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Gonadal transcriptome analysis reveals *MAG* participates in ovarian suppression of intersex red claw crayfish (*Cherax quadricarinatus*)

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Abstract

Background The red claw crayfish (*Cherax quadricarinatus*) is a commercially and ecologically significant species that displays a unique intersex model with an ovotestis gonad and was identified to have functional testes and a vestigial ovary, which was inhibited by insulin-like androgenic gland hormone (*IAG*), but the underlying molecular mechanisms are still unclear.

Results In this study, the structure and transcriptomic profiles of ovotestis and female and male gonad was analysis and compared, 406 differentially expressed genes were identified, among which membrane-anchored AG-specific factor (*MAG*) exhibited significantly greater expression in ovotestis gonads than in male or female gonads. The localization of MAG in type I or II cells of androgenic gland revealed its potential function of IAG hormone synthesis. Furthermore, the analyses of gene regulation relationship revealed that *IAG* positively regulates *MAG* expression, while *MAG* negatively regulates vitellogenin gene (*VTG*) expression.

Conclusions Our research suggesting *MAG* participates in the *IAG* regulated ovarian suppression in the intersex red claw crayfish, which provides important information on the regulatory mechanism of the ovarian dysplasia in the ovotestis of intersex red claw crayfish. These results will enhance the knowledge of *IAG*-related pathways in the female reproductive axis, as well as the mechanisms of sexual differentiation in crustaceans.

Keywords Intersex, Ovotestis, Ovarian suppression, IAG hormone, MAG

Background

Crustaceans widely exhibit diverse reproductive strategies and robust sexual plasticity [1], with intersexual phenotypes observed in numerous gonochoristic species across various taxa [2, 3]. Researchers have endeavored to elucidate the precipitating factors underlying intersex formation through various clues [4–7], including genetic factors [8], endocrine abnormalities [9], environmental effects [10], and parasitic manipulation [11]. However, a consensus has yet to be reached. *Cherax quadricarinatus*, commonly referred to as the red claw crayfish, is a gonochoristic decapod with ZZ/ZW-type sex determination [12], and exhibits sexual dimorphism, with males displaying significantly larger body sizes than females [13]. Thus, the sex determination studies of red claw crayfish are important for improving the economic benefit of aquaculture.

Red claw crayfish possess a bilaterally symmetrical reproductive system and openings. The male openings are located at the base of the fifth walking leg, while the female openings are situated at the base of the third

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walking leg. However, in both cultured and wild populations, intersex individuals exhibit various types of genital openings and typically display male secondary sex traits but exhibit distinct differences in their reproductive systems [8]. The intersex individuals with one male opening on one side and one female opening on the other side have ovotestis, which contains one side of the active male reproductive system alongside a consistently arrested ovary [14]. It is reported that in China, the percentage of intersex in the natural population was 1.5%, and the percentage of intersex possess ovotestis in all kinds of intersex is 22% [15]. So far, there have been no reports indicating successful artificial induction of normal female or male red claw crayfish to become intersex. However, intersex red claw crayfish can be used as a sex reversal model itself due to their remarkable sexual plasticity. It has been reported that the ablation of androgenic gland (AG) or knockdown of AG-specific insulin-like factors (*IAG*) in intersex individuals can induce morphological and physiological changes, including degeneration of male reproductive organs and ovarian activation. This ultimately leads to a sex reversal from physiological male to physiological female in intersexual individuals[2]. This unique sex reversal model making it an exceptional subject for investigating the molecular mechanisms underlying sexual shifts and the homeostasis of bisexual gonads [16].

Crustacean male-specific endocrine AG, which is responsible for the secretion of AG-specific insulin-like hormone (IAG), exerts a negative effect on ovarian development and acts as a key upstream regulator of ovarian suppression in the ovotestis of intersex red claw crayfish [17–21]. However, the molecular mechanisms underlying its ovarian suppression function remain to be elucidated. The recent research in crayfish found the silencing of *IAG* gene led to the up-regulation of ovary-related genes. Meanwhile the membrane-anchored AG-specific factor (MAG), which has been reported to be potentially involved in the transport process of the IAG hormone [22], was found to be down-regulated [23]. Therefore, *MAG* may mediate the mechanism by which *IAG* inhibits ovarian development. Vitellogenin (VTG) is a precursor of yolk proteins in decapod crustaceans, and *VTG* gene expression is positively correlated with ovarian development [24] and has been widely utilized as a biomarker for ovarian development in both vertebrates and invertebrates [25].

In this research, to explore the underlying molecular mechanisms of *IAG*-mediated inhibition of ovarian development, the transcriptome of intersex and normal sex gonads were compared, through which *MAG* gene was found to be positively related to the ovotestis gonad. The spatial and temporal expression profiles of *MAG* and *IAG* were analyzed, and using *VTG* as a biomarker, the role of *MAG* in the ovarian suppression in red claw crayfish was investigated. This study could enrich the understanding of the molecular basis for intersex formation and inform continued research on sex reversal mechanisms in crustaceans.

