play in sexual differentiation in crustaceans.

In the present study, a masculinizer gene (Mr-Masc) was newly identified in the freshwater prawn *M. rosenbergii*. The molecular characteristic and the expression pattern of Mr-Masc were expounded. *In vivo* knockdown of Mr-Masc by RNAi was carried out to explore the effects on sexual differentiation and induction of the full and functional sex reversal. This finding offered a novel insight regarding the biology and evolution of Masc proteins in sexual differentiation and sexual regulation in crustaceans.

RESULTS

The characterization and the expression pattern of Mr-Masc gene

A Masc homolog was newly identified in the prawn *M. rosenbergii* and designated as Mr-Masc. The ORF of Mr-Masc was 1314 bp encoding a deduced protein of 437 amino acid residues with a predicted molecular weight of 47.67 kDa and pI of 7.51. The deduced amino acid sequence of Mr-Masc contained three conserved ZnF domains (aa 21–45, E-value 0.00209; aa 59-83, E-value 0.781 and aa 117-144, E-value 1.35), four low complexity regions (LCR) and a coil region domain (CRD) (Figure 1A). Additionally, a monopartite nuclear localization signal (NLS; aa PDPKRQRYE) and a leucine zipper binding domain (aa LRKQIDDLKKDNANLKKENSDL) were predicted (Figure 1A). The putative Mr-Masc protein presented six predicted glycosylation sites, six methyladenosine sites, and 35 phosphorylation sites, respectively.

Intuitively, a diagram was presented to illustrate the composition of Mr-Masc. Briefly, Mr-Mas consisted of three ZnF domains, four LCR domains, and a CRD (Figure 1B). All three conserved ZnF domains were arranged in the form of the "C-X7-C-X4-6-C-X3-H" type, which belonged to the CCCH-type of the ZnF proteins (Figure 1B).

On the other hand, the mRNA expressions of Mr-Masc gene in various tissues of adult male and female *M. rosenbergii* were detected by the qPCR method. It was found that Mr-Masc mRNA was highly expressed in the thoracic ganglia, intestines, and reproductive system rather than in other organs (Figure 1C). The male reproductive system consists of the testis, vas deferens, and the terminal ampullae in the prawn *M. rosenbergii*. Significantly, the expressions of Mr-Masc were predominant in the testis and vas deferens, but not in the terminal ampullae (Figure 1C). Meanwhile, the higher expression of Mr-Masc was also presented in the ovary. It was indicated that the Mr-Masc may play important roles in the physiological functions of reproduction and sexual regulation in prawns. In the present study, its potential functions in sexual differentiation and gonad development were primarily focused.

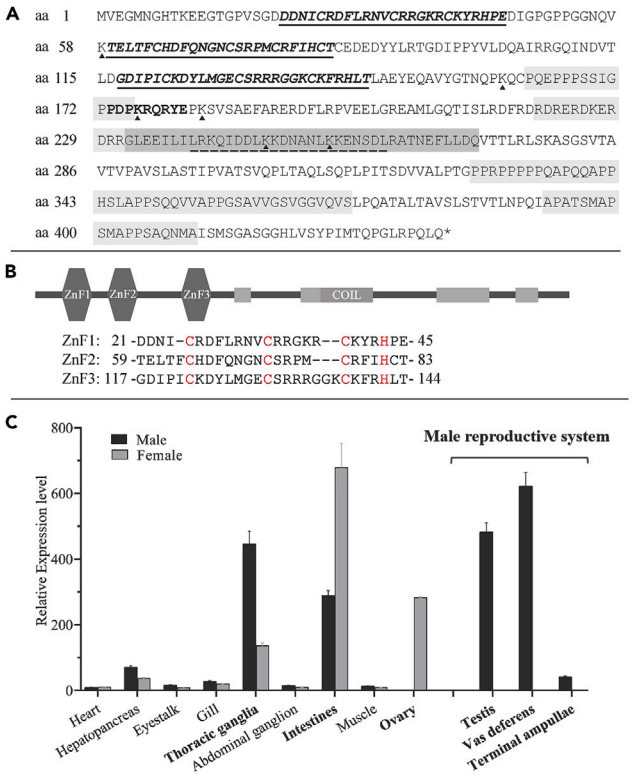

Figure 1. Molecular identification and tissue expression of Mr-Masc

(A) The deduced amino acid sequences of Mr-Masc. The deduced Mr-Masc protein of 437 amino acid residues consisted of three zinc finger (ZnF) domains (bold and italicized, underline), four low complexity region domains (light gray background), and a coil region domain (COIL, dark gray background). A predicted monopartite nuclear localization signal (aa PDPKRQRYE) was in bold and a predicted Leucine zipper binding domain (aa LRKQIDDLKKDNANLKKENSDL) was presented dashed. Six glycosylation sites were shown in the black triangle at 58, 159, 176, 183, 247, and 254 aa sites, respectively. The stop codon was indicated in an asterisk.

(B) Predicted various domains of Mr-Masc. A brief diagram was presented to illustrate the composition of Mr-Masc. Three zinc finger domains (ZnF1, ZnF2, and ZnF3) were shown in dark gray hexagons. Four complexity regions domains were displayed with light gray boxes, respectively. The coil region domain (COIL) was in a dark gray rectangle. The amino acid sequences of each ZnF domain were presented and the conserved C-C-C-H motifs were shown in red.

(C) Expression pattern of Mr-Masc in different tissues of *M. rosenbergii*. All of the tissues including the heart, hepatopancreas, heart, eyestalk, gills, thoracic ganglion, abdominal ganglion, intestines, muscle, ovary, and male reproductive system (testis, vas deferens, and the terminal ampullae) of prawns were ,respectively, done by qPCR and data analysis. Mr-18S rRNA gene was used as the internal reference. All data were represented as the means ± SEM. The transcripts of Mr-Masc were widely expressed in all tissues and highly expressed in thoracalia ganglia, intestines, and reproductive system (testis and vas deferens of males and ovary of females).

Similarities, SMAR, and phylogenetic analysis of Mr-Masc and representative ZnF proteins

Firstly, the results of multiple sequence alignment revealed the similarities of Mr-Masc with the reported Masc proteins and these representative ZnF proteins (Table 1). The whole amino acid sequence of Mr-Masc showed the highest similarity (77.67%) with ZnF protein from the white shrimp *Penaeus vannamei*, the higher similarity (57.60%) with that from the brine shrimp *Armadillidium vulgare*, followed by those from other species with similarities ranging from 11.38% to 38.90%.

Both the first and the second ZnF domains of Mr-Masc present much more conserved among these representatives ZnF proteins from insects to mammals. The highly conserved CCCH sequences of these two ZnF domains and the short conservative sequences, i.e., CRDF/Y sequences in the first ZnF domain, FCH/RDF/YQN and F-H sequences in the second ZnF domain, were congruously presented among various species (Figure 2A).

Table 1. Multiple alignments of whole amino acid sequences among Masc proteins and ZnF proteins from invertebrates to vertebrates

Species	GenBank accession no.	Similarity (%)
Artemia franciscana	ARB66312.1	31.28
Artemia parthenogenetica	ARB66313.1	31.01
Bombyx mori	BAO79517.1	17.11
Trilocha varians	BAS02075.1	16.80
Ephestia kuehniella	QXE45293.1	11.38
Ostrinia furnacalis	BAS02074.1	18.72
Penaeus vannamei	XP_027213468.1	77.67
Armadillidium vulgare	RXG73331.1	57.60
Bombus terrestris	XP_003402296.1	37.36
Bombus impatiens	XP_003485712.1	37.36
Apis mellifera	XP_624646.1	37.36
Apis florea	XP_003692393.1	37.36
Megachile rotundata	XP_003706648.1	37.36
Solenopsis invicta	EFZ20709.1	38.90
Acromyrmex echinatior	EGI61457.1	37.99
Microplitis demolitor	JO958161.1	34.15
Papio anubis	XP_003906632.2	33.42
Macaca mulatta	AFE64182.1	29.75
Homo sapiens	NP_116175.1	33.42
Pan troglodyte	XP_509133.3	33.42
Oryctolagus cuniculus	XP_002711115.1	33.07
Tupaia chinensis	ELV12038.1	32.81
Octodon degus	XP_004647136.1	33.33
Heterocephalus glaber	XP_004890300.1	33.60
Cavia porcellus	XP_005006470.1	33.07
Mesocricetus auratus	XP_005079768.1	33.33
Jaculus jaculus	XP_004650028.1	32.81
Myotis brandtii	EPQ14238.1	33.07
Loxodonta africana	XP_003405601.1	33.60
Trichechus mananus latirostris	XP_004379998.1	33.60
Condylura cristata	XP_004692691.1	33.07
Lctidomys tridecemlineatus	XP_005335678.1	30.00
Canis lupus famlilaris	XP_538227.1	33.60
Chilo suppressalis	GAJS01009478.1	22.70
Manduca sexta	XP_030026651.1	36.96
Antheraea assama	GBZC01022662.1	22.69
Actias selene	GBZL01025705.1	19.45
Antheraea yamamai	GBZJ01013465.1	23.75
Danaus plexippus	BAS02074.1	21.45
Helicoverpa armigera	QCD63870.1	18.67
Spodoptera exigua	GARL01061422.1	17.91

Secondly, SMART representations of Mr-Masc and the complete sequences of representative ZnF proteins from the white shrimp *P. vannamei* (Decapoda, Crustacea), *A. vulgare* (Isopoda, Crustacea), the brine shrimps *A. franciscana* and *Artemia parthenogenetica* (Anostrac, a Crustacea), *Microplitis demolitor* (Hymenoptera, Insecta), *Apis florea* and *Apis mellifera* (Hymenoptera, Insecta), *Bombyx moxi*, *Actias selene*,

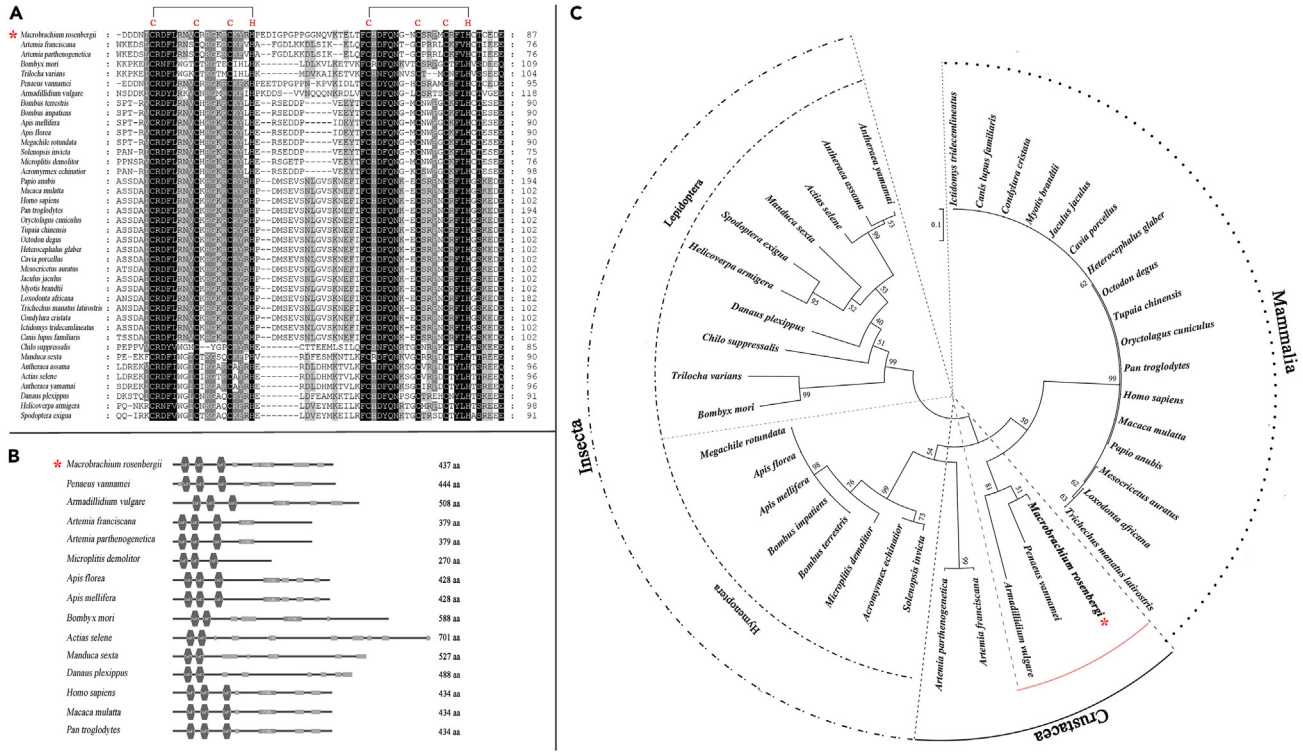

Figure 2. Multiple sequence alignments, SMART, and phylogenetic analysis of Mr-Masc and representative ZnF proteins from invertebrates and vertebrates

(A) Multiple sequence alignments of Mr-Masc and representative ZnF proteins. The ZnF1 and ZnF2 domains of Mr-Mas revealed a high degree of similarity with those from representative ZnF proteins. The conserved identical residues were highlighted among the sequences with a dark background. Residues with strongly similar properties were shown in gray. The conserved C-C-C-H motifs of each ZnF domain were shown in red. ZnF: zinc finger.

