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Inhibitory effects of RNAi-mediated knockdown of *EsDmrt-like* gene on testicular development in the Chinese mitten crab *Eriocheir sinensis*

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ABSTRACT

EsDmrt-like is a novel testis-specific *Dmrt* gene identified from the Chinese mitten crab *Eriocheir sinensis*. To explore its biological functions, we performed RNA interference (RNAi) by injection of long double-stranded RNA (dsRNA) targeting the coding region of the *EsDmrt-like* gene. At 24 h post-injection with *EsDmrt-like*-dsRNA, the relative expression level of *EsDmrt-like* mRNA significantly decreased by 80% ($P < 0.01$) compared to negative control of PBS group, while no RNAi effect was detected using a dsRNA of the green fluorescent protein (GFP), indicating target sequence specificity of this interference effect. Although the EsDMRT-like protein could be obviously detected in the PBS group, its expression in the *EsDmrt-like*-dsRNA group disappeared in four of the five tested individuals. Taken together, the expressions of *EsDmrt-like* mRNA and its protein were successfully inhibited by RNAi. After extending RNAi treatment for one month, the crab testicular development exhibited inhibitory effects. The average testis size was reduced and the gonadosomatic index (GSI) was significantly decreased by 75% compared with the controls. The histological and immunohistochemical analysis showed that the spermatogenesis was blocked, with rare spermatozoa in the seminiferous tubules, and the immunohistochemical signal in the spermatogonia and spermatocytes became extremely weak in the *EsDmrt-like*-dsRNA group. These data demonstrated a critical role for *EsDmrt-like* in testicular development and spermatogenesis in *E. sinensis*, and *in vivo* repetitive injection of *EsDmrt-like*-dsRNA could work efficiently to inhibit male testicular development.

Statement of relevance: The Chinese mitten crab *Eriocheir sinensis* is one of the most important aquaculture species and has high commercial value as a food source. *EsDmrt-like* is a novel testis-specific *Dmrt* gene identified from the *E. sinensis*. We performed RNAi by repetitive injection of long double-stranded RNA (dsRNA) targeting the coding region of the *EsDmrt-like* gene, and certified that *EsDmrt-like* plays a critical role during spermatogenesis and testicular development.

1. Introduction

RNA interference (RNAi) was first observed as a natural phenomenon of gene-silencing that occurs post-transcriptionally and was revealed as a sequence-specific degradation of mRNA mediated by double stranded RNA (dsRNA) (Yoshinari et al., 2004). By means of introducing dsRNA molecules into organisms or cells to inhibit the expression of a gene of interest, RNAi has become a very powerful tool for reverse genetics to characterize the function of a novel gene, and was widely used in many model organisms such as worm (Fire et al., 1998), fruit fly (Misquitta and Paterson, 1999) and zebrafish (Wargelius et al., 1999). In recent years, RNAi was also exploited to study the function of a gene or a protein in aquatic commercial invertebrates. For example, in the oyster, injecting dsRNA of germline-specific gene *vasa* into the gonad could provoke a knockdown phenotype corresponding to germ cells under proliferation, and prematurely arrest meiosis throughout the organ (Fabioux et al., 2009). In decapod crustaceans, silencing the expression of sexual regulator gene *IAG* could lead to sex reversal from male towards female, revealing a key role of *IAG* in male sexual differentiation in crayfish and giant freshwater prawn (Rosen et al., 2010; Ventura et al., 2012). In addition, RNAi of *IAG*-binding protein (*IAGBP*) could significantly reduce the transcription of *IAG*, and injection of *IAG*-dsRNA also decreases the expression of *IAGBP* transcripts in the testis, suggesting that *IAGBP* may act as a modulator and plays a role in the *IAG* signaling pathway in the river prawn (Li et al., 2014). In *Eriocheir sinensis*, a study showed that dsRNA treatment could significantly increase the activities of phenoloxidase, acid phosphatase and superoxide dismutase. And the mRNA expression of an antibacterial peptide gene *ALF* was significantly up-regulated 12 h after dsRNA treatment, indicating that dsRNA injection could induce broad-spectrum immune response in the Chinese mitten crab (Dong et al., 2009).