Methods

Samples, RNA extraction, tissue section

The red claw crayfish used in this research were obtained from the breeding center of the Zhejiang Academy of Agricultural Sciences, Institute of Hydrobiology, China. The crayfish were subjected to cold shock to death according to the guidelines of the Committee of Laboratory Animal Experimentation at Zhejiang Academy of Agricultural Sciences (No. 2023ZAASLA35). The genital organ of each crayfish was identified. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, German), following the manufacturer's instructions. The quality and quantity of RNA were assessed by agarose gel electrophoresis and a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, USA). Ovary and testis tissues from male, female, and intersex individuals were dissected, fixed overnight in 4% paraformaldehyde, dehydrated, embedded in paraffin blocks, and sectioned into 4 μm thick slices for hematoxylin and eosin staining or immunofluorescence analysis.

Library generation, sequencing and data analysis

A total of 26 libraries (including four androgenic glands and four testes from males, five ovaries from females, four androgenic glands, four testes and five ovaries from intersex individuals) were constructed using the NEBNext Ultra RNA Library Prep Kit (Illumina), followed by sequencing on the BGISEQ-500 platform. The raw data of RNA-seq reads were filtered by removing the reads containing adapter, low quality, or poly-N. The clean data were mapped to the reference genome (https://identifiers.org/ncbi/insdc.gca:GCA_026875155.2) [26] using HISAT2 [27].The FPKMs were calculated by RSEM [28]. Differential expression analysis was performed using DEseq2 in R package [29], significantly differentially expressed genes (DEGs) threshold for |log2FoldChange|>1 and all DEGs were classified and enriched by GO and KEGG analyses.

Validation of the RNA-Seq results

Complementary DNA (cDNA) was synthesized using the Hifair® III 1st Strand cDNA Synthesis SuperMix for qPCR (gDNA digester plus) HOT kit (Yeasen, China), following the provided instructions. Six differentially expressed genes (DEGs) were selected for validation of the RNA-Seq results, and their primer sequence information is listed in Supplementary Table 1.

Detection of gene expression patterns

To investigate gene expression patterns during development of red claw crayfish, gonad tissues were sampled from male and female crayfish in preliminary differentiation stage, early development stage and maturation stage. To compare gene expression among sexes, the ovary, AG, testis and ovotestis were sampled from female, male and intersex red claw crayfish. The primer information is listed in Supplementary Table 1. The qRT-PCR was determined using Hieff® qPCR SYBR Green Master Mix (Yeasen, China) on QIAquant 96 2Plex (QIAGEN, Germany) as follows: 95 °C for 5 min; 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s; and 72 °C for 10 min. Beta-actin was used as the internal control gene, and each reaction was repeated three times. The relative expression of each gene was determined references for $2^{-\Delta\Delta CT}$ method.

Antibody preparation and immunofluorescence

The 438 bp fragment of *MAG* was inserted into pET-30a by the double enzyme digestion method, and subsequently transformed into BL21 (DE3) competent *E. coli* cells (TransGen, China). The fusion protein expression was induced by 0.5 mM IPTG and purified using a His·Bind purification kit (Novagen, Germany). Polyclonal antibody against MAG was generated by Zoonbio Biotechnology Co., Ltd (Nanjing, China), through immunization of New Zealand rabbits. Immunofluorescence staining was performed by Servicebio Technology Co. Ltd. (Wuhan, Hubei, China). For immunofluorescence staining, firstly, MAG (1:1000 dilution) was visualized using a CY3-coupled goat anti-rabbit secondary antibody (1:300) (red), nuclei were labeled with DAPI for blue fluorescence detection.

DsRNA, plasmid prepared and injection

The gene-specific primers and T7 primers utilized for generating RNA synthesis templates are listed in Supplement Table 1. Single-stranded RNA (ssRNA) was synthesized using the RiboMAX™ Large Scale RNA Production System T7 (Promega, Beijing, Biotech Co., Ltd.) according to the manufacturer's instructions. Subsequently, ssRNA was quantified and diluted to a concentration of 1 μg/μl with distilled deionized water (DDW), and annealed to form double stranded RNA (dsRNA). The overexpression system used in this study was based on the published research [20].The pcDNA3.1(+)-C-eGFP plasmid was used as the backbone plasmid for gene overexpression, and the CDSs of *MAG* (438 bp), *IAG* (531 bp) and *VTG* (7755 bp) were inserted into the backbone plasmid by double enzyme digestion.

Adult red claw crayfish were collected and temporarily reared in the laboratory for one week before injection. In the RNAi analysis, *IAG* silencing was conducted in males, *VTG* silencing was conducted in females, and *MAG* silencing was conducted in both sexes. The experimental group and control group were injected with dsRNAs and DDW [18, 19], respectively. The injection site is the genital opening, which is located in base of the third pereiopoda of females and the fifth of males. The injected dosages of the dsRNA were 1 μg/g of body weight. In the gene overexpression analysis, the experimental group were injected with pcDNA-*MAG*-eGFP plasmid (or pcDNA-*IAG*-eGFP, pcDNA-*VTG*-eGFP plasemid) and lipo8000 (1:2.5) (Beyotime, China), the control group were injected with pcDNA-eGFP plasmid and lipo8000. The injected dosages of pcDNA plasmids were 3 μg/g of body weight. AG and ovary tissues were sampled 24 h after injection, from which total RNA was extracted and transcribed into cDNA for subsequent qRT-PCR analysis. The relative expression of each gene was calculated using the $2^{-\Delta\Delta CT}$ method.