(B) SMART representation of predicted domains in Mr-Masc and representative ZnF proteins. Two decapod members *Macrobrachium rosenbergii* and *Penaeus vannamei*, one isopod *Armadillidium vulgare*, two Anostraca representatives *Artemia franciscana* and *Artemia parthenogenetica*, one Neoptera representative *Microplitis demolitor*, two Insecta members *Apis florea* and *Apis mellifera*, four Lepidoptera representatives *Bombyx mori*, *Actias selene*, *Manduca sexta*, and *Danaus plexippus*, three representative vertebrates *Homo sapiens*, *Macaca mulatta*, and *Pan troglodytes* were used for analysis. Various domains were represented as follows: zinc finger domains (ZnF, dark gray hexagons), coil region domain (COIL, dark gray rectangle), and low complexity region domains (light gray boxes). Mr-Masc consisted of three ZnF domains and a coiled region, which was concordant with that of ZnF proteins among the representative species.

(C) Phylogenetic analysis of Mr-Masc and representative ZnF proteins. Various homologous ZnF proteins used for phylogenetic analysis were listed in Table 1. Mr-Masc was firstly clustered into a separate branch with ZnFs from decapoda *P. vannamei* and isopoda *A. vulgare* in the crustacean, and then clustered together with ZnFs from mammals. Bootstraps were performed with 1000 replicates to ensure reliability.

Manduca sexta, and *Danaus plexippus* (Lepidoptera, Insecta) and the distant *Homo sapiens*, *Macaca mulatta*, and *Pan troglodytes* (Primates, Mammalia) demonstrated that the domain composition and organization were conserved from invertebrates to mammals. The SMART results showed that all of the predicted ZnF proteins from various species contained conserved ZnF domains. Starting from the N-terminal end of the putative sequence, the Mr-Masc consisted of three ZnF domains and a coiled region, which was concordant with most of the ZnF proteins among the representative species (Figure 2B). Differently, the ZnF proteins of *M. demolitor* and *D. plexippus* lacked a coiled region domain near the C-terminal end.

By comparison, two ZnF domains were presented in four representative lepidoptera species, *B. moxi*, *A. selene*, *M. sexta*, and *D. plexippus*, while three ZnF domains were shown in other representative species (Figure 2B).

Thirdly, various representative ZnF proteins from invertebrates to vertebrates were used for phylogenetic analysis. The clade of the phylogenetic analysis showed that Mr-Masc was firstly clustered into a separate branch with ZnF proteins from decapoda *P. vannamei* and then clustered together with ZnF proteins from isopoda *A. vulgare* in crustaceans (Figure 2C). Mascs from two Artemia of anostraca crustacean,

A. parthenogenetica, and *A. franciscana*, were clustered with ZnF proteins from hymenoptera, while the ZnF proteins from lepidoptera were clustered alone (Figure 2C). Moreover, the ZnF proteins from mammal species were also clustered into a separate branch alone (Figure 2C).

Similarities, SMART and phylogenetic analysis of Mr-Masc and representative Masc proteins

The results of multiple sequence alignment revealed the similarities of Mr-Masc with the reported Masc proteins showed the highest similarity with the Ar-Mascs from *A. franciscana* (31.28%) and *A. parthenogenetica* (31.01%) followed by those from other species with similarities ranging from 11.38% to 18.72% (Table 1).

On one hand, both the first ZnF and the second ZnF domains of Mr-Masc presented much more conserved among these representative Mascs in arthropods. The highly conserved CCCH sequences of these two ZnF domains and the short conservative sequences, i.e., CRD/NF/Y sequences in the first ZnF domain, FCH/RD/NFQN, and F-H sequences in the second ZnF domain, were congruously presented among various species except for the Masc of *Ephestia kuehniella* (Figure 3A).

SMART results of Masc ortholog representatives showed that the domain composition of these reported Mascs was diversiform among arthropods. There were three ZnF domains in the Mascs of *M. rosenbergii* and Artemia, two ZnF domains in that of *B. Mori*, and only one ZnF domain in that of *Trilocha varians* (Figure 3B). Nevertheless, the Mascs of *E. kuehniella* just have the coiled region but lack expectant ZnF domain. The Masc of the crambid *O. furnacalis* did not contain the ZnF domain and the coiled region (Figure 3B). Significantly, these Mascs presented various patterns of domain composition.

On the other hand, the results of phylogenetic analysis of Masc orthologs briefly revealed that the Mr-Masc was clustered into a separate branch with two Masc proteins of Artemia, and then clustered into the branch with *B. mori* and *T. varians* among these reported Masc proteins (Figure 3C).

Mr-Masc silencing in postlarvae induced a fully functional sex reversal into neo-female

In the freshwater prawn *M. rosenbergii*, it has been reported that the manipulation of some sexual-related genes' silencing obtained full and functional sex reversal species, leading to the production of all-male monosex populations.[13,15,32] Thus, similar gene silencing was attempted to expound whether Mr-Masc participated in male sexual differentiation in *M. rosenbergii*. In Figure 4A, a brief strategy of the animal experiment study was illustrated. The neo-female prawn was expected to be induced by Mr-Masc silencing and applied for the breeding of all-male progeny. Meanwhile, the comparative transcriptomic analyses of the reproductive systems were implemented to enrich differently expressed genes after Mr-Masc knockdown in adult prawns.

Firstly, Mr-Masc silencing was carried out in postlarvae and the Mr-Masc transcript level was significantly reduced to less than 10% with the control group at the end of the experiment (Figure 4B). Then, both the appearance characteristics observation and the sex genetic identification method were used to confirm the sex of Mr-Masc knockdown prawns. On one hand, the appearance features of pereiopods and pleopods were used as reference indexs to distinguish sex for prawn individuals. As shown in Figure 4C, one pair of the male genital pores (MGP) was located at the coxopodite of the fifth pereiopods (Figure 4C-Male) and one pair of the female genital pores (FGP) was located at the coxopodite of the third pereiopods (Figure 4C Female) in *M. rosenbergii*. Meanwhile, there was a pair of particular rod-shaped protruding accessory known as the male appendage (MA) at the inner edge of the second pleopods of male (Figure 4C-Male) while the female did not have this structure (Figure 4C–Female). Thus, the males were firstly distinguished from the prawns by the appearance characteristics observation. On the other hand, the sex genetic identifications of normal female and neo-female were further estimated according to the female-specific sex marker method.[32] As shown in Figure 4D, the female-specific band was only detected in the female but not in the male by the sex marker tests (Figure 4D control). In the present study, some Mr-Masc silenced male individuals (Figure 4D Neo-female) with neither MGP nor MA (Figure 4C Neo-female) compared with individual male and female controls were obtained. That was to say clear evidence for sex reversal was observed. In more detail, the sex reversal male resembled the control female, having FGP at the third pereiopods, but neither MGP at the fifth pereiopods nor MA at the second pleopods (as evident in the control male) (Figure 4C Neo-female), whereas it was identified to genetic male lacking the female-specific sex band (Figure 4D Neo-female) and termed neo-female.

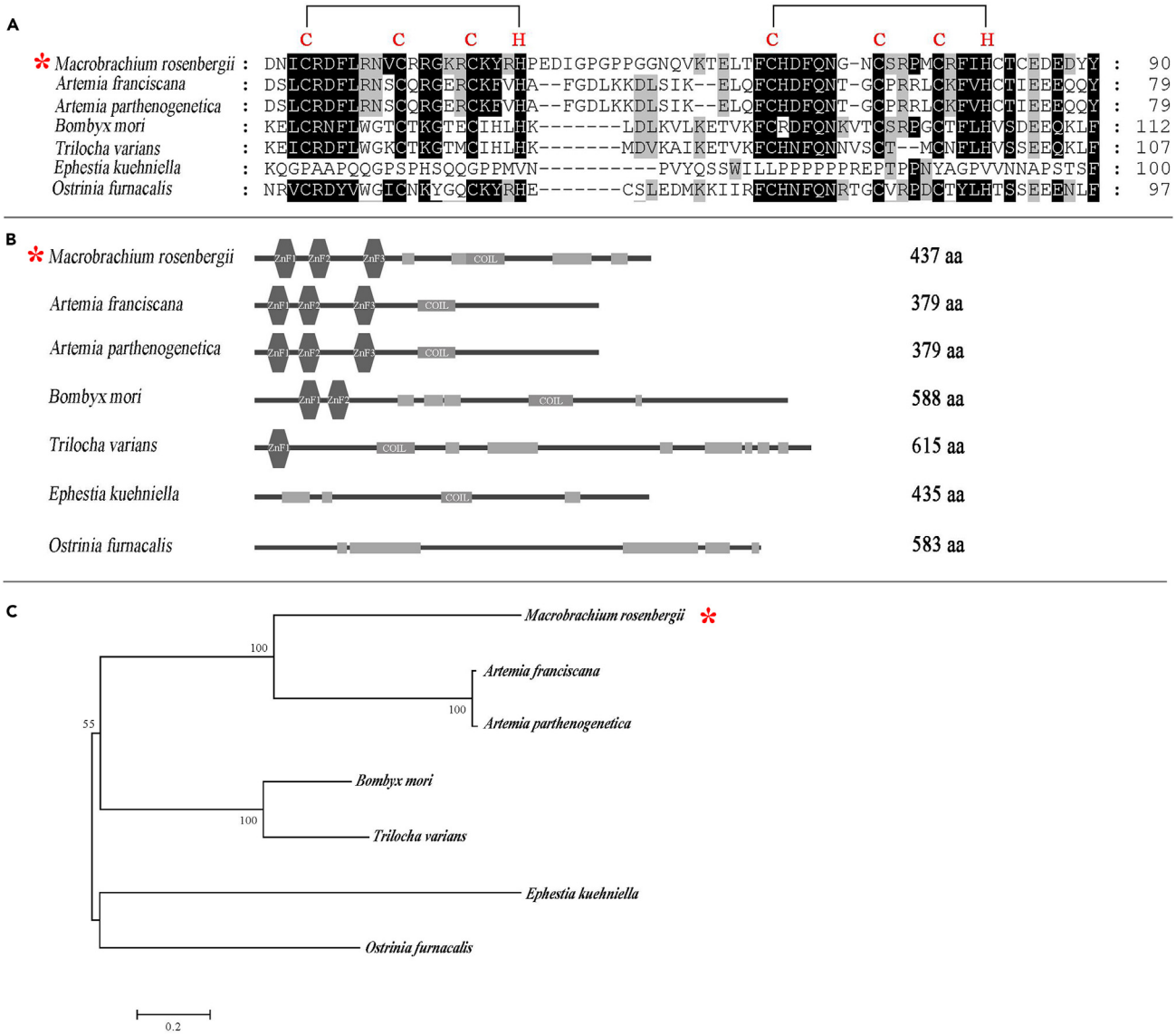

Figure 3. Multiple sequence alignments, SMART, and phylogenetic analysis of Mr-Masc and representative Masc proteins in arthropods

(A) Multiple sequence alignments of Mr-Masc and representative Masc proteins. The ZnF1 and ZnF2 domains of Mr-Mas revealed a high degree of similarity with those from representative Masc proteins. The conserved identical residues were highlighted among the sequences with a dark background. Residues with strongly similar properties were shown in gray. The conserved C-C-C-H motifs of each ZnF domain were shown in red. The Masc of the crambid *Ostrinia furnacalis* did not contain the C-C-C-H motif.

(B) SMART representation of predicted domains in Mr-Masc and representative Masc proteins. The domain compositions of representative Mascs were diversiform among crustaceans and insects. Various domains were represented as follows: zinc finger domains (ZnF, dark gray hexagons), coil region domain (COIL, dark gray rectangle), and low complexity region domains (light gray boxes). Mr-Masc consisted of three ZnF domains and a coiled region, which was concordant with that of ZnF proteins among the representative species. Nevertheless, the Mascs of *Ephestia kuehniella* just have the coiled region and lack expectant ZnF. The Masc of the crambid *Ostrinia furnacalis* did not contain the ZnF domain and the coiled region.

(C) Phylogenetic analysis of Mr-Masc and representative Masc proteins. The Mascs of decapod *M. rosenbergii*, two Anostraca representatives *A. franciscana* and *A. parthenogenetica*, four Lepidoptera representatives *B. mori*, *Trilocha varians*, *E. kuehniella* and *O. furnacalisone* were used for analysis. Mr-Masc was clustered into a separate branch with Mascs from isopoda *A.vulgare* in crustaceans. Bootstraps were performed with 1000 replicates to ensure reliability.

