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Short communication

Cell division cycle 2 participates in eyestalk ablation-induced ovarian maturation of *Procambarus clarkii*

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ABSTRACT

Eyestalk ablation is commonly used to promote ovarian development and maturation in captive economic crustaceans. Understanding the molecular mechanism of eyestalk ablation in *Procambarus clarkii* is essential in finding alternative measures to trigger ovarian maturation on this species without undesirable effects. The cell division cycle 2 (Cdc2) is a catalytic subunit of the maturation-promoting factor, which is a central factor for inducing oocyte meiotic maturation. This study analyzed the Cdc2 gene expression patterns and the progesterone level in the *P. clarkii* ovary during its eyestalk ablation-induced maturation course with or without synthetic pcGnRH injection. The RNA interference technique was used to elucidate the role of Cdc2 in the eyestalk ablation mechanism. A combination of the present and previous results revealed that the progesterone-mediated oocyte maturation pathway could exist in the ovary. Furthermore, this pathway could play a crucial role in the eyestalk ablation-induced ovarian maturation in *P. clarkii*.

Statement of relevance: The results of this study enable the utilization of the Cdc2 function to control oocyte maturation in Procambarus clarkii.

1. Introduction

Eyestalk ablation can be used to manipulate the ovarian development and maturation of the red swamp crayfish *Procambarus clarkii*, which is an important economic species held in captivity (Rodríguez et al., 2002; Martín-Díaz et al., 2006; Guan et al., 2013). Understanding the molecular mechanism of eyestalk ablation in *P. clarkii* is essential in finding alternative measures to trigger ovarian maturation without undesirable effects (e.g., poor egg quality and high mortality from the ablation) (Uawisetwathana et al., 2011; Varalakshmi and Reddy, 2010). Knowledge on the molecular mechanisms and functional involvements of reproduction-related genes in ovarian development is necessary to better understand reproductive maturation and resolve the major constraint of this species in captivity (Guan et al., 2013).

For the first time, transcriptomic analysis combined with bioinformatics analysis reveals that the activation of the gonadotropin releasing hormone (GnRH) signaling, calmodulin (CaM) calcium signaling, and progesterone-mediated oocyte maturation pathways are putatively crucial in ovarian maturation induced by eyestalk ablation in decapod crustacean (Uawisetwathana et al., 2011). However, these findings that exhibit potentially important molecular mechanisms of eyestalk ablation need to be experimentally examined further. In one of our previous studies, a GnRH peptide was isolated and characterized first from the ovaries of the eyestalk-ablated *P. clarkii* using chromatographic and immunological methods. The synthetic *P. clarkii* GnRH (pcGnRH) shows stimulative effects on the ovarian development of crayfishes. This result indicates the existence of the GnRH signaling pathway, which may play an important role in regulating the oocyte development in *P. clarkii* (Guan et al., 2014). The results of another previous study we conducted revealed that the expression level of ovarian CaM was significantly induced to 8.7-fold one day after eyestalk ablation in *P. clarkii* ($P < 0.05$) and gradually decreased on the following days. The CaM knockdown by RNA interference (RNAi) inhibits the ovarian maturation induced by ablation, which suggests the importance of the CaM calcium signaling pathway in inducing ovarian maturation by ablation in crayfish (Guan et al., 2013). Therefore, we assumed that pcGnRH could activate the calcium signaling pathway to increase the intracellular calcium ion levels through GnRH and calcium signaling pathways. These pathways subsequently activate steroid hormone folliculogenesis, estradiol, and progesterone production in the ovary. Progesterone may then induce a progesterone-mediated oocyte maturation process of *P. clarkii* from oocyte stages 1 to 4. This study intended to provide evidence on the involvement of the progesterone-mediated oocyte maturation pathway in inducing ovarian maturation by eyestalk ablation in *P. clarkii*.

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The cell division cycle 2 (Cdc2), which is a 34 kDa protein, functions as a catalytic subunit by binding with its regulatory subunit cyclin B to form maturation or an M-phase promoting factor (MPF) for inducing the meiotic oocyte maturation or the G2/M phase transition in the eukaryotic cell cycle (Masui and Markert, 1971; Gautier et al., 1990; Phinyo et al., 2013). The signal transduction pathways that activate the Cyclin/Cdc2 complex and subsequent processes in eukaryotes are the key pathways in the meiotic oocyte maturation. Steroid hormones are functionally involved in the reproductive process in crustaceans. Progesterone or its derivatives is recognized as the maturation-inducing hormone that causes the signal transduction of meiotic oocyte maturation (Miura et al., 2006; Lazar et al., 2002; Rodríguez et al., 2002).

This study analyzed the Cdc2 gene expression patterns and progesterone abundance during ovarian maturation in both eyestalk-ablated and eyestalk-intact *P. clarkii* with or without synthetic pcGnRH peptide. The following RNAi experiment clearly revealed the key role of the Cdc2 gene in ovarian maturation induced by eyestalk ablation in *P. clarkii*. These data, coupled with our previously acquired results, indicated the putative existence and participation of the progesterone-mediated oocyte maturation pathway in inducing ovarian maturation by eyestalk removal in *P. clarkii*.