Statistics

All the statistical tests were performed using SPSS 22.0 (IBM). Difference in gene expression were analyzed using one-way analysis of variance followed by Duncan multiple comparison tests. Data were expressed as the mean ± standard error (SEM). Significance was set at P, and different letters indicate significant differences.

Results

Structural analysis of gonads

According to morphoanatomical analysis, the intersex testis and ovary were integrated together, and the sperm efferent duct extended into the interior of the ovary (Fig. 1a). Mounts of mature sperm were observed in the male testis, while in the intersex testis, there were many spermatozoa but no mature sperm (Fig. 1b, e). There were only primary oocytes and follicular cells in the intersex ovary, in contrast, the ovary of female red claw crayfish at the same body length were filled with early vitellogenic oocytes (Fig. 1c, f). The spermatophores in the sperm efferent duct of males were full of mature sperm, whereas those in the sperm efferent duct of intersex testis were empty (Fig. 1d, g).

Transcriptomic analysis and validation

The morphologies of gonads sampled from different sexes of red claw crayfish used for RNA-seq are shown in Fig. 1a. An average of 7.863 GB of clean data was obtained from 26 samples (including 4 male testes, 4 intersex testes, 4 male AGs, 4 intersex AGs, 5 female ovaries and 5 intersex ovaries). The Q20 and Q30 ratios were determined to be 98.52% and 96.21%, respectively. Approximately 86.07% of the reads were successfully mapped to the reference genome (GCA_026875155.2). The numbers of up and down regulated DEGs in three comparative groups are presented in Fig. 2a. The KEGG and GO enrichment results are presented in Supplement