Secondly, the Mr-Masc silenced genetic males were raised to maturity and developed ovaries. The full and inflated orange-green ovary was displayed throughout the carapace or extended from behind the eyestalk down to the first abdominal segment (Figure 4E, Neo-female). Histologically, the mature ovary was enwrapped by an ovarian membrane (OM) and full of late vitellogenic oocytes or mature oocytes (termed Oc4 period oocytes) that were intensely eosinophilic (reddish orange (Figure 4E inset). The cytoplasm of the

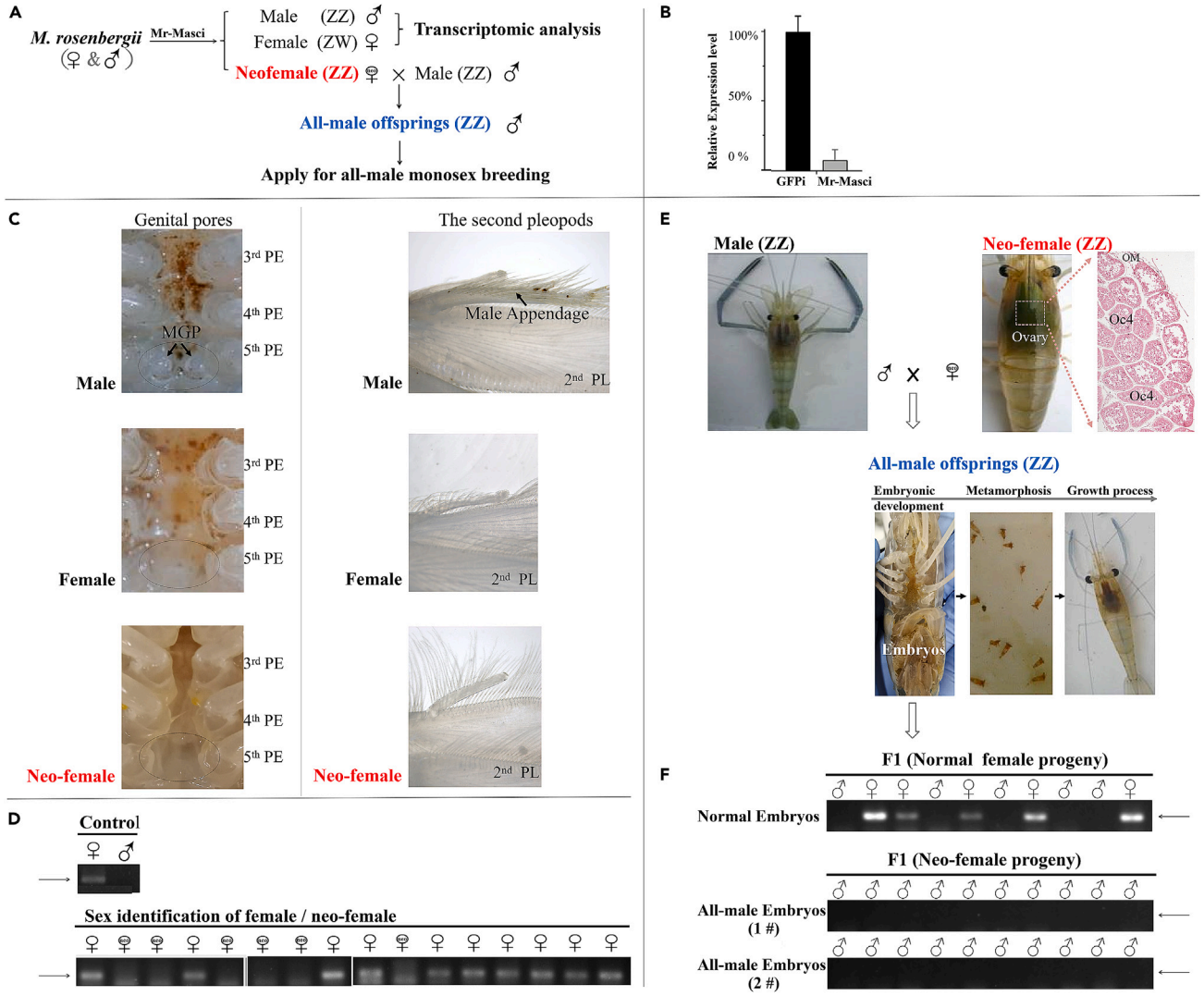

Figure 4. *In vivo* knockdown of Mr-Masc by RNAi in *M. rosenbergii*

(A) The brief diagrammatic sketch of *in vivo* knockdown of Mr-Masc by RNAi. Mr-Masc dsRNA injection was performed in postlarvae. The Mr-Masc RNAi-mediated male and female were collected for comparative transcriptomic analysis, respectively. The neo-female with Mr-Masc-silencing genetic male was identified and utilized to all-male progeny breeding. The brief graphic strategy was illustrated that the induced neo-female was mated with normal male, and its progeny was all-male monosex population.

(B) Detection of the relative mRNA level in the Mr-Masc RNAi induced and the control groups. The data were represented as the means ± SEM. The Mr-Masc transcript level was significantly reduced to less than 10% with the control group at the end of the experiment.

(C) The appearance and sexual characteristics of individuals in neo-female and normal prawns. The Mr-Masc-silenced male individual with neither MGP nor MA compared with individual male and female controls and termed neo-female. PE: pereiopods; PL: pleopods; MGP: male genital pores; FGP: female genital pores; MA: male appendage.

(D) The sex identification and selection of neo-females by the female-specific sex marker method. The unique fragment was identified from genetic female prawns but no similar band was amplified in genetic males. The sex reversal individual of Mr-Masc-silenced male was identified and confirmed. The position of arrowhead indicated that of the female-specific band.

(E) The preparation strategy of all-male monosex progeny by the full functional sex reversal neo-females. The brief strategy of all-male monosex progeny preparation was illustrated. Firstly, the appearance of a yellow-green ovary was seen from the dorsum in the mature female. Histologically, the mature ovary was mostly full of mature oocytes (Oc4). The Mr-Masc-silenced genetic males (neo-females) mated with normal males, and spawned and incubated the embryos in their intumescent abdomens.

(F) The sexual identification of all-male monosex progeny. The embryos were collected from the abdomens of females or neo-females. The sex progeny of the normal females was found to comprise both males and females (normal female-F1), whereas the sex progeny of the neo-female was all-male and monosex (Neo-female-F1). Thus, the embryos of the neo-females were identified by the female-specific sex marker method and confirmed to all-male. The arrowhead indicated position of the female-specific band.

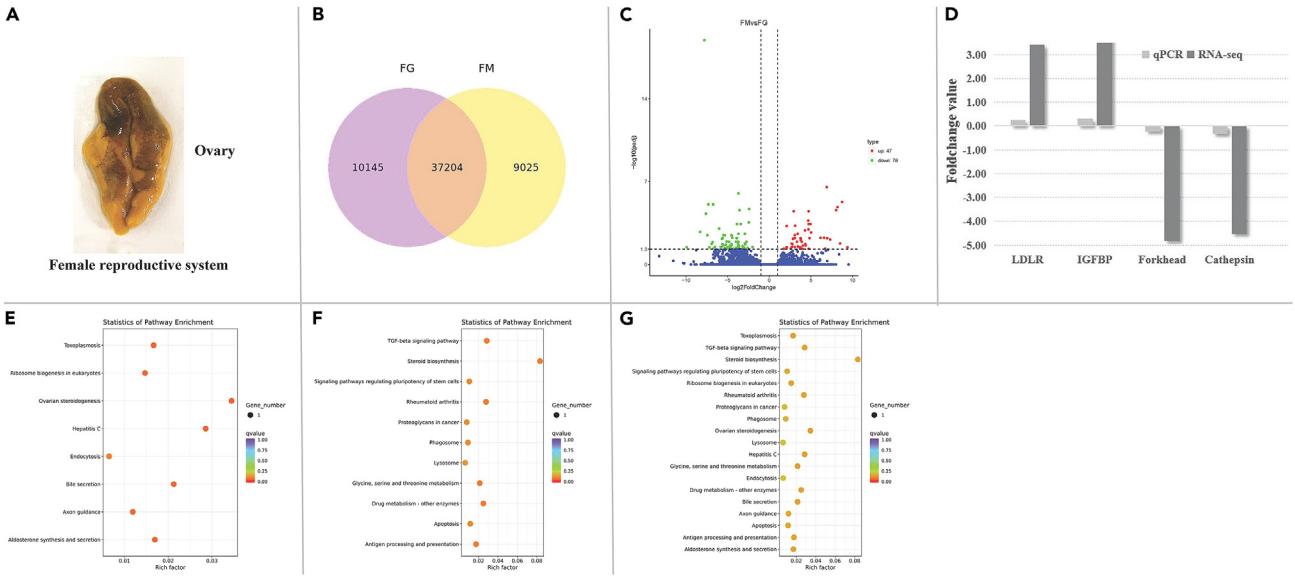

Figure 5. The comparative transcriptomic analysis of Mr-Masc knockdown in female

(A) The appearance of the mature ovary in *M. rosenbergii*.

(B) Venn diagram showing the numbers of expressed genes and differentially expressed genes (DEGs) in the transcriptomic library of the ovary in the Mr-Masc RNAi group vs. GFP RNAi group. FM: the Mr-Masc RNAi group of females; FG: the GFP RNAi group of females.

(C) The significant differentiation of upregulated and downregulated genes in the Mr-Masc RNAi group vs. the GFP RNAi group in the ovary. The red dots indicated the upregulated genes and the green dots indicated the downregulated genes, respectively.

(D) Verification of the expression patterns both in real-time quantitative reverse transcription PCR (qPCR) and RNA-seq. The four detected genes showed similar upregulated or downregulated expression patterns, respectively. The results indicated the reliability and accuracy of our transcriptome analysis. LDLR: low-density lipoprotein receptor; IGFBP: insulin-like growth factor binding protein.

(E) Gene Ontology (GO) classification of DEGs in the ovarian transcriptome. Biological process (BP) enrichment of DEGs in the Mr-Masc RNAi group vs. the GFP RNAi group.

(F) Cellular component (CC) enrichment of DEGs. Ovarian steroidogenesis of biological process and steroid biosynthesis were found to be dominant.

(G) Molecular function (MF) enrichment of DEGs.

mature ovary was full of numerous large yolk granules and smaller-sized lipid droplets (Figure 4E inset). The Mr-Masc silenced genetic males (neo-females) could mate with normal males. In the reproductive season, the neo-females spawned and incubated the embryos in their intumescent abdomens. The embryos adhered tightly to the setae of the pleopods of the female prawns and go through embryonic development before hatching (Figure 4E).

Furthermore, ten embryos from each neo-female were sampled for genetic sex determination and confirmation. As expected, the sex of the progeny from the normal females were comprised male and female (Figure 4F, top, Normal female-F1), whereas the sex of the progeny from the neo-female was all-male (Figure 4F bottom, Neo-female-F1). It confirmed that silencing the transcript encoding Mr-Masc in male individuals caused a complete and functional sex reversal into neo-female that could be applied to produce all-male populations.

The differential gene expression (DGE) analysis of Mr-Masc silencing in the comparative transcriptomic sequencing

Despite the success of sexual reversal through Mr-Masc knockdown, there have some questions to explore about the effects of Mr-Masc knockdown on the signal pathways or the expression levels of related genes in the molecular mechanism of sexual differentiation. Therefore, the comparative transcriptomic profiling of the female or male reproductive system of adult *M. rosenbergii*, in response to Mr-Masc silencing, was enriched, respectively.

On one hand, the transcriptomic libraries of the ovaries (Figure 5A ovary) obtained about 50.23 million clean reads and 7.54G clean bases in the Mr-Masc RNAi group, while 57.59 million clean reads and 8.64G clean bases in the GFP RNAi group. The values of Q20 and Q30 were more than 96.3% and 90.6%

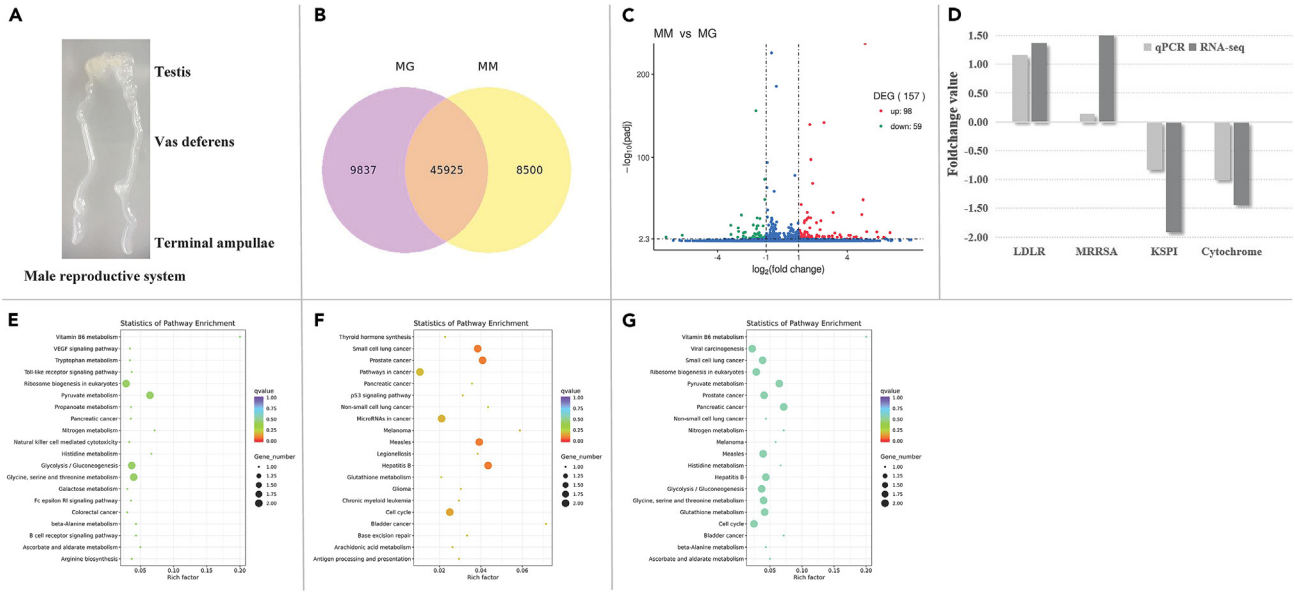

Figure 6. The comparative transcriptomic analysis of Mr-Masc knockdown in male

(A) The appearance of the male reproductive system which consists of the testis, vas deferens, and the terminal ampullae in *M. rosenbergii*.