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As a transcription factor, *Dmrt* shares a widely conserved Doublesex/Mab-3 domain (DM domain) from invertebrate to human, but shows little conservation in sequence outside the DM domain region (Kopp, 2012; Smith et al., 1999). The DM domain gene *Doublesex* was first discovered in the fruit fly *Drosophila melanogaster* (Baker and Ridge, 1980), and then another DM domain gene *Mab-3* in the nematode *Caenorhabditis elegans* (Shen and Hodgkin, 1988). As major sex regulators, *Doublesex* and *Mab-3* function in controlling sex-specific differentiation (Baker and Ridge, 1980; Burtis and Baker, 1989). Similarly, in vertebrates, *Dmrt1* is required for male sexual differentiation and development, and has been extensively characterized in humans (Ottolenghi et al., 2000) as well as birds (Raymond et al., 1999; Smith et al., 2003; Smith et al., 1999), reptiles (Smith et al., 1999) and teleosts (Guan et al., 2000; Kobayashi et al., 2004).

Most animals are sexually dimorphic in nature, but different taxa bear diverse sex-specific traits, generating major differences in the genetic control of sexual development (Kopp, 2012). *Sry* is a well-known master sex-determining gene in mammals, but absent in non-mammals (Wallis et al., 2008). In chicken, the role of sex-determining gene is taken over by *Dmrt1* in Z chromosome (Raymond et al., 1999; Smith et al., 2003). In teleosts, *DMY* is a dominant male-determining gene in medaka (Kobayashi et al., 2004), but not found in other fishes. Instead, *Dmrt1*, a gonad-specific gene, was deduced to be a sex-determining gene in tilapia and rainbow trout (Guan et al., 2000; Marchand et al., 2000). These data implied that the sex-determining genes rarely remain at the top of the hierarchy in different lineages for very long since sex-determination signals and mechanisms evolve (Kopp, 2012). In decapod crustaceans, we previously identified a novel testis-specific *Dmrt* gene, termed *EsDmrt-like* gene, in the Chinese mitten crab *E. sinensis* (Zhang and Qiu, 2010). The transcript profiles of testis-specific *EsDmrt-like* is very similar to the rainbow trout *Dmrt1* (Marchand et al., 2000), suggesting that the *EsDmrt-like* gene has a potential role in crab testicular development and differentiation, as *Dmrt1* does in vertebrates (Zhang and Qiu, 2010).

To functionally characterize the *EsDmrt-like* gene, herein we developed a repetitive dsRNA-injection method for the Chinese mitten crab, and further examined the effect of RNAi-mediated knockdown of *EsDmrt-like* gene on testicular development. Our RNAi data clearly showed that the expression of *EsDmrt-like* mRNA and its protein was successfully inhibited in the testis, which led to the defects in crab testicular development, demonstrating an essential role of *EsDmrt-like* in spermatogenesis and testicular development.

2. Materials and methods

2.1. Animals and tissue sampling

Healthy and active male Chinese mitten crabs, with wet body weight of approximately 50 g, were collected from a local aquaculture farm in Shanghai and transferred alive to our laboratory. All the crabs were cultured in freshwater at 20 °C for five days and fed a commercial crab diet once a day to acclimate to the laboratory conditions prior to dsRNA injection. The testicular development was preliminary at this time. The crab testis was white in color and approximately 0.06 g in weight on average. At the end of the injection, each of the testes and crab bodies was weighted, and tissues, including the testis, heart, hepatopancreas, muscle, thoracic ganglion and gill, were collected for the following western blot, gene expression or histological and immunohistochemistry examinations.