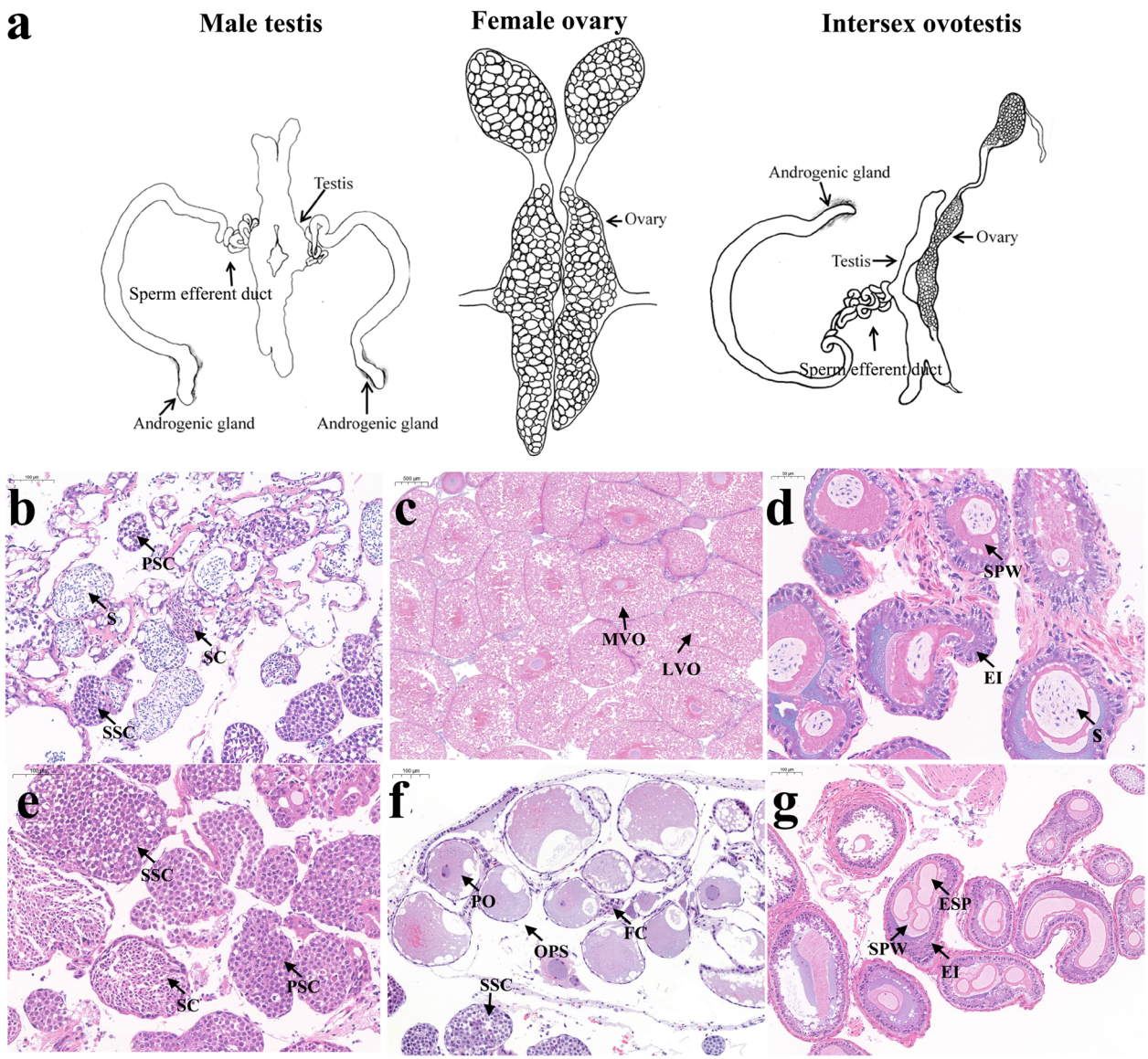

Fig. 1 Morphoanatomical and histological sections of gonads from male, female and intersex red claw caryfish. **a** is a schematic representation of the reproductive system of male, female and intersex red claw crayfish; **b-g** are paraffin sections of gonads stained with HE. **b** is the testis from an adult male, which contains primary spermatocytes (PSC), secondary spermatocytes (SSC), spermatocytes (SC) and sperm (S); **c** is the ovary from an adult female, which has a dense arrangement with early vitellogenic oocytes (EVO). **d** is the sperm efferent duct of an adult male, and the spermatophore is full of sperm. **e** is testis from an adult intersex, and no sperm was observed. **f** shows ovaries from intersex adults; only primary oocytes (PO) were observed, and the oocyte perivascular space (OPS) was large and rich in follicular cells (FC) and was attached to the testis. **g** is the sperm efferent duct, which extends into the ovaries of intersex adults, and only empty spermatophores (ESP) were observed. SPW is the spermatophore wall, EI is the epithelial layer

Fig. 1 and Supplement Fig. 2. The down-reghulated DEGs between intersex ovary and female ovary were enriched in the sterioid hormone biosynthesis, endocytosis pathway.

Six DEGs, including *IAG*, *MAG*, *Mucin-5AC-like*, *Tenascin C*, *secernin*, and *HSP70* were selected for qRT-PCR analysis to validate the RNA-Seq results (Fig. 2b, Supplement Table 1). The results of genes expressions in transcriptome and qRT-PCR were consistent, *Tenascin C* was not differentially expressed between intersex and female or male, *secernin* and *Mucin-5AC-like* only and highly expressed in male AG, and *HSP70* display higher expression in intersex AG than male AG. Among these genes, *MAG* (membrane-anchored AG-specific factor) expressed in both AG and ovary, and exhibited