(B) Venn diagram showing the numbers of expressed genes and DEGs in the transcriptomic library of the male reproductive system in the Mr-Masc RNAi group vs. the GFP RNAi group. MM: the Mr-Masc RNAi group of males; MG: the GFP RNAi group of males.

(C) The significantly differentiated upregulated and downregulated genes in the the Mr-Masc RNAi group vs. the GFP RNAi group in the male reproductive system. The red dots indicated the upregulated genes and the green dots indicated the downregulated genes, respectively.

(D) Verification of the expression patterns both in qPCR and RNA-seq. The four detected genes showed similar upregulated or downregulated expression patterns, respectively. The results indicated the reliability and accuracy of our transcriptome analysis. LDLR: low-density lipoprotein receptor; MRRSA: male reproductive-related serum amyloid A; KSPI: kazal-type serine protease inhibitor.

(E) Gene Ontology (GO) classification of DEGs in the ovarian transcriptome. Biological process (BP) enrichment of DEGs in the Mr-Masc RNAi group vs. the GFP RNAi group.

(F) Cellular component (CC) enrichment of DEGs.

(G) Molecular function (MF) enrichment of DEGs.

in both groups, respectively. As a result, 46229 unigenes were significantly differentially expressed in the Mr-Masc RNAi group and 47349 unigenes were found differentially expressed in the GFP RNAi group, respectively (Figure 5B). Differential gene expression (DGE) analysis between the Mr-Masc silencing and the control group generated enriched 125 genes of which, 47 unigenes were upregulated and 78 unigenes were downregulated in females (Figure 5C). To gain insights into the biological processes being operative during Mr-Masc knockdown including other differentially expressed genes, we have subjected transcripts (with significant differentially expressed) to gene ontology (GO) enrichment analysis. The GO terms were significant (adjusted p value < 0.05) in biological process (Figure 5D), cellular component (Figure 5E), and molecular function (Figure 5F). Within three main categories of GO classification, ovarian steroidogenesis of biological process, and steroid biosynthesis were found to be dominant. Furthermore, four DEGs (two upregulated unigenes, low-density lipoprotein receptor (LDLR) and insulin-like growth factor binding protein (IGFBP), and two downregulated unigenes, forkhead, and cathepsin were chosen to confirm their expressions in the female transcriptomic profiling. As a result, all the detected genes showed similar trends of expression patterns with those of RNA-seq (Figure 5G), which indicated the reliability and accuracy of our transcriptome analysis.

On the other hand, the male reproductive system consists of testis, vas deferens, and the terminal ampullae in *M. rosenbergii* (Figure 6A). The transcriptomic libraries of the male reproductive system obtained about 53.02 million clean reads and 7.95G clean bases in the Mr-Masc RNAi group, while 55.69 million clean reads and 8.35G clean bases in the GFP RNAi group. The values of Q20 and Q30 were more than 97.8% and 93.5% in both groups, respectively. There were 54425 significantly differentially expressed unigenes enriched in the the Mr-Masc RNAi group and 55762 unigenes found differentially expressed in the GFP RNAi group, respectively (Figure 6B). DGE analysis generated 157 genes of which, 98 unigenes were upregulated and 59

Table 2. Nucleotide sequences and positions of primers used in PCRs

Gene Description	Gene ID	Primer description	Primer sequence (5′-3′)	Primer length (bp)
Mr-Masc qPCR	GenBank: OQ786857.1	Mr-Masc-qF	GCAAAGCAAGTGGAAGTGTGACA	23
		Mr-Masc-qR	CACTTGTTGCAACAGGGATAGTAG	24
Mr-Masc dsRNA	GenBank: OQ786857.1	Mr-Masci F	CGCGGATCCTCACAGCCCCTCCC AATTACA	30
		Mr-Masci R	CCCAAGCTTATAGGACACAAGATG ACCACCAGA	33
Mr-18S qPCR	GenBank: DQ642856.1	Mr-18S-qF	GAGAAACGGCTACCACATCCA	21
		Mr-18S-qR	GTGCTCATTCCAATTACGCAGACT	24
Insulin-like growth factor binding protein	Cluster-11746.11665	IGFBP-qF	AAAGGAGAGAGGGAGAAAGAGT	22
		IGFBP-qR	ACATGGGACACAGGACAGG	19
Low-density lipoprotein receptor	Cluster-11746.7113	F-LDLR-qF	CTCCAATAAAGACCCAATGCT	21
		F-LDLR-qR	GCTAAGGAGTAGGATGAACCAA	22
Peptidase C1-like family/cathepsin L	Cluster-11746.36371	Cath-qF	TCATCAAGAACTCGTGGG	18
		Cath-qR	CATTAGGCAAGGGCATAG	18
Forkhead	Cluster-5279.1	Fork-qF	GTAATCGTGCTGCGGTCA	18
		Fork-qR	GCATTCTCCTGTATCCAAAG	20
Low-density lipoprotein receptor	Cluster-19843.18391	M-LDLR-qF	TGGAGGTCTGACTCGCAAT	19
		M-LDLR-qR	TTTCTCCGAACACCTACTGG	20
Male reproductive-related serum amyloid A	Cluster-19843.23876	MRRSA-qF	AGGATTACCACCTCGTCGTC	20
		MRRSA-qR	GAGAAGCCAACTGGAAGAACT	21
Cytochrome *b*559	Cluster-19843.12112	Cyt-qF	AGAATCGGTTGGGTATGG	18
		Cyt-qR	ATTCATCACAACTCTTGCTACT	22
Kazal-type serine protease inhibitor	Cluster-19843.29031	KSPI-qF	TTCCTTCACGCTCAGACG	18
		KSPI-qR	TTGTGGTGTAAGCCCGA	17

F and R indicated the forward and reverse directions, respectively.
The underlines represented the restriction sites for *Bam*H I and *Hind* III restriction enzymes, respectively.

downregulated in males (Figure 6C). The GO terms were significant (adjusted p value < 0.05) in biological process (Figure 6D), cellular component (Figure 6E), and molecular function (Figure 6F). Furthermore, four DEGs (two upregulated unigenes, LDLR and male reproductive-related serum amyloid A (MRRSA), and two downregulated unigenes, KSPI and cytochrome were selected to confirm their expressions in the male transcriptomic profiling. As a result, all the detected genes showed similar trends of expression patterns with those of RNA-seq (Figure 6G), which indicated the reliability and accuracy of our transcriptome analysis.

In addition, the highest numbers of significantly up- and downregulated unigenes were focused on various ZnF proteins in the female transcriptomic library (Table 3). Meanwhile, the highest numbers of significantly differentially expressed unigenes were focused on ZnF proteins and integrase homologs in the male transcriptomic library (Table 4). Despite subjecting transcripts (with significant differentially expressed) to DEG enriched KEGG pathway analysis, low percentage of up- and downregulated unigenes, belonging to the categories of response to various pathways, were identified.

The response of sexual-related candidate genes to Mr-Masc silencing

To gain insights into the biological processes being operative during sexual differentiation, the effect of Mr-Masc on the crucial signal pathways or the expression levels of related genes was evaluated from the comparative transcriptomic profiling of *M. rosenbergii*. The influences of some significant differentially upregulated and downregulated transcripts were illustrated in Figure 7.

Firstly, upregulated LDLR unigene, downregulated heat shock cognate 70 (Hsc70), significant differentially expressed epidermal growth factor (EGF) homologs were enriched in the endocytosis (Figure 7A).

Table 3. The primary significantly differential expression genes and sexual-related transcripts in comparative transcriptomic analysis of Mr-Masc silenced females

Gene Description	Gene ID	FM Readcount	FG Readcount	log2FoldChange	p Value	p Value	Results	GenBank No.	NR Evalue	Species
C2H2-type zinc finger	Cluster-11746.12527	13.66354028	307.5037848	−4.4891	1.81E-06	0.0037799	Down	KFM62220.1	4.90E-15	*Stegodyphus mimosarum*
C2H2-type zinc finger	Cluster-11746.3724	35.78292152	477.3986816	−3.7403	3.56E-05	0.030336	Down	XP_018024654.1	3.10E-46	*Hyalella azteca*
C2H2-type zinc finger	Cluster-11746.8947	178.6860626	26.44987928	2.7531	3.79E-05	0.031928	Up	CAA36225.1	1.40E-109	*Drosophila melanogaster*
CCCH-type zinc finger	Cluster-15032.0	187.5980442	13.80732579	3.7683	4.85E-05	0.03532	Up	OWF42210.1	7.00E-23	*Mizuhopecten yessoensis*
C4-type zinc finger	Cluster-11746.11139	179.0549625	716.9016251	−2.0006	4.06E-05	0.032651	Down	XP_011333567.1	5.60E-22	*Ooceraea biroi*
P-11 zinc finger	Cluster-11746.22948	45.85387807	720.1035361	−3.9718	6.12E-05	0.041497	Down	–	–	–
Ligand-gated ion channel	Cluster-17959.0	66.63742911	363.9433888	−2.4483	5.71E-08	0.000279	Down	XP_018019483.1	2.20E-78	*Hyalella azteca*
Ligand-gated ion channel	Cluster-17431.0	0	20.93106443	−6.7989	1.12E-05	0.013423	Down	CEF34393.1	5.90E-35	*Coenobita clypeatus*
Neurotransmitter-gated ion-channel	Cluster-11746.22149	260.9974577	36.12266557	2.856	4.37E-06	0.0066761	Up	AGK89909.1	4.10E-94	*Pandalopsis japonica*
Trypsin	Cluster-11746.42212	974.0923722	8.048326828	6.9224	8.12E-12	2.97E-07	Up	XP_018802350.1	2.90E-100	*Bactrocera latifrons*
Trypsin	Cluster-11746.11099	4.594592437	494.2815738	−6.7373	7.05E-10	8.61E-06	Down	PFX20186.1	1.50E-30	*Stylophora pistillata*
Trypsin	Cluster-10398.0	156.2085996	2.135657969	6.1752	2.85E-06	0.0056034	Up	XP_013112103.1	4.00E-101	*Stomoxys calcitrans*
Secretin	Cluster-11746.42508	75.71956736	2.373762026	5.0363	7.15E-07	0.002016	Up	–	–	–
Secretin	Cluster-11746.21024	3.165079842	38.00218351	−3.6116	2.73E-05	0.025551	Down	–	–	–
Proteasome subunit A	Cluster-11746.44354	156.9753955	20.26053623	2.9473	5.33E-09	3.30E-05	Up	–	–	–
Proteasome assembly chaperone 4	Cluster-11746.20370	1859.836103	168.2928513	3.4669	3.43E-05	0.029587	Up	–	–	–
BESS motif	Cluster-11746.28109	2.243413355	111.8745582	−5.6116	1.85E-05	0.019407	Down	XP_022164934.1	7.90E-29	*Myzus persicae*
BESS motif	Cluster-11746.3446	2.286261093	93.93209726	−5.3421	6.35E-05	0.042292	Down	XP_022160391.1	4.30E-26	*Myzus persicae*

(*Continued on next page*)

Table 3. *Continued*

Gene Description	Gene ID	FM Readcount	FG Readcount	log2FoldChange	p Value	p Value	Results	GenBank No.	NR Evalue	Species
Sexual-related significantly differential expression transcripts										
Epidermal growth factor	Cluster-13419.0	83.10714073	0.701624432	6.9737	3.70E-06	0.0060993	Up	CEL71274.1	1.90E-13	*Neospora caninum Liverpool*
Epidermal growth factor	Cluster-11746.4250	156.3967339	23.8962534	2.7089	1.53E-05	0.017516	Up	XP_022310806.1	8.20E-11	*Crassostrea virginica*
Epidermal growth factor	Cluster-11746.9347	23.90006249	172.1020025	−2.8517	1.77E-05	0.019241	Down	BAM18606.1	2.80E-10	*Papilio xuthus*
Insulin-like growth factor binding protein	Cluster-11746.11665	1256.574839	92.33478461	3.767	3.69E-06	0.0060993	Up	XP_019631600.1	4.50E-13	*Branchiostoma belcheri*
Low-density lipoprotein receptor	Cluster-11746.7113	124.2894118	11.58427703	3.4284	9.84E-07	0.0025551	Up	XP_002740307.1	1.40E-15	*Saccoglossus kowalevskii*
Sex-lethal	Cluster-11746.10941	1128.513155	256.961302	2.135	4.77E-05	0.03532	Up	AGI44577.1	1.50E-22	*Macrobrachium nipponense*
Peptidase C1-like family/cathepsin L	Cluster-11746.36371	14.96640852	346.2732302	−4.5324	1.42E-06	0.0033007	Down	AGJ03550.1	1.10E-139	*Palaemon carinicauda*
Forkhead	Cluster-5279.1	4.430214901	124.6726288	−4.8162	9.49E-06	0.012168	Down	KZS06335.1	2.80E-77	*Daphnia magna*
Transforming growth factor beta-2	Cluster-22584.0	2.761807837	37.82200809	−3.7895	9.96E-06	0.012366	Down	KXJ22303.1	2.50E-08	*Exaiptasia pallida*
Heat shock protein 21	Cluster-11746.28442	27.11344796	320.4945037	−3.562	3.49E-09	2.56E-05	Down	AET34915.1	1.10E-30	*Macrobrachium rosenbergii*

FM: the Mr-Masc RNAi group in females; FG: GFP RNAi group in females.