2. Materials and methods

2.1. Animal collection and eyestalk ablation

Domesticated female *P. clarkii* broodstock (12.4–13.6 cm length and 30.5–36.2 g weight) were collected from the Jiangsu Baolong Breed Aquatics Company in Dafeng City, Jiangsu Province, China, on January. The brooders were maintained in 40 L aquaria in filtered and aerated water at room temperature (25 °C). The crayfish broodstock were fed with a commercially available diet twice daily. The animals were classified into four groups ($n = 40$ each) as follows: (1) group with intact eyestalk, (2) group injected with 50 ng/g BW synthetic pcGnRH, (3) group with eyestalk ablation, and (4) group with eyestalk ablation + injected with 50 ng/g BW synthetic pcGnRH. The eyestalks were cut near the base with a scalpel. The wounds were cauterized with a soldering iron before returning the animals to the water. The brooder ovaries from each group were collected before (day 0; $n = 10$) and after the unilateral eyestalk ablation and/or injected with pcGnRH for 1, 7, and 15 days ($n = 10$ for each time point). Each ovary was weighed. Measurements were recorded to calculate the gonadosomatic index (GSI, ovary weight/crayfish BW) × 100%. The ovaries were then stored at −80 °C until use. The progesterone concentrations in the ovary samples were estimated using radioimmunoassay according to previous reports (Oreczyk et al., 1974; Warrier et al., 2001; Meunpol et al., 2007). The vitellin protein expression level was determined from the ovary samples using the enzyme-linked immunosorbent assay described in detail in our previous studies (Guan et al., 2014).

2.2. RNA preparation and reverse-transcriptase quantitative PCR (RT-qPCR)

RNA samples were extracted from frozen ovaries using TRIzol™ reagent according to the manufacturer's instruction (Invitrogen, USA). The contaminating genomic DNA was removed using DNase I at 0.15 U/mg total RNA treatment at 37 °C for 30 min. The RT-qPCR experiment was performed to detect *P. clarkii* Cdc2 transcripts (GenBank accession no. KR135176) in different ovary samples. The first-strand cDNAs were synthesized using Reverse Transcriptase XL (Takara, Japan). A pair of Cdc2 primers (cdc2-1 and cdc2-2) was used to amplify a 117 bp product (Table 1). The 18S rRNA was used as internal control to verify the RT-qPCR reaction. Two primers (P3 and P4) were used in the process (Table 1). Diethylpyrocarbonate-treated water was used to replace the RNA template as a negative control.

The SYBR Green RT-qPCR assay was conducted in an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, USA). Each sample was run in triplicate along with the internal control gene in a 96-well plate. One PCR product was amplified and detected using curve analysis at the end of each PCR reaction. The RT-qPCR data analysis was performed using SDS software V2.0 (Applied Biosystems) as described in detail in our previous study (Guan et al., 2013). The relative Cdc2 expression level could be calculated using $2^{-\Delta\Delta Ct}$, where the value stands for an *n*-fold difference relative to the calibrator. The obtained data were subjected to one-way analysis of variance followed by Duncan's test ($P < 0.05$).

2.3. RNAi knockdown

The PCR-generated partial DNA fragments of the *P. clarkii* Cdc2 cDNA and the green fluorescent protein (GFP) gene served as the linearized DNA template for single-stranded (ss) RNA synthesis. The T7 promoter sequence was incorporated into the DNA templates using appropriate primers (CR-1, CR-2, P7, and P8) with a T7 promoter at their 5′-ends (Table 1). The T7 Ribo-MAX™ Express RNAi System (Promega, USA) that synthesized the ssRNAs was used according to the manufacturer's instructions. The complementary ssRNA strands were mixed and incubated at 70 °C for 20 min after synthesis. The Cdc2 dsRNA (670 bp) was generated according to our previous study (Guan et al., 2013). The dsRNA (655 bp) specific for the GFP gene was generated using the same procedure and used as a control to exclude the non-specific dsRNA effect. The dsRNAs were verified by agarose gel electrophoresis, quantified by ultraviolet spectrophotometry, and stored at −80 °C for later use. Table 1 presents the primers used for the dsRNA preparation.

The Cdc2 gene expression in the eyestalk-ablated *P. clarkii* was inhibited by the intramuscular injection (injected immediately after eyestalk ablation) of 50 μL phosphate buffer saline (PBS) containing Cdc2 dsRNA. This solution was introduced at a dosage of 0.5 μg/g crayfish body weight after the unilateral eyestalk ablation. The injections were made with a 27-gauge needle through the arthroidal membrane at the base of a walking leg. Cdc2 silencing was performed by injecting with Cdc2-dsRNA twice over a 24 h interval. The crayfish received an

Table 1
Primer sequences used in this study.

Primers	Direction	Nucleotide sequences (5′-3′)[a]	Usage
cdc2-1	Forward	5′-CTTGGGGAGGGAACCTATG-3′	Cdc2 RT-qPCR
cdc2-2	Reverse	5′-AGCAGTGGATGGAACACCT-3′	Cdc2 RT-qPCR
P3	Forward	5′-TGGTGCATGGCCGTTCTTA-3′	18S rRNA RT-qPCR
P4	Reverse	5′-AATTGCTGGAGATCCGTCGAC-3′	18S rRNA RT-qPCR
CR-1	Forward	5′-TAATACGACTCACTATAGGGGAGG ACTACCTACGCATAGAG-3′	Cdc2 dsRNA synthesis
CR-2	Reverse	5′-TAATACGACTCACTATAGGGCTGT AGGAGTTGTCAGGGTTC-3′	Cdc2 dsRNA synthesis
P7	Forward	5′-TAATACGACTCACTATAGGGCGAC GTAAACGGCCACAAGT-3′	GFP dsRNA synthesis
P8	Reverse	5′-TAATACGACTCACTATAGGGCTTG TACAGCTCGTCCATGC-3′	GFP dsRNA synthesis

[a] The T7 promoter sequence is underlined.