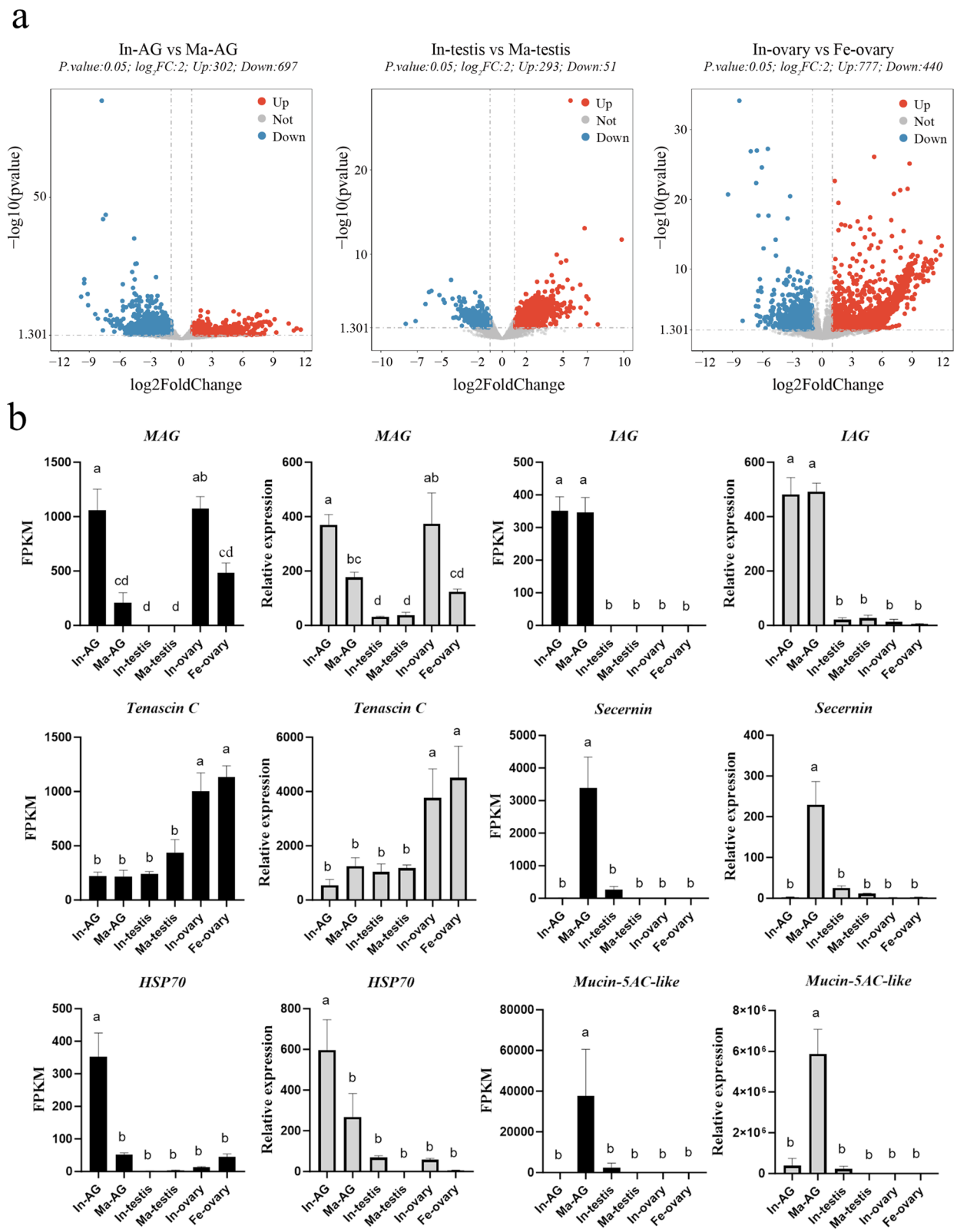

Fig. 2 Volcano plot of differentially expressed genes between the comparison groups and qRT–PCR validation of the RNA–Seq results. Note: **a** is information on the differentially expressed genes identified between intersex AG and male AG, intersex testis and male testis, intersex ovary and female ovary. **b** is validation of six differentially expressed genes identified by RNA-Seq, the black bars represent gene expression levels obtained by mRNA-seq, and gray bars represent gene expression levels obtained by qRT-PCR

higher expression in intersex gonads than that of male and female.

Spatiotemporal expression analysis of *MAG* and *IAG*

To explore the relationship between *MAG* and *IAG*, the gene expression patterns during individual development and among sexes were detected and compared. In male and female crayfish, *MAG* was expressed in the AG, testis and ovary tissue, and its expression was positively related to the developmental stage. Among the adult crayfish of different sexes, *MAG* was expressed at significantly greater levels in the intersex AG and ovary than in the male and female (Fig. 3a, b), indicating that *MAG* is related to gonadal development and intersex ovotestis formation in red claw crayfish. In contrast, *IAG* was specifically expressed in the AG but was not significantly correlated with the developmental stage or sex of red claw crayfish (Fig. 3c, d).

Immunofluorescence analysis revealed that in ovary of ovotestis, MAG localized on the oocytes nuclear and follicular cells around oocytes (green arrows in Fig. 4). In AG of ovotestis, MAG signals appear in the types I or II AG cells (yellow arrows in Fig. 4). The AG cell stages reference the reported research [30–32]. The H&E-stained sections of ovotestis were showed in Supplement Fig. 3.