Table 4. The primary significantly differential expression genes and sexual-related transcripts in comparative transcriptomic analysis of Mr-Masc silenced males

Gene Description	Gene ID	MM Readcount	MG Readcount	log2FoldChange	p Value	q Value	Results	GenBank No.	NR Evalue	Species
C3HC4-type zinc finger	Cluster-19843.14206	53.86925595	17.66501984	1.6086	8.41E-06	0.0014355	Up	XP_018017284.1	2.60E-30	*Hyalella azteca*
C3HC4-type zinc finger	Cluster-19843.25697	76.30641643	30.37373982	1.329	3.95E-06	0.00074078	Up	XP_018014480.1	1.70E-07	*Hyalella azteca*
C2H2-type zinc finger	Cluster-19843.16467	107.4066811	45.69871807	1.2329	2.32E-07	5.63E-05	Up	KNC87863.1	2.20E-19	*Sphaeroforma arctica JP610*
C2H2-type zinc finger	Cluster-19843.30045	47.84991097	8.534269324	2.4872	3.00E-08	8.81E-06	Up	–	–	–
C2H2-type zinc finger	Cluster-19843.17039	57.51418803	144.0272656	−1.3244	1.33E-09	5.04E-07	Down	XP_022309566.1	4.00E-64	*Crassostrea virginica*
B-box zinc finger	Cluster-19843.21162	48.80422046	102.8700636	−1.0757	1.53E-05	0.0024765	Down	XP_021924291.1	5.90E-43	*Zootermopsis nevadensis*
DBF zinc finger	Cluster-19843.30651	205.2536994	23.43115761	3.1309	4.93E-38	1.43E-34	Up	KOX74132.1	4.00E-46	*Melipona quadrifasciata*
Integrase	Cluster-19843.23266	185.3926065	92.02328256	1.0105	6.02E-09	1.98E-06	Up	KXZ75731.1	5.30E-163	*Tribolium castaneum*
Integrase	Cluster-19843.17272	146.7435455	57.78572112	1.3445	1.09E-10	4.99E-08	Up	XP_006815925.1	3.10E-154	*Saccoglossus kowalevskii*
Integrase	Cluster-19843.19249	68.96968886	239.5826345	−1.7965	8.37E-23	1.10E-19	Down	XP_006817263.1	0.00E+00	*Saccoglossus kowalevskii*
Integrase	Cluster-19843.22128	42.224408	243.7937917	−2.5295	4.75E-35	1.25E-31	Down	PIK39827.1	2.50E-140	*Apostichopus japonicus*
Integrase	Cluster-19843.25178	38.30018892	125.6675746	−1.7142	5.77E-12	2.95E-09	Down	KXJ06669.1	1.80E-247	*Exaiptasia pallida*
Integrase	Cluster-19843.21844	185.7144966	424.0237949	−1.1911	1.26E-21	1.45E-18	Down	XP_022290521.1	4.10E-183	*Crassostrea virginica*
Integrase	Cluster-19843.22507	105.0649305	298.0556855	−1.5043	6.07E-22	7.27E-19	Down	XP_021366095.1	4.80E-169	*Mizuhopecten yessoensis*
Integrase	Cluster-19843.28546	221.3477313	7.020124766	4.9787	1.48E-53	5.43E-50	Up	XP_022292871.1	7.80E-56	*Crassostrea virginica*
Lipoprotein leucine-zipper	Cluster-19843.11132	31.46996489	2.550645332	3.625	8.37E-08	2.24E-05	Up	–	–	–
Lipoprotein leucine-zipper	Cluster-19843.14088	197.8005233	38.19039639	2.3728	5.36E-28	9.24E-25	Up	–	–	–

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Table 4. *Continued*

Gene Description	Gene ID	MM Readcount	MG Readcount	log2FoldChange	p Value	q Value	Results	GenBank No.	NR Evalue	Species
Lipoprotein leucine-zipper	Cluster-19843.13503	377.7475066	123.8386715	1.609	3.95E-32	9.47E-29	Up	XP_021346730.1	2.70E-10	*Mizuhopecten yessoensis*
leucine zipper	Cluster-19843.20431	43.73918497	102.9618299	−1.2351	1.25E-06	0.00026439	Down	–	–	–
ATPase family	Cluster-19843.22863	52.82784678	112.0274263	−1.0845	5.54E-06	0.00099525	Down	–	–	–
ATPase family	Cluster-19843.14610	147.5501642	72.58671359	1.0234	1.59E-07	4.01E-05	Up	XP_018015187.1	0.00E+00	*Hyalella azteca*
ATPase family	Cluster-19843.28488	168.6202385	72.75235183	1.2127	1.56E-10	7.02E-08	Up	PAA84769.1	1.20E-109	*Macrostomum lignano*
anti-lipopolysaccharide factor 1	Cluster-19843.22118	384.945064	139.6215638	1.4631	9.23E-29	1.70E-25	Up	AOF80307.1	3.70E-44	*Macrobrachium rosenbergii*
anti-lipopolysaccharide factor 1	Cluster-19843.15381	67.17704499	23.26276638	1.5299	1.58E-06	0.00032467	Up	AOF80303.1	7.60E-31	*Macrobrachium rosenbergii*
anti-lipopolysaccharide factor 1	Cluster-19843.9009	42.70724316	5.964811893	2.8399	1.96E-08	6.11E-06	Up	ADI80707.1	6.60E-57	*Macrobrachium rosenbergii*
Histone 1	Cluster-19843.21687	22.39029707	66.20941201	−1.5642	2.97E-06	0.00058267	Down	CDI59403.1	4.10E-22	*Macrobrachium rosenbergii*
Histone 1	Cluster-19843.21889	668.2059903	2075.295707	−1.635	3.92E-161	5.40E-157	Down	CDI59403.1	1.80E-23	*Macrobrachium rosenbergii*
Fibrinogen alpha/beta chain family	Cluster-19843.27432	1001.633016	169.138206	2.5661	9.37E-147	1.03E-142	Up	EAY20417.1	1.40E-08	*Trichomonas vaginalis G3*
Fibrinogen alpha/beta chain family	Cluster-19843.18740	202.4698233	62.58785353	1.6938	3.47E-19	3.54E-16	Up	–	–	–
BTB/POZ domain	Cluster-19843.10586	110.7207291	36.43123572	1.6037	1.86E-10	8.07E-08	Up	XP_018020163.1	6.70E-36	*Hyalella azteca*
BTB/POZ domain	Cluster-19843.3565	18.12998684	1.14707921	3.9823	2.70E-05	0.0041722	Up	XP_015602328.1	1.40E-10	*Cephus cinctus*
Ras family	Cluster-19843.10591	159.1935921	55.19745159	1.5281	1.54E-13	1.00E-10	Up	XP_023234890.1	1.50E-54	*Centruroides sculpturatus*

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Table 4. *Continued*

Gene Description	Gene ID	MM Readcount	MG Readcount	log2FoldChange	p Value	q Value	Results	GenBank No.	NR Evalue	Species
Sexual-related significantly differential expression transcripts										
Insulin-like growth factor binding protein	Cluster-19843.29094	87.61706671	33.81451862	1.3736	4.03E-07	9.38E-05	Up	AGV98853.1	6.00E-15	*Androctonus bicolor*
Low-density lipoprotein receptor	Cluster-19843.18391	411.1279839	158.8874474	1.3716	5.56E-28	9.29E-25	Up	AHA83583.1	1.30E-76	*Marsupenaeus japonicus*
Crustacean calcium-binding protein	Cluster-19843.8907	23.10034877	2.707106936	3.0931	1.70E-05	0.0027036	Up	P80363.1	5.40E-40	
Male reproductive-related serum amyloid A	Cluster-19843.23876	383.8582115	132.413318	1.5355	1.23E-30	2.51E-27	Up	ABQ41247.1	1.90E-25	*Macrobrachium rosenbergii*
Flotillin	Cluster-19843.25954	330.4107263	157.4159742	1.0697	4.52E-16	3.66E-13	Up	AEN94568.1	4.80E-185	*Scylla paramamosain*
Heat shock protein	Cluster-19843.21609	5.964434314	32.48528323	−2.4453	9.82E-06	0.0016464	Down	AKB96227.1	5.70E-297	*Cherax destructor*
Cytochrome *b*559	Cluster-19843.12112	25.29488191	68.6334198	−1.4401	7.88E-06	0.0013531	Down	–	–	–
Kazal-type serine protease inhibitor	Cluster-19843.29031	13.06400462	49.29274665	−1.9158	3.13E-06	0.00061069	Down	ATU83053.1	4.60E-63	*Pristhesancus plagipennis*
Cell cycle-related significantly differential expression transcripts										
Cyclin-dependent kinase inhibitor	Cluster-19843.25635	54.48463409	12.70963765	2.0999	8.81E-08	2.34E-05	Up	–	–	–
Cyclin-dependent kinase	Cluster-19843.23048	56.6147892	21.01449113	1.4298	2.72E-05	0.0041904	Up	KXJ26870.1	6.40E-65	*Exaiptasia pallida*
Cell division protein FtsL	Cluster-19843.9761	80.94589426	31.79703571	1.3481	1.56E-06	0.00032255	Up	XP_024220371.1	2.70E-188	*Bombus impatiens*
Cyclin E	Cluster-19843.23974	43.17114361	95.80405563	−1.15	1.03E-05	0.001705	Down	AGW23550.1	9.00E-180	*Penaeus monodon*
E2F transcription factor	Cluster-19843.24803	101.679404	209.2295421	−1.0411	1.99E-09	7.26E-07	Down	ARW29624.1	7.00E-183	*Penaeus monodon*
PCNA-associated factor	Cluster-19843.13219	55.80959057	121.3169326	−1.1202	1.19E-06	0.00025415	Down	XP_022251473.1	5.70E-08	*Limulus polyphemus*

MM: the Mr-Masc RNAi group in males; MG: the FP RNAi group in males.

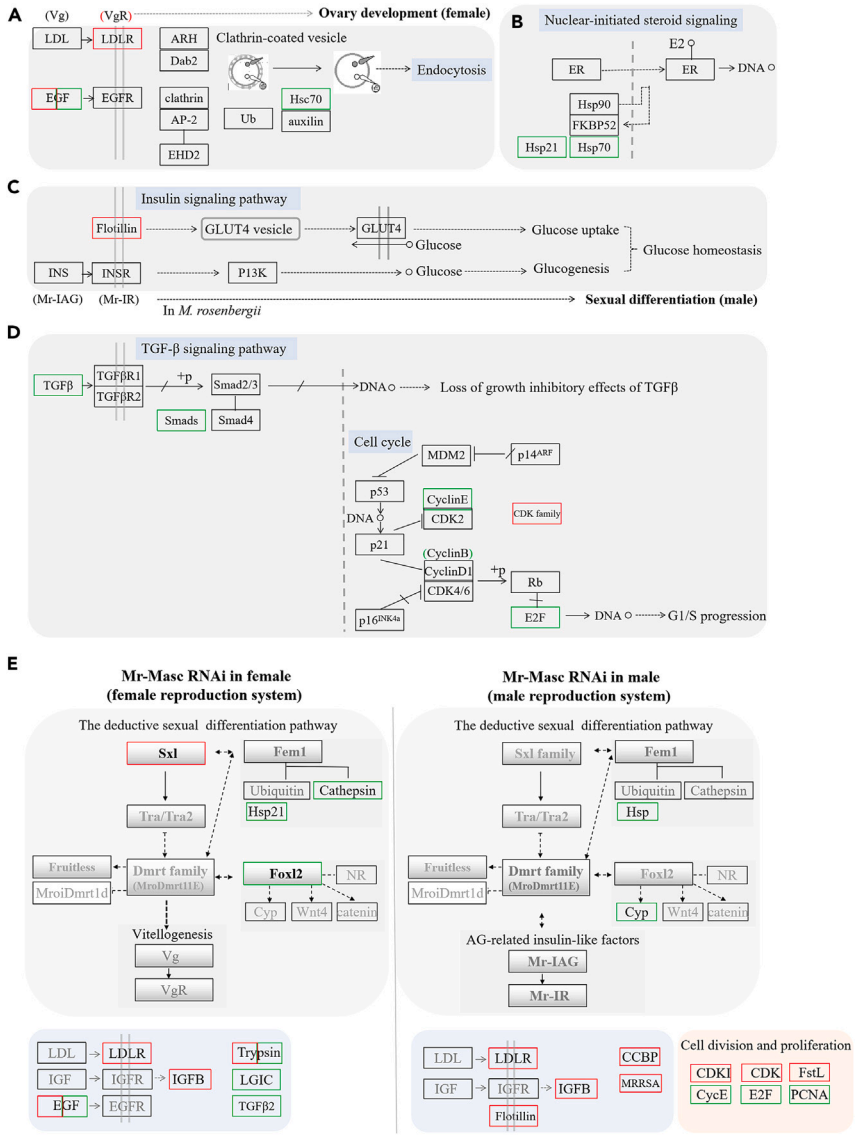

Figure 7. The gonad-related candidate unigenes enriched in putative sexual differentiation pathway and reproduction by the comparative transcriptomic analysis of Mr-Masc knockdown

(A) The summary of endocysis which may participate in ovary development. The upregulated transcripts (red color rectangle) and the downregulated transcripts (green color rectangle) were briefly profiled in the signaling pathway.

