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Argonaute 1 is indispensable for juvenile hormone mediated oogenesis in the migratory locust, *Locusta migratoria*

Jiasheng Song, Wei Guo, Feng Jiang, Le Kang, Shutang Zhou*

State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, 1 Beichen West Road, Chaoyang District, Beijing 100101, China

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ABSTRACT

Juvenile hormone (JH) is the primary hormone controlling vitellogenesis and oocyte maturation in the migratory locust *Locusta migratoria*, an evolutionarily primitive insect species with panoistic ovaries. However, molecular mechanisms of locust oogenesis remain unclear and the role of microRNA (miRNA) in JH mediated locust vitellogenesis and oocyte maturation has not been explored. Using miRNA sequencing and quantification with small RNA libraries derived from fat bodies of JH-deprived versus JH analog-exposed female adult locusts, we have identified 83 JH up-regulated and 60 JH down-regulated miRNAs. QRT-PCR validation has confirmed that transcription of selected miRNAs responded to JH administration and correlated with changes in endogenous hemolymph JH titers. Depletion of Argonaute 1 (Ago1), a key regulator of miRNA biogenesis and function by RNAi in female adult locusts dramatically decreased the expression of *vitellogenin* (*Vg*) and severely impaired follicular epithelium development, terminal oocyte maturation and ovarian growth. Our data indicate that Ago1 and Ago1-dependent miRNAs play a crucial role in locust vitellogenesis and egg production.

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1. Introduction

Juvenile hormone (JH) regulates various aspects of insect reproduction including vitellogenesis, oocyte maturation and ovarian growth, but its effects vary among insect species (Raikhel et al., 2005; Raikhel and Dhadialla, 1992; Riddiford, 2012; Wyatt and Davey, 1996). In *Drosophila melanogaster*, both JH and ecdysteroid hormone (20-hydroxyecdysone, 20E) are required for yolk protein production and ovarian development with JH primarily regulating vitellogenin (Vg) uptake into oocytes (Berger and Dubrovsky, 2005; Bownes, 1989; Carney and Bender, 2000; Soller et al., 1999). In the mosquito *Aedes aegypti*, JH stimulates fat body competence for Vg synthesis and controls oocyte development, while 20E regulates Vg expression after the blood meal (Raikhel et al., 2002; Shin et al., 2012). In the red flour beetle *Tribolium castaneum,* JH regulates Vg synthesis in the fat body and 20E regulates ovarian growth and oocyte maturation (Parthasarathy et al., 2010a, 2010b; Sheng et al., 2011). Either JH or JH with 20E has been reported to be involved in vitellogenesis and oocyte maturation of lepidopteran insects (Swevers and Iatrou, 2003; Telfer, 2009). In the migratory locust *Locusta migratoria* and the cockroach *Blattella germanica*, vitellogenesis and oocyte maturation are controlled by JH (Belles, 2005; Cruz et al., 2003; Wyatt and Davey, 1996).

The migratory locust is a representative of an evolutionarily primitive insect with an incomplete metamorphosis (hemimetaboly) and panoistic ovarioles. Although there are two major types of insect ovarioles (panoistic ovarioles with no nurse cells and meroistic ovarioles with nurse cells), research on oogenesis has been limited mostly to insects, such as *Drosophila* with meroistic ovarioles. Similar to the cockroach, the locust oocyte within each ovariole matures synchronously during a particular gonadotrophic cycle (Raikhel and Dhadialla, 1992; Tanaka and Piulachs, 2012). JH controls locust Vg synthesis in the fat body, secretion into hemolymph and uptake by the developing oocytes through patency initiation in the follicular epithelium and receptor-mediated endocytosis at the oocyte membrane (Wyatt and Davey, 1996). However, the molecular mechanisms underlying locust vitellogenesis and oocyte maturation remain elusive.

In recent years microRNA (miRNA) has emerged as an important regulator in insect development, metamorphosis and reproduction (Behura, 2007; Bellés et al., 2012; Lucas and Raikhel, 2013). miRNAs are short (~22 nucleotides) non-coding RNAs that target the 3′-untranslated region (UTR) of complimentary mRNAs and regulate gene expression by translational inhibition or mRNA degradation (Ghildiyal and Zamore, 2009). While miRNA biogenesis and function in insects have been studied in detail only in

* Corresponding author. Tel.: +86 10 64806293; fax: +86 10 64807099.
E-mail address: szhou@ioz.ac.cn (S. Zhou).

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D. melanogaster, a number of conserved and specific miRNAs have been sequenced and characterized in various orders of insects (Cristino et al., 2011; Jagadeeswaran et al., 2010; Legeai et al., 2010; Li et al., 2009; Luo et al., 2008; Rubio et al., 2012; Stark et al., 2007; Wei et al., 2009). Certainly, 20E appears to have a role in miRNA expression. 20E increases the expression of *let-7*, *miR-100* and *miR-125* which are involved in the metamorphosis of both holometabolous and hemimetabolous insects (Chawla and Sokol, 2012; Rubio et al., 2012; Sempere et al., 2003). Levels of *miR-34* and *miR-252* are reduced in response to 20E in the development of the fruit fly and cockroach (Rubio et al., 2012; Sempere et al., 2003). In *Drosophila*, miR-14 targets the ecdysone receptor (EcR). 20E down-regulates *miR-14*, which consequently elevates EcR levels and amplify the 20E signaling (Varghese and Cohen, 2007). 20E also decreases *miR-8* expression levels, which results in enhanced levels of U-shaped (USH), a PI3 kinase inhibitor in insulin pathway to control the body size of *Drosophila* (Jin et al., 2012). In *Drosophila* S2 cells, the expression of *let-7* and *miR-125* is robustly induced by 20E, and such induction appears to be repressed by the subsequent application of the JH analog, methoprene (Garbuzov and Tatar, 2010). 20E treatment of final female cockroach nymphs mimics the nymph–adult transition and results in the down-regulation of *miR-252* and the up-regulation of *miR-1* and *miR-100*, whereas 20E plus JH which represents that of nymph–nymph transition significantly decreased expression of *miR-252*, *miR-100*, *miR-276*, *miR-190*, *miR-14*, *let-7*, *miR-125* and *bantam* (Rubio et al., 2012). However, although there are descriptions of 20E or JH effects on miRNAs, there are no experiments directly linking miRNA regulation and JH signaling in insect vitellogenesis and oocyte maturation.

Dicer 1 (Dcr1) and Argonaute