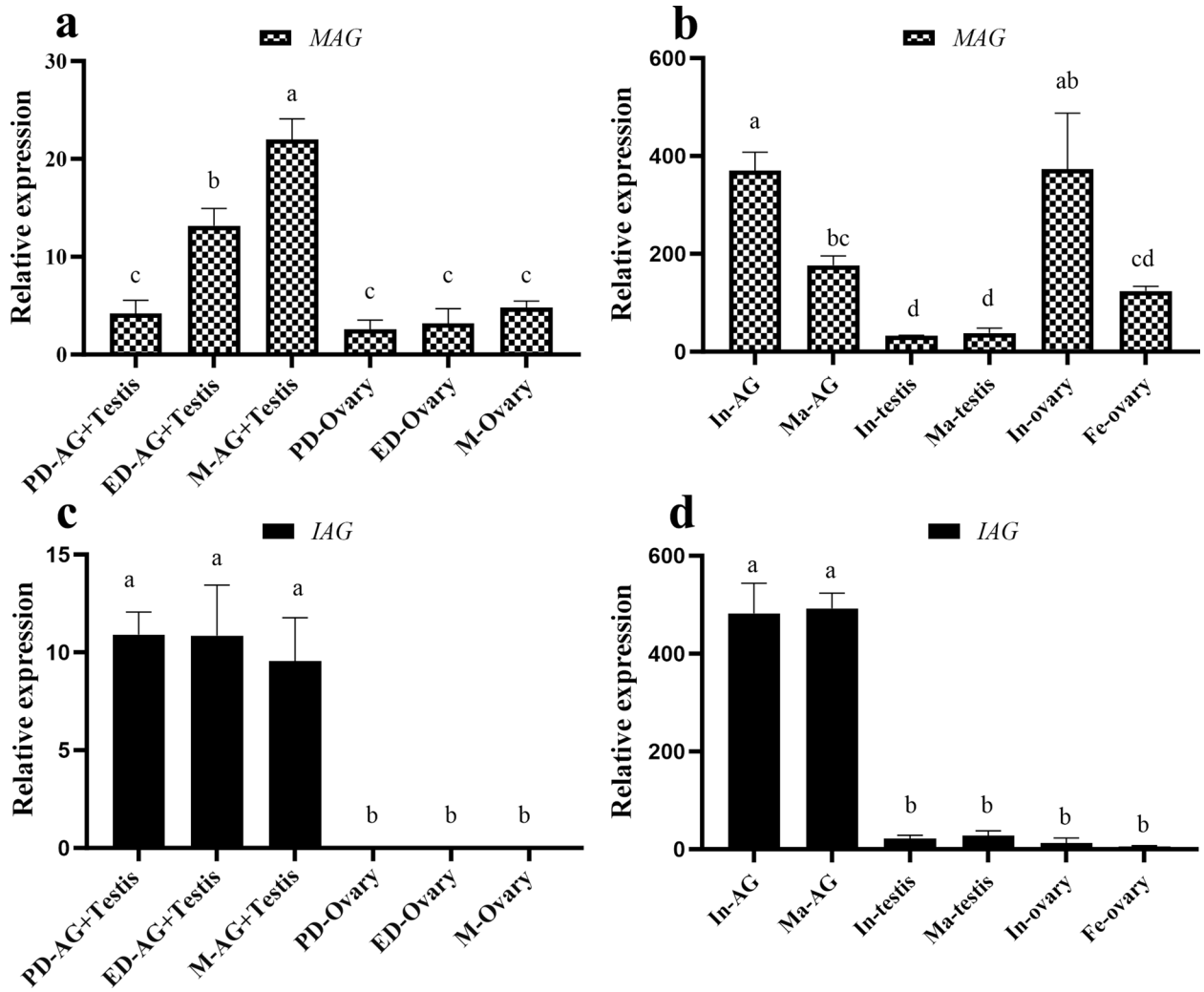

Fig. 3 Gene expression patterns during crayfish development and between sexes. Note: **a** and **c** show the expression patterns of *MAG* and *IAG* during crayfish development. **b** and **d** show the differential expression of *MAG* and *IAG* in the gonads of intersex males and females. In **a** and **c**, PD is short for preliminary differentiation stage, ED is short for early development stage, M is short for maturation stage. In-AG, In-testis and In-ovary indicate the AG, testis and ovary from adult intersex individuals, Ma-AG and Ma-testis are the AG and testis from adult male individuals, and Fe-ovary is the ovary from adult female individuals