(B) The brief illustration of nuclear-initiated steroid signaling.

(C) The diagrammatic sketch of the insulin signaling pathway which was possibly involved in male sexual differentiation.

(D) The simple process of TGF-β signaling pathway and cell cycle. The significantly differentially expressed unigenes in brackets were indicated as the possible candidate homologs in *M. rosenbergii*.

(E) The comparison of the transcriptomic profiling of the female and male reproductive system after Mr-Masc knockdown. There were significant similarities and differences of upregulated and downregulated homologs found in the deductive regulation axis or networks of sexual differentiation and gonad development. The upregulated transcripts (red color rectangle) and the downregulated transcripts (green color rectangle) were briefly illustrated. The genes with gray color font indicated the possible homologs involving in the deductive regulation axis or networks. CCBP: crustacean calcium-binding protein; CDK: cyclin-dependent kinase; Dmrt: doublesex and mab-3-related transcription factor; E2F: E2 transcription Factor; EGF: epidermal growth factor; EGFR: EGF receptor; Foxl2: forkhead box L2; Fru: fruitless; Hsc70: heat shock cognate 70; Hsp70: heat shock protein 70; IAG: insulin-like AG-related factors; IGF: insulin-like growth factor; IGFBP: insulin-like growth factor binding protein; IGFR: IGF receptors; IR: insulin-like receptor; LDL: low-density lipoprotein; LDLR: low-density lipoprotein receptor; LGIC: ligand-gated ion channel; MRRSA: male reproductive-related serum amyloid A; PCNA: proliferating cell nuclear antigen; Sxl: sex-lethal; TGFβII: transforming growth factor beta-2; Tra/Tra2: transformer/Transformer 2; Vg: vitellogenin; VgR: Vg receptor.

Meanwhile, the downregulated heat shock protein 70 (Hsp70) unigene that participated in nuclear-initiated steroid signaling was enriched (Figure 7B). That meant the Mr-Masc knockdown had significant effects on the endocytosis process and the steroid hormone signaling in ovarian development.

Then, the upregulated flotillin unigene was enriched in the insulin signaling pathway which is important for glucose homeostasis and male sexual differentiation in crustaceans (Figure 7C). The downregulated transforming growth factor beta-2 (TGFβII) unigene and Smad homologs in the TGF-β signaling pathway were also identified (Figure 7D). As for the cell cycle, downregulated Cyclin B, Cyclin E, and E2F transcription factor, and upregulated Cyclin-dependent kinase (CDK) members were enriched (Figure 7D cell cycle).

Moreover, further analysis provided novel clues to clarify the potential role of Mr-Masc in the regulation axis or networks of sexual differentiation and gonad development. In comparison, there were significant similarities and differences of upregulated and downregulated homologs found in the transcriptomic profiling of the female and male reproductive system after Mr-Masc knockdown (Figure 7E).

DISCUSSION

Zinc finger proteins involve in sexual differentiation in insects and crustaceans

A novel transcription factor related to sexual differentiation, named as Mr-Masc, was identified in *M. rosenbergii* and characterized as ZnF protein in the present study. ZnF proteins are generally thought of as DNA-binding transcription factors, however, they are now recognized to bind DNA, RNA, protein, and/or lipid substrates.[33,34] ZnF domains are relatively small protein motifs which contain multiple finger-like protrusions that make tandem contacts with their target molecule.[34] Meanwhile, ZnF domains are often found in clusters, where fingers can have different binding specificities.[34] In the present study, the deduced protein of Mr-Masc was similarly predicted to encode three C3H-tandem ZnF domains having a high degree of shared identities with the ZnF proteins characterized in other species. The N-terminus of Mr-Masc contained three C3H ZnF domains probably responsible for the binding capacity of the protein, and the C-terminus may present transcriptional activation activity. Moreover, there was a monopartite nuclear localization signal presented in its structure. Consistent with its role as a transcription factor, Mr-Masc has been shown to be a nuclear protein.

C3H-type ZnF proteins commonly bind RNA by recognizing specific sequences or secondary structures in their mRNA targets or interacting with microRNA (miRNA) processing and effector pathways.[33] For instance, the lepidopteran-specific C3H-tandem ZnF protein gene (BmMasc) is required for masculinization and dosage compensation in embryonic *B. mori*.[7,27] Masc and Fem piRNA coordinate in a ping-pong amplification partnership in regulating Bmdsx splicing to generate the female isoform, which prevail in the regulation of sexual differentiation in the silkworm.[7] Due to the similar molecular characteristics of Mascs and the use of the similar putative WZ-ZZ sex-determining system in *B. mori* and *M. rosenbergii*, we suggested that there may be a similar piRNA in *M. rosenbergii*. While the existence of such piRNA could neither be confirmed nor denied, it was difficult to predict the sequence directly by bioinformatics in *M. rosenbergii*.

Nevertheless, evidence for the importance and evolutionary conservation of Masc proteins in the context of sexual differentiation was scarce in crustaceans. The available data were reported from brine shrimp Artemia. Ar-Masc was identified in brine shrimp *A. franciscana* and proved to be essential for male-specific cleavage as well as participated in the sex determination process in Artemia, in the case of Ar-Masc silencing altered the ratio of males to females in offspring and caused a high female-male ratio of progeny.[31] Moreover, the phylogenetic analysis showed that the Mr-Masc was clustered into a separate branch with the Masc proteins of Artemia among the reported Masc genes in the present study. It suggested that there was a closed evolutionary relationship between the Mr-Masc and the Ar-Mascs. To our knowledge, Mr-Masc was the first functional Masc homolog that has been identified to date in the decapod. Thus, we carried out *in vivo* knockdown of the Mr-Masc to evaluate its potential function in sexual differentiation.

The sexual differentiation and sex reversal in crustaceans

It is known that the sexual differentiation of crustaceans is primarily regulated by the male-specific AG. The IAGs and their receptors are known as vital factors in regulating male sexual differentiation and maintaining male characterization over the past several decades. For instance, Mr-IAG[11,13] and its receptor Mr-IR[14,15] participated in the male sexual differentiation whereas either of their silencing caused a full and functional

sex reversal in *M. rosenbergii*. Silencing Cq-IAG feminized male-related phenotypes in *Cherax quadricarinatus*.[10] However, the mechanism of this insulin-like signal pathway involved in sexual differentiation is still unclear. The absence of an underlying common effector that triggers sexual differentiation and male reproductive physiology is puzzling. In the present study, the most interesting finding was that silencing the Mr-Masc transcript in male individuals caused complete and functional sex reversal into neo-female individuals which produced all-male progeny. This is another novel finding of a single gene manipulation inducing full functional sex reversal in *M. rosenbergii*. It indicated that Mr-Masc played a crucial role in the complex regulatory axis or network of sexual differentiation. The study suggested a new viewpoint concerning the evolution of Masc proteins emerging in the cascade of sexual differentiation in crustaceans.

On the other hand, recent research documented that a member of Dmrt gene, MroDmrt11E, played a crucial role in sexual differentiation in the prawn *M. rosenbergii*. It was reported that *in vivo* knockdown of MroDmrt11E at the postlarva stage in male prawns also induced a complete and functional sex reversal and achieved the production of an all-male monosex population.[24] Meanwhile, MroDmrt11E silencing was continuously related to the decrease of the Mr-IAG transcript and the increase in the expression of MroiDmrt1d, another member of the Dmrt family.[24] Moreover, Sv-iDMY, the heterogametic sex-linked (Y-linked) Dmrt gene, was identified in an invertebrate species, the Eastern spiny lobster, *Sagmariasus verreauxi*.[35] It is demonstrated that the dominant negative suppression of iDMY over its autosomal iDmrt1 paralog by employing a GAL4-transactivation assay, suggesting the mechanism with which iDMY determines sex.[36] Structurally, both MroDmrt11E and Sv-iDMY share the unique DNA binding motif, the DM domain. DMRT genes encode a deeply conserved family of transcription factors and they appear to have controlled sexual differentiation for hundreds of millions of years.[37]

In the present study, Mr-Masc was known as transcription factor and participated in sexual differentiation via transcriptional regulation and signaling modulation. Our result provides a novel proof of concept for biotechnology of crustacean monosex population culture based on Mr-Masc gene manipulation in commercial decapod. It is probably an interesting event during the Masc evolutionary history of the decapod and is likely to have similarly occurred in different species. Notwithstanding, how the Mr-Masc is recruited into the decapod sexual differentiation pathway remained unclear.

The comparative transcriptomic analysis of the reproductive system in Mr-Masc knockdown

To elucidate the mechanism of sexual differentiation in crustaceans, the effect of Mr-Masc knockdown-mediated gene expression was evaluated by the comparative transcriptomic analysis of the reproductive system. It is noteworthy that several reproduction and sexual differentiation-related genes have been enriched as significantly differentially expressed unigenes by DEG analysis. Some of them were probably considered to involve in the gonad development and sexual regulation cascades in *M. rosenbergii*.

Insulin-like signal pathway in sexual differentiation in crustaceans

In crustaceans, some IAGs and IAG receptors regulate male sexual differentiation and are involved in insulin-like signal pathways. In the present study, there are several crucial elements of the insulin-like signal pathway have been identified in Mr-Masc knockdown prawns. Firstly, three significantly expressed EGF unigenes and one upregulated insulin-like growth factor binding protein (IGFBP) homolog were enriched in the ovary library of Mr-Masc knockdown. Meanwhile, two upregulated transcripts, follitin, and IGFBP homologs were identified in the male reproductive system library of Mr-Masc silencing.

Epidermal growth factor (EGF). EGF, a member of the EGF family of ligands and leading to activation of the EGF receptor (EGFR), in several mammalian species, is believed to mediate the luteinizing hormone signal as it induced oocyte maturation and cumulus expansion *in vitro*.[38] The previous studies have not only implicated EGF-like peptide signaling in granulosa and cumulus cells critical for oocyte meiotic maturation but also provided insight into how this signaling network coordinates oocyte cytoplasmic maturation and thus influences the developmental capacity of the oocyte.[38] Moreover, it is reported that the temporary silencing of epidermal growth factor receptors Mr-EGFR resulted in a significant reduction in growth and a delay in the appearance of a male secondary sexual characteristic, namely the appendix masculina in *M. rosenbergii*.[39] Meanwhile, Mr-EGFR-silenced individuals developed abnormal eyes that presented irregular organization of the ommatidia.[39] In the present study, three significantly expressed EGF unigenes were enriched in the ovary library of Mr-Masc knockdown, which indicated the Mr-Masc may play various roles in regulating the expression of EGF unigenes in the ovary development in females.

Insulin-like growth factor binding protein (IGFBP). Insulin-like growth factor (IGF) and insulin signaling systems are ancient and involved in growth, development, cell differentiation, and metabolism.[40] The IGF system comprises ligands of IGF-I/II, IGF receptors (IGFR), IGF binding proteins (IGFBPs), and IGFBP hydrolases.[40] IGFBPs can bind to IGFs and have wider functions through IGF-independent mechanisms.[40] IGFs play a key role in sensitizing ovarian granulosa cells to follicle-stimulating hormone (FSH) action during terminal follicular growth.[40] Concentrations of IGFBPs in follicular fluid strongly decrease during follicular growth, leading to an increase in IGF bioavailability.[41]

It is known that ZnF proteins play a significant role in gene regulation.[33,34] For instance, pleomorphic adenoma gene 1 (PLAG1) belongs to the PLAG family of ZnF transcription factors and is involved in cell proliferation by directly regulating a wide array of target genes, including a number of growth factors, such as IGF2.[42] In detail, IGF2 is a direct target of PLAG1, but IGF1 also appears to have a connection to PLAG1.[42] It suggested an important role for PLAG1 in postnatal growth and reproduction, as PLAG1 deficiency causes growth retardation and reduced fertility in mice.[42] These data have led to the conclusion that PLAG1 exhibits biological functions, at least in part, via the mitogenic action of IGF2, most likely via the activation of IGF1R and the Ras/Raf/MAPK signaling pathway.[42] Significantly, Mr-Masc knockdown generated the upregulated transcripts of IGFBP homologs in both the transcriptomic analyses of the ovary and the male reproductive systems in the present study.

It is known that IAGs and IGFs belong to the insulin-like peptide superfamily. In *M. rosenbergii*, Mr-IAG has a high similarity with insulin in structure,[12] is bound to its receptor Mr-IR,[15] and plays a pivotal role in the regulation of male sexual differentiation.[13,14] Moreover, the insulin-like androgenic gland hormone-binding protein from the oriental river prawn *Macrobrachium nipponense,* Mn-IAGBP, is prominently expressed in the testis.[43] Mn-IAGBP silencing significantly reduced the transcription of Mn-IAG, while Mn-IAG knockdown significantly reduced the transcription of Mn-IAGBP.[43] These results demonstrate the involvement of the IAGBP gene in IAG signaling in *M. nipponense*. Based on the above studies, it is deduced that a similar regulatory mechanism ([IGF and IGFBP]IGF receptor) might apply to IAG, i.e., (IAG and IAGBP)-IAG receptor signaling schemes may exist in crustaceans.[43] Despite there being no significant change of the Mr-IAG transcription observed in Mr-Masc silencing, the upregulated transcripts of IGFBP homologs were enriched in both ovary and male reproductive systems. It indicated that the Mr-Masc plays a role in regulating the expression of IGFBP homologs and possibly participated in the gonad development through insulin or insulin-like signal pathway.