2.2. Preparation of polyclonal antibody of anti-EsDmrt-like

The crab *EsDmrt-like* cDNA was ligated into an expression vector pET-32a (Novagen, USA) and expressed in BL21 strain (Tiangen, China). The recombinant protein was purified and injected into rabbits for production of polyclonal antibodies, as described by Qiu and Chen (Qiu and Chen, 2013). Briefly, the abundant recombinant protein of EsDMRT-like was purified by Ni affinity purification column. Thereafter, the purified protein was emulsified with Freund's adjuvant and injected into three healthy adult New Zealand rabbits. Seven days after initial immunization, these rabbits were continuously injected at intervals of two weeks for four times, then bled, and serum samples were collected and purified by antigen affinity (Qiu and Chen, 2013). The specificity of the resultant antibody of the *EsDmrt-like* protein was tested by western blot.

2.3. Synthesis of dsRNAs

Based on the *EsDmrt-like* cDNA sequence (GenBank accession number, HM051384.1), a fragment of nucleotide sequences from position 347 to 870 was used to design (Table 1) and amplify the target site of the dsRNA for RNAi. Then the silencing effect of this fragment was evaluated on the Promega website (http://www.promega.com/siRNADesigner/promegam/). The 524-bp fragment, containing the DM domain, was subcloned to pGM-T vector (Tiangen) to obtain the recombinant plasmid. As a negative control, a 289-bp fragment of green fluorescent protein gene (*GFP*, GenBank accession number, EU716633.1) was amplified from a template of PSC2-eGFP plasmid (Table 1) and then subcloned to pGM-T vector. Both of the two plasmids were purified by the plasmid purification kit (Tiangen), and linearized by the restriction enzymes *Sac* II and *Spe* I, respectively. The linearized plasmids were transcribed to produce the sense and antisense RNAs using SP6 and T7 RiboMAX large-scale RNA production systems (Promega, USA) according to the manufacturer's instructions. Equimolar amounts of sense and antisense RNAs of *EsDmrt-like* or *GFP* were separately mixed, heated at 72 °C for 10 min, and cooled to room temperature over a period of 2 h for annealing. The synthesized *EsDmrt-like*-dsRNA and *GFP*-dsRNA were then purified using phenol/chloroform. After ethanol precipitation, the two dsRNAs were suspended with RNase-free water. The quality of dsRNA was assayed with running 1.5% agarose gel electrophoresis, and the concentration was measured using a Nanodrop 2000c spectrophotometer (Thermo, USA), and then kept at −80 °C until use.

2.4. Injection of dsRNAs

The injection was performed intramuscularly into the fourth pereopodcoxa of each crab. Generally, a total of 45 healthy crabs were divided into three groups: *EsDmrt-like*-dsRNA group (n = 20), *GFP*-dsRNA (n = 5) group, and phosphate buffer saline (PBS) group (n = 20). The first group was the experimental group, injected with *EsDmrt-like*-dsRNA with a concentration of 100 μg in 100 μL of PBS buffer (pH 7.6) per 50 g body weight of a crab. Meanwhile, based on body weight of the crabs, the *GFP*-dsRNA and PBS groups were taken as two types of negative controls and separately injected with volumes equivalent to those applied to the experimental group. At 24 h post-injection, five crabs' testes from each group were sampled for RNA extraction. Forty-eight hours after injection, two testes from the PBS group and five testes from the *EsDmrt-like*-dsRNA group were dissected for protein

Table 1
Primers used in this study.

Gene	Primer sequence (5′-3′)	Application
EsDmrt-like	F: AACGGTATCAGCGCACGCCAAAAT	RNAi
	R: TAGACATGCATGACGGTGATGTGG	
	F: AAGGGCCACAAGCGTTACTG	qPCR
	R: TTCACGCACCTCGTTCTCCT	
GFP	F: CAGTGCTTCAGCCGCTACC	RNAi control
	R: AGTTCACCTTGATGCCGTTCTT	
β-actin	F: GCATCCACGAGACCACTTACA	qPCR
	R: CTCCTGCTTGCTGATCCACATC	

Note: F, forward; R, reverse.