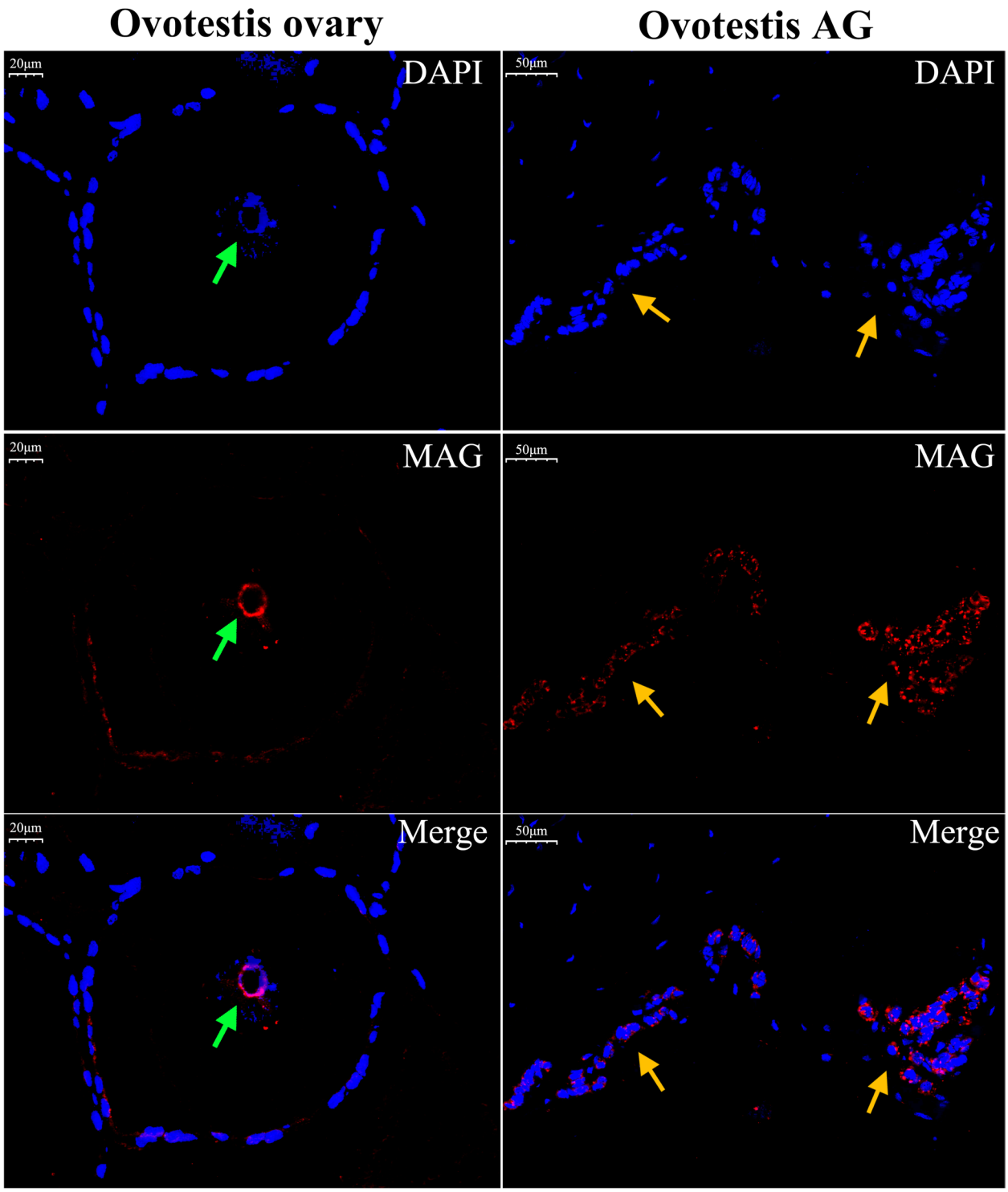

Fig. 4 Immunofluorescence assays of MAG in the ovotestis ovary and ovotestis AG of intersex red claw crayfish. Note: The green arrow points to the oocyte nuclear of the ovotestis ovary, the yellow arrow points to the types I or II AG cells of ovotestis

Gene silencing and overexpression

After dsRNA injection, the expression of the target gene was significantly downregulated, indicating that the genes were successfully silenced. In the AG of male red claw crayfish, *IAG* silencing resulted in a significant decrease in the expression of *MAG*, and *IAG*

overexpression induced an increase in the expression of *MAG*. In addition, *MAG* silencing increased the expression of *IAG*, while *MAG* overexpression barely affected *IAG* expression (Fig. 5a), suggesting that *IAG* positively regulates *MAG* expression and that *MAG* silencing has a feedback effect on *IAG*. In the ovaries of female red claw crayfish, *MAG* silencing led to increased expression of *VTG*, whereas *MAG* overexpression led to decreased expression of *VTG* (Fig. 5b), suggesting that *MAG* significantly inhibited *VTG*. *VTG* silencing or overexpression had no significant effect on *MAG* expression, indicating that *VTG* is the downstream gene of *MAG* and that there is no feedback regulation of *MAG by VTG*.

Discussion

The evolutionary status of crustaceans is relatively primitive, which contributes to the complexity and variability of their sexual differentiation systems. Intersex conditions have been observed in numerous crustacean species [33]. In red claw crayfish, intersex individuals exhibit multiple forms, with varying proportions across different regions. For instance, previous studies have documented a high prevalence of intersex red claw crayfish individuals in Israel, where individuals often display one male and two female genital openings with ovotesticular characteristics [8]. In contrast, these intersex occurrences are less frequent in the red claw crayfish population introduced to China from Australia in the 1990s [15, 34]. Genetic