Flotillin. Flotillins are considered scaffold proteins in lipid rafts and marker proteins of lipid microdomains.[44] Flotillin-dependent endocytosis (FDE) is an uptake mechanism, similar to the well-recognized clathrin-mediated endocytosis (CME) and results in the uptake of the cargo into vesicles enriched in flotillins, shortly after flotillins become phosphorylated by Fyn kinase.[45] Flotillin-1 and flotillin-2 are transmembrane proteins that mediate a peculiar endocytic pathway that is independent of clathrin.[45] It is reported that flotillin-1 stimulates the activation of glucose transporter 4 in response to insulin and is a regulator of insulin function.[46] In the clinic, polycystic ovary syndrome is a common endocrinopathy and its pathogenesis has been linked to the development of insulin resistance and hyperinsulinemia.[46] In the present study, the Mr-Masc has remarkably suppressed effects on the transcription of flotillin. It indicated that Mr-Masc played important roles indirectly or indirectly regulating glucose homeostasis through the insulin pathway or endocytosis process and participated in the gonad development.

Sexual differentiation related candidate genes

Sex-lethal (Sxl). In Drosophila, the primary sex determination and somatic sexual differentiation are controlled by a genetic hierarchy X: A > Sex-lethal (Sxl) > Transformer/Transformer 2 (Tra/Tra2) > Doublesex (Dsx) and Fruitless (Fru).[16,47,48] In Drosophila species, it is known that the action of Sxl results in the active splice variant of Tra in females. What is noteworthy is that several sexual differentiation-related candidate genes were probably considered to involve the sex regulation cascade in crustaceans. There are four Sxl isoforms, named MroSxl1, MroSxl2, MroSxl3, and MroSxl4, in both males and females in *M. rosenbergii*.[49] In the present study, one upregulated Sxl unigenes was enriched in Mr-Masc knockdown, which indicated that Mr-Masc had a significant promotion effect on the transcription of the Sxl gene and may play a complex regulatory role in sexual differentiation. Moreover, the Tra2 and Dsx (known as the Dmrt gene family in *M. rosenbergii*) homologs were also identified in the comparative transcriptomic library but they were not enriched as significantly differentiated transcripts in the present study.

Forkhead box L2 (Foxl2). Forkhead box L2 (Foxl2) maintains the fates of ovaries in adult mammals, and its mutations might have a dramatic effect on the gonadal phenotype.[20] Ablation of Foxl2 from the adult ovary caused transdifferentiation of granulosa cells to Sertoli-like cells and transformation of ovarian follicles into testis-like structures.[50] Foxl2 is essential for ovarian determination and FOXL2 mutations are often associated with female infertility.[51] In crustaceans, the identification of Mn-Foxl2 confirmed its important roles in early gonad development and may be involved in sexual differentiation in *M. nipponcuse*.[52] In mud crab *Scylla paramamosain*, knockdown of Spfoxl2 increased the expression of vitellogenin (Spvtg) in the ovary, suggesting Spfoxl2 might be the upstream negative regulator of Spvtg in ovary development.[53] In the present study, Mr-Masc silencing decreased the transcription of forkhead, which indicated that Mr-Masc probably had a promotional effect on the expressions of forkhead and may play a regulatory role in ovarian development.

Heat shock protein. Heat shock protein (Hsp) family is a universally conserved chaperone with important functions in protein folding and disaggregation. Hsp contributes to the interaction with steroid hormone receptors, temperature, estrogen signaling, etc., and several Hsps are critical for successful embryogenesis and reproduction.[54,55] It reported that two Hsps (Hsp27 and Hsp70), and heat shock protein 70 cognate3 (Hsc70) exhibited upregulated expression patterns in the testis in the transcriptomic database of gonads of *M. rosenbergii*.[56] In the present study, Hsp70 and Hsc70 homologs in the male reproductive system and heat shock protein 21 (Hsp21) homolog in the ovary were identified as downregulated transcripts in Mr-Masc knockdown, respectively.

It is known that Hsp70 and Hsp90 serve as the central chaperones that mediate this process in conjunction with a variety of co-chaperones and function as a complex of Hsp90-FKBP52-Hsp70 in the nuclear-initiated steroid signaling.[57] Steroid hormone receptors are ligand-dependent transcription factors that require the dynamic, ordered assembly of multimeric chaperone complexes to reach a functional conformation.[57] Meanwhile, heat shock cognate 70 (Hsc70) is known to be involved in CME by which cells take up various extracellular materials.[58] More specifically, Hsc70 promotes the disassembly of clathrin-coated vesicles (CCVs) by directly binding to clathrin during CME.[58] Therefore, it was indicated that Mr-Masc probably had promotion effects on the expressions of Hsps (Hsp70 and Hsp21) and Hsc70, and played a regulatory role in steroid signaling in gonad development.

Vitellogenesis and ovarian development

The process of yolk synthesis or vitellogenesis is the key event in oocyte development. In *M. rosenbergii*, vitellogenesis begins with the synthesis of vitellogenin (Vg) in the hepatopancreas, and then it is released into the hemolymph and cleaved into vitellin, which is taken up through receptor-mediated endocytosis (RME) and incorporated into the yolk granules in the late oocytes, i.e., Oc3 and Oc4 in ovary.[59,60,61] As Vg interacts with Vg receptor (VgR) during RME, a clathrin-coated pit is formed around the Vg-VgR complex allowing the oocyte membrane to further invaginate into a vesicle that is pinched-off.[62] In the present study, one LDLR homolog thought to be VgR homolog was identified as significantly upregulated transcripts in the comparative transcriptomic library of males.

In crustaceans, many researchs have been carried out to explore the function of Vg and VgRs in vitellogenesis during ovarian development. It was reported that MnVgR RNAi led to vitellin depletion in the oocytes and dramatically delayed the maturation of the ovary in *M. nipponense*.[63] In *P. monodon*, the VgR is likely to be involved in the endocytosis of extra ovarian Vg into developing egg cells, whereas VgR RNAi led to a decrease in VgR protein content in the ovary but an increase in the hemolymph level of Vg.[64] Moreover, Lv-VgR specifically mediated Lv-Vgs transport into oocytes during oocyte maturation and ovarian development in *P. vannamei*.[65] Lv-VgR silencing was effective in stunting ovarian development.[65] In the present study, the identified Vg and MrVgR of *M. rosenbergii* were not enriched as significantly differentiated transcripts but one LDLR homolog was generated as an upregulated transcript in Mr-Masc silencing. It suggested that Mr-Masc may have distinct effect on the vitellogenesis in ovarian development.

On the other hand, cathepisin homolog, a proteinase hydrolyzing vitellogenin into vitellin, was enriched as significantly downregulated transcripts in the ovary, which suggested that the Mr-Masc probably regulated vitellogenin degradation in ovarian development. Meanwhile, trypsin is a hydrolytic enzyme and could inhibit the expression of vitellogenin synthesis hormone (VSH) to regulate the release of gonadotropin (gonad hormone). In the present study, three trypsin homologs were enriched as significantly expressed

unigenes in the ovary. All of these results suggested that Mr-Masc participated in the vitellogenesis and ovarian development of female prawns.

Cell division and cell cycle pathway

As to the data of the male transcriptomic library of Mr-Masc knockdown, some critical regulators of cell cycle progression, such as upregulated transcripts, Cyclin-dependent kinase (CDK), Cyclin-dependent kinase inhibitor and cell division protein FtsL, and downregulated transcripts, Cyclin E, E2F transcription factor, and proliferating cell nuclear antigen (PCNA)-associated factor, were also enriched in the cell cycle signaling pathway. It indicated that Mr-Masc was involved in cell cycle progression and possibly participated in the spermatogenesis process in the male reproductive. Mr-Masc may be responsible for regulating reproduction and development by increasing or inhibiting key regulatory factors in gonad development.

In addition, Mr-Masc mRNA was highly expressed in thoracalia ganglia, intestines, and reproductive system. Mr-Masc may have various effects on several crucial signaling pathways. In the present study, several crucial elements involved in common signal pathways, such as upregulated Ras in the MAPK signaling pathway, upregulated Rac in the phosphatidylinositol 3-kinase/AKT/mammalian target of the rapamycin signaling pathway (PI3K/AKT/mTOR), were identified from the male transcriptomic library of Mr-Masc knockdown. Moreover, the downregulated transcriptions of TGF-βII and Smad homolog in females were enriched in the TGF-β signaling pathway. Thus, it suggested that the complex cross talk among the multiple interacting cell signaling cascades would further promote or repress the diverse physiological functions.

The comparative analysis of sexual differentiation gene silencing

This finding provided another sexual manipulation technique through silencing of Mr-Masc gene family for achieving a complete and functional sex reversal besides the knockdown of Mr-IAG or Mr-IR or MroDmrt11E. On one hand, we have compared the results of significantly up- and downregulated unigenes in both the Mr-Masc silencing group and the MroDmrt11E silencing group.[24] Several factors, namely, CDK, Hsp, Cyclin E, and PCNA were achieved and involved in the cell division and proliferation. Significantly, both the upregulated gene CDK and downregulated gene Hsp were enriched from two comparative transcriptomic analyses. They presented consisted change tendency in the Mr-Masc silencing group and the MroDmrt11E silencing group.[24] While, Cyclin E and PCNA were enriched but showed contrast regulated tendency, while they were upregulated in the MroDmrt11E silencing group[24] and downregulated in the Mr-Masc silencing group.

On the other hand, Dmrt gene family probably participated in the transcriptional activation of Mr-IAG and possibly played great roles in the switch of Mr-IAG-related regulatory signaling of male differentiation.[26] Moreover, Mr-IAG directly or indirectly influenced the expression of partial genes of Dmrt family.[66] These comparative analyses of gene knockdown offered new insights regarding the mechanisms in the sexual differentiation of crustaceans.

In conclusion, the present study documented the Mr-Masc silencing in male postlarvae resulted in functional sex reversal females and provided new insight into the Mr-Masc evolution in sexual differentiation in *M. rosenbergii*. The comprehensive analysis of gene transcriptions, and the related signaling pathways in Mr-Masc knockdown gave useful clues for the understanding of the molecular mechanisms of sexual differentiation in *M. rosenbergii*.

Limitations of the study

One concern about the findings was that sexual reversal and gene expression of the male postlarvae were affected by the silence of Mr-Masc, so the major limitation of the present study is an exploration of gene overexpression in female prawns. Meanwhile, the findings of the comparative transcriptomic analyses provide the following insights on more sexual differentiation-related candidate genes and their sex regulation pathways for future research.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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AUTHOR CONTRIBUTIONS

Conceptualization: W.M. and J.Y.; data curation: S.S., J.L., and H.X; drawing figures: S.S., J.L., and W.M.; writing original draft: S.S., J.L., and W.M.; funding acquisition: J.L. and W.M.; validation: J.L. ; revision: J.L. and W.M.; all authors reviewed the whole work and approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

We worked to ensure sex balance in the selection of non-human subjects. We worked to ensure diversity in experimental samples through the selection of the genomic datasets.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
DH5α	Sangon Biotech	Cat#B528411
E. coli HT115	Biovector NTCC	HT115(DE3) strain
Biological samples		
Male reproductive system in RNAi group	This paper	N/A
Female reproductive system in RNAi group	This paper	N/A
Chemicals, peptides, and recombinant proteins		
Trizol Reagent	BBI	Cat#B610409
SpeedyCut *Bam* HI	BBI	Cat#B600729
SpeedyCut *Hind* III	BBI	Cat#B600741
Critical commercial assays		
3′-RACE Kit	BBI	Cat#B605101
5′-RACE Kit	BBI	Cat#B605102
PrimeScript™ 1st Strand cDNA Synthesis Kit	TaKaRa	Cat#6110A
SYBR® Premix Ex Taq ™	TaKaRa	Cat#RR420L
Column Trizol Total RNA Isolation Kit	Sangon Biotech	Cat#B511321
Illumina HiSeq	Sangon Biotech	N/A
Deposited data		
Raw and analyzed data of Mr-Masc interfered female reproduction system comparative transcriptome	This paper	BioProject ID: PRJNA954167
Raw and analyzed data of Mr-Masc interfered male reproduction system comparative transcriptome	This paper	BioProject ID: PRJNA954150
Mr-Masc sequence	This paper	GenBank: OQ786857
Oligonucleotides		
Primers for dsRNA preparation, see Table 2	This paper	N/A
Primers for qPCR, see Table 2	This paper	N/A
Recombinant DNA		
Plasmid: pET-T7-Mr-Masc vector	This paper	N/A
Software and algorithms		
Prism 8	GraphPad	https://www.graphpad.com/
Other		
PredictProtein	PredictProtein	http://www.predictprotein.org/
SMART	SMART	http://smart.embl-heidelberg.de/smart
NetNGlycate-1.0	Department of Health Technology	https://services.healthtech.dtu.dk/services/NetNGlyc-1.0/
NetPhos3.1	Department of Health Technology	https://services.healthtech.dtu.dk/services/NetPhos-3.1/
Swiss-model	Swiss-model	https://swissmodel.expasy.org
Clustal Omega	Clustal Omega	http://www.ebi.ac.uk/Tools/msa/clustalo/
Phylogenetic analysis	phylogeny	http://www.phylogeny.fr

RESOURCE AVAILABILITY

Lead contact

Further information and request for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Wen-Ming Ma (m-wm02@163.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

All data reported in this paper will be shared by the lead contact upon request.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animals

The prawns at the postlarvae (PL) 15–30 stage with 1.0–1.5 cm body length were purchased from the Yonggang prawn culture facility in Ningbo, Zhejiang for the Mr-Masc silencing in animal experiments. Then, 5 μg Mr-Masc dsRNA in 500 nL SDW was injected into the PL (n = 100) through the arthrodial membrane at the basis of the fifth walking legs using microinjection needles. The controls (n = 100) received an equal amount of GFP dsRNA injection. Then, the PL was cultured in 2-3m^3 tanks until being sacrificed to obtain samples for further analysis. During animal experiments, all prawns were monthly injected with 5 μg dsRNA/each prawn during the 90 days gene silencing.