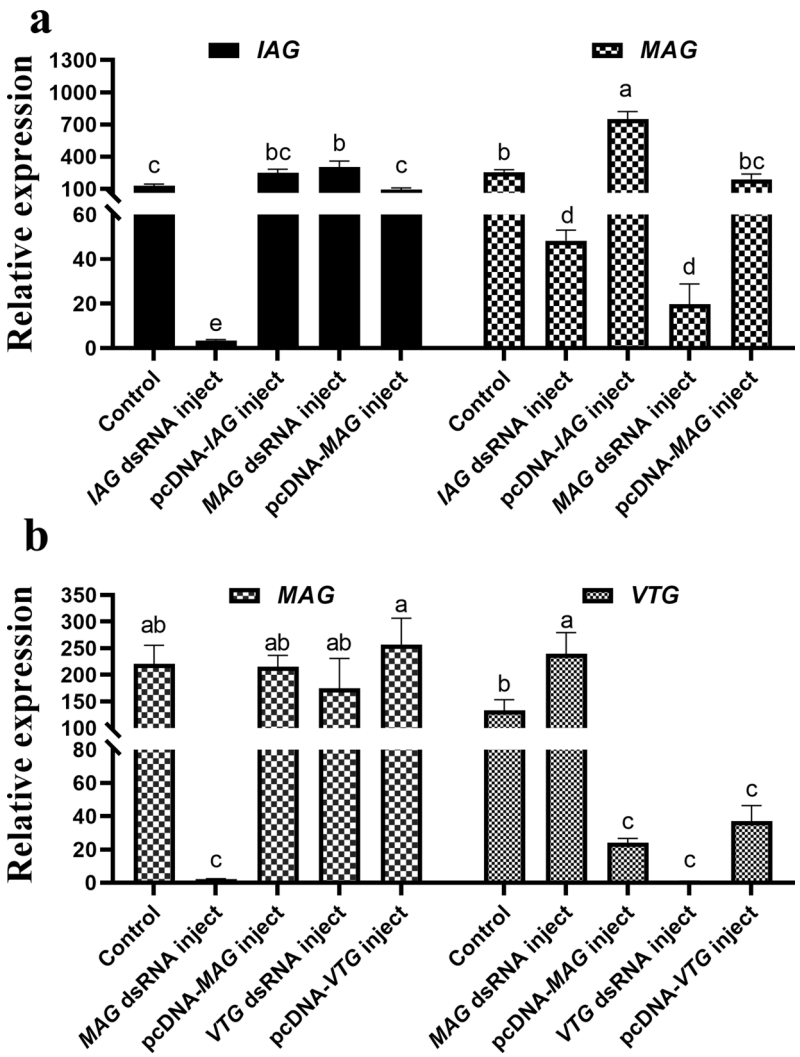

Fig. 5 Regulatory interactions between *IAG* and *MAG* and between *MAG* and *VTG* in red claw crayfish. Note: **a** shows *IAG* and *MAG* expression before (control) and after gene silencing (dsRNA inject) or after overexpression (pcDNA inject) in the AG of male red claw crayfish. **b** shows *MAG* and *VTG* expression before (control) and after gene silencing (dsRNA inject) or overexpression (pcDNA inject) in the ovaries of female red claw crayfish

identification has revealed that intersexes with ovotestis are genetic females (ZW) [12] but functional male [16], possessing an underdeveloped ovary on one side and a well-developed testis on the other. This unique reproductive system provides valuable insights into the molecular mechanisms underlying ovarian suppression by male hormones. In this study, it was found that the spermatophores in the sperm efferent ducts of intersex individuals lack mature sperm compared to those in males of the same age, suggesting that intersex individuals may have a longer cycle for producing mature sperm than males. This extended cycle could represent a reproductive cost associated with intersexuality [35, 36].

The crucial role of the *IAG* gene in ovarian dysplasia in the ovotestis gonads of intersex red claw crayfish has been confirmed by previous studies [18, 37, 38]. In *Scylla paramamosain*, *IAG* expressed in female ovary and plays a role in suppressing oocyte growth and vitellogenesis [39]. However, in red claw crayfish, *IAG* is specifically expressed in the AG [40], and it is speculated there may be other factors mediating inhibitory effect of IAG on the ovary. MAG was reported involved in the transport process of the IAG hormone. In this study, it was found that *MAG* expressed in both AG and ovary, and the expression levels of *MAG* in intersex were higher compared to male and female, in spite of intersex individuals possess only one AG, indicating MAG might be a factor mediating inhibitory effect of IAG on the ovary. In immunofluorescence results, MAG signal was detected in Type I or Type II AG cells, which are rich in rough endoplasmic reticulum (RER) [32, 33], the abundance of RER is an indicator of secretory activity in the androgenic gland, suggesting that MAG may play a potential role in the synthesis of IAG hormones.

Previous studies have shown that both *IAG* and *MAG* are negatively regulated by the neuroendocrine [22]. In the gonadal transcriptome following *IAG* gene silencing, *MAG* transcripts was significantly reduced [23]. In this study, the positively regulation from *IAG* to *MAG* indicating *MAG* might be a downstream gene of *IAG*. Additionally, *MAG* negatively regulates *VTG* expression, provides further evidence that *MAG* play a role in ovarian suppressing by inhibit *VTG* expression. Therefore, it can be inferred that *MAG* mediates the process of *IAG* inhibiting ovarian development, possibly by participating in the secretion of IAG hormone and other processes. This provides important information on the regulatory mechanism of the ovarian dysplasia in the ovotestis of intersex red claw crayfish. It was also observed that the efficiency of *MAG* silencing is relatively lower in males compared to females, which may be attributed to the feedback regulation between *IAG* and *MAG*. This finding also explains the challenges encountered during *MAG* silencing in intersex red claw crayfish as reported in previous studies [22]. Moreover, although there were no significant changes in gene expression before and after pcDNA plasmid injection, downstream gene expression was significantly affected, similar to findings in studies involving gene overexpression in *Macrobrachium rosenbergii* [20].

Conclusions

In summary, the intersex phenomenon of red claw crayfish has been well-documented for several decades, with *IAG* being recognized as a pivotal factor in ovarian dysplasia of intersex ovotestis gonads. This study presents detailed structural features of intersex ovotestis and normal testes or ovaries, compares transcriptomic characteristics among males, females, and intersex gonads, and unveils *MAG* participates in the *IAG* regulated ovarian suppression in the intersex red claw crayfish. These results will enhance the knowledge of *IAG*-related pathways in the female reproductive axis. The transcriptomic library and findings from this research will significantly enhance our comprehension of sex determination in red claw crayfish while also advancing sex manipulation techniques specific to this species.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11249-x.

Supplementary Material 1.

Acknowledgements
Not applicable.

Authors' contributions
H.C conceived and designed the study, and drafted the manuscript: M.OY and H.Z carried out the sampling, genetic identification, qRT-PCR, RNAi and gene overexpression studies. F.L draws the schematic. H.C conduct data statistics. B.L provided supervision.

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Data availability
RNA reads generated in this study were deposited into the NCBI SRA with the BioProject ID PRJNA968004.

Declarations

Ethics approval and consent to participate
The study was approved by the Committee of Laboratory Animal Experimentation at Zhejiang Academy of Agricultural Sciences (No. 2023ZAASLA35).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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