On the other hand, the sex reversal male (termed neo-female) was identified and screened by the appearance sex features and sex molecular marker method. The breeding capacity of neo-females (n = 6) was further estimated. The neo-females of prawn *M. rosenbergii* were cultured in the Yonggang prawn hatchery in Ningbo, Zhejiang, China. They were reared in aquaria with circulating freshwater at 25°C with an artificial photoperiod of 14-h light: 10-h dark per day. Prawns were fed with compound foods twice a day. The neo-females with mature ovaries were mated with normal males and spawned. Then, the embryo-bearing neo-females were separately transferred to closed tanks with saltwater for embryonic incubation in a hatching facility.

METHOD DETAILS

Gene characterization and bioinformatics analysis

A transcript identified as a putative Masc gene (designated as Mr-Masc) was obtained from the embryonic transcriptome of *M. rosenbergii* and the full length of the cDNA sequence was amplified by rapid amplification of cDNA ends (RACE) method following the manufacture's protocol of the RACE Kit (BBI, Shanghai, China). Then, the Mr-Masc nucleotide sequence (GenBank accession number OQ786857.1) was uploaded to the NCBI website (http://www.ncbi.nlm.nih.gov/blast). The deduced amino acid sequence of the protein was predicted by the PredictProtein (http://www.predictprotein.org/). The translated sequence was further analyzed by SMART (http://smart.embl-heidelberg.de/smart) for the prediction of conserved domains. Sites of N-linked glycosylation were predicted by the NetNGlycate1.0 of the CBS prediction server (https://services.healthtech.dtu.dk/services/NetNGlyc-1.0/). The NetPhos3.1 server was used to predict serine, threonine, or tyrosine phosphorylation sites (https://services.healthtech.dtu.dk/services/NetPhos-3.1/). The ZnF domain of Mr-Masc homology model was predicted by the Swiss-model web server (https://swissmodel.expasy.org/).

On the other hand, multiple sequence alignments of Mr-Masc with several reported Masc proteins and representative members of ZnF proteins were conducted (Table 1). The result of percent identity created by Clustal was employed to analyze sequence similarity among the Masc proteins and various ZnF proteins. Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) was then used for multiple sequence alignment of Mr-Masc sequence against the known Masc proteins and representative ZnF proteins. Phylogenetic

analysis was constructed by the neighbor-joining method and presented as a cladogram (http://www.phylogeny.fr).

Tissue expression pattern of Mr-Masc

Health adult prawns (body length 14.40 ± 0.50 cm, body weight 21.30 ± 2.50 g) were placed in an ice bath for 2–3 min until they were lightly anesthetized. Heart, hepatopancreas, eyestalk, gills, thoracic ganglion, abdominal ganglion, intestines, muscle, female reproductive system (ovary) (n = 3), and male reproductive system (testis, vas deferens, and the terminal ampullae) (n = 3) were dissected, snap-frozen in liquid nitrogen, and stored at −80°C until RNA preparation, respectively. The sample of each organ was homogenized in Trizol Reagent (BBI) and total RNA was prepared according to the manufacturer's instructions. Next, 1 μg of each total RNA was used to synthesize the first-strand cDNA using oligo (dT) and M-MLV reverse transcriptase (PrimeScript first Strand cDNA Synthesis Kit, TaKaRa Bio Inc., Japan) in a 20 μl reaction system. Then, the expression level of Mr-Masc was analyzed by real-time quantitative reverse transcription-polymerase chain reaction (qPCR).

The qPCR reactions were performed on the Bio-Rad MiniOpticon Real-Time PCR System using SYBR Premix Ex Taq (TaKaRa Bio Inc., Japan) and specific primers, Mr-Masc-qF and Mr-Masc-qR (Table 2). Cycling parameters were: 40 cycles of 15 s at 95°C (1 min only for the first cycle), 25 s at 63°C, and 10 s at 72°C (5 min only for the last cycle). Dissociation curves were analyzed at the end of each run to determine the purity of the product and the specificity of the amplification. The relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method with Mr-18S cDNA (GenBank accession number DQ642856.1) as the internal reference. All data were given as the means ± SEM of independent experiments from three separate RNA pools and analyzed using Graph-Pad Instat (GraphPad Software Inc.).

In vivo knockdown of Mr-Masc by RNAi

The preparation of Mr-Masc dsRNA for RNAi

The dsRNA of Mr-Masc was conducted at the aa 311–424 region near the C terminal of protein and *in vivo* knockdown of Mr-Masc by RNAi was carried out in *M. rosenbergii*. A pair of primers (Mr-Masci F and Mr-Masci R) was designed for the preparation of Mr-Masc dsRNA (Table 2). A 342bp cDNA fragment was subcloned into the plasmid pET-T7 vector at the *Bam*H I and *Hind* III restriction sites. For negative control, a pET-T7 vector with a 359 bp GFP cDNA fragment was previously prepared and preserved in the laboratory.[24] The recombinant plasmids were transformed into *Ephestia coli* HT115 and the dsRNAs were produced and purified as described before.[24] *In vivo* knockdown was performed by dsRNA injection into *M. rosenbergii* prawns.

Full sex reversal neo-female induction and all-male progeny breeding

For the Mr-Masc silencing in animal experiments, the prawns at the postlarvae (PL) 15–30 stage with 1.0–1.5 cm body length were purchased from the Yonggang prawn culture facility in Ningbo, Zhejiang. Then, 5 μg Mr-Masc dsRNA in 500 nL SDW was injected into the PL (n = 100) through the arthrodial membrane at the basis of the fifth walking legs using microinjection needles. The controls (n = 100) received an equal amount of GFP dsRNA injection. Then, the PL was cultured in 2-3m^3 tanks until being sacrificed to obtain samples for further analysis. During animal experiments, all prawns were monthly injected with 5 μg dsRNA/each prawn during the 90 days gene silencing. Finally, the qPCR was done to estimate the interference efficiency. The RNA of the male (n = 3) or female reproductive system (n = 3) of RNAi individuals was extracted, respectively. Relative abundances were expressed as the ratio of Mr-Masc transcript levels to those of Mr-18S. The peak values of the GFP RNAi group were set to 100 and the values of the Mr-Masc RNAi group were normalized. All data were from three separate RNA pools.

On the other hand, to evaluate the potential role of Mr-Masc in sexual regulation, the sex of RNAi prawns was identified and confirmed through both the genetic sex identification method[32] and the appearance characteristics of individuals.[2] The male prawn *M. rosenbergii* was distinguished from the female by the appearance of genital pores of pereiopods and the male-unique appendage of second pleopods in both the Mr-Masc RNAi group and the GFP RNAi group. Then, the genetic gender of these collected female prawns (or neo-females) was confirmed by the sex molecular marker method.[32] The female-unique fragment is identified from a genetic female prawn but no similar band is amplified in genetic males.[32] Thus, the sex reversal male (termed neo-female) could be identified and screened by the appearance sex features and sex molecular marker method.

Furthermore, the breeding capacity of neo-females (n = 6) was further estimated. The neo-females with mature ovaries were mated with normal males and spawned. Then, the embryo-bearing neo-females were separately transferred to closed tanks with saltwater for embryonic incubation in a hatching facility. The embryos with dark compound eyes were collected from the setae of the pleopods of neo-females and the sex of each embryo was checked by sex molecular marker method.[32] The sex of normal offspring consists of females and males, whereas the progeny sex of neo-females was expected all-male according to the previously reported data.

Comparative transcriptomic analyses

RNA isolation for transcriptomic sequencing

To evaluate the potential role of Mr-Masc in the regulation of sexual differentiation and gonad development in *M. rosenbergii*, the effect of RNAi-mediated Mr-Masc knockdown on the gene transcriptions was investigated by comparative transcriptomic analyses. Adult females (body length 9.49 ± 0.80 cm, body weight 20.5 ± 4.80g)) and male prawns (body length 10.21 ± 0.40 cm, body weight 26.7 ± 3.80 g) were selected for gene knockdown, respectively. Each prawn was injected with 5 μg Mr-Masc dsRNA every five days for ten days in the Mr-Masc RNAi group. The prawns have injected with an equal dosage of GFP dsRNA in the GFP RNAi group. Then, the ovaries in the Mr-Masc RNAi group (n = 3) and the GFP RNAi group (n = 3) were dissected to provide sufficient RNA for the transcriptomic sequencing, respectively. Meanwhile, the male reproductive system (contained testis, vas deferens, and the terminal ampullae) in the Mr-Masc RNAi group (n = 3) and the GFP RNAi group (n = 3) were collected for the transcriptomic sequencing, respectively. The total RNA of each sample was extracted by using the column Trizol total RNA isolation kit (Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China) following the manufacturer's protocol. The OD260/280 should range from 1.8 to 2.0, to ensure the purity of the RNA sample.

Identification and classification of differentially expressed unigenes

The transcriptome of each sample was sequenced using the Illumina HiSeq (Sangon Biotech, Shanghai, China). The raw reads were cleaned by removing adaptor sequences, empty reads, and low-quality sequences. The clean reads were assembled into non-redundant transcripts, which have been developed specifically for the *de novo* assembly of the transcriptome using short reads. The resulting unigene sequences were then annotated using homology search (BLASTX) with an E-value cut-off of 10^{-5} against an NCBI non-redundant (Nr) database (http://ncbi.nlm.nih.gov/), Uniprot database (http://www.uniprot.org/), Cluster of Orthologous Groups database (COG) (https://www.ncbi.nlm.nih.gov/COG/), and Kyoto Encyclopedia of Genes and Genome (KEGG) database (http://www.kegg.jp). The coding sequence and the direction of the annotated unigenes were determined based on the BLAST results from the four above-mentioned databases. For the differential expression analysis, the transcript expression level of the unigenes was measured using the FPKM method (Fragments Per kb per Million fragments). Genes were considered differentially expressed in the given library when the p value was less than 0.05 and a greater than 2-fold change (with $|\log_2 \text{foldchange}|>1$). Furthermore, the effect of RNAi-mediated gene knockdown of Mr-Masc on the expression of sexual- or gonad-related genes was also screened and analyzed. The putative cascade regulation axis of these differentially expressed transcripts involved in crucial signal pathways of sexual differentiation, reproduction, or cell proliferation was briefly illustrated.

Real-time quantitative reverse transcription PCR analysis

To validate the accuracy of gene expression data obtained by RNA-seq, some DEGs were selected to be verified by qRT-PCR using the same samples for RNA-seq. Four sexual-related significantly differential expression transcripts, namely, insulin-like growth factor binding protein (IGFBP), low-density lipoprotein receptor (LDLR), peptidase C1-like family/cathepsin L (Cathepsin), and forkhead were chosed to comfirm their expressions in the female transcriptomic profiling. Meanwhile, four sexual-related significantly differential expression transcripts, namely, LDLR, male reproductive-related serum amyloid A (MRRSA), Cytochrome, and kazal-type serine protease inhibitor (KSPI) were selected to be verified their expressions in the male transcriptomic profiling. qPCR primers were designed and listed in the Table 2. Then Mr-18S was used as a housekeeping gene to normalize the mRNA levels of DEGs. For qPCR analysis, total RNA from transcriptome sequencing samples was reverse-transcribed using PrimeScript first Strand cDNA Synthesis Kit (TaKaRa). The SYBR Green RT PCR assay was carried out in an CFX384 multiple real-time fluorescence quantitative PCR Detection System (Bio-Rad). The qRT-PCR was carried out as described above and performed in triplicate. The relative expression level was calculated using the $2-\Delta\Delta Ct$ method. The data

obtained from qPCR analysis were analyzed for statistical significance using Graph-Pad Instat (GraphPad Software Inc.).

QUANTIFICATION AND STATISTICAL ANALYSIS

The qRT-PCR was carried out as described above and performed in triplicate. The relative expression level was calculated using the $2-\Delta\Delta Ct$ method. The data obtained from qPCR analysis were analyzed for statistical significance using Graph-Pad Instat (GraphPad Software Inc.). Graphs were generated and statistical analysis was performed with the use of GraphPad Prism 8. Quantitative data are presented as means $\pm$ SEM.

ADDITIONAL RESOURCES

This work is not a part of a clinical trial and does not use any clinical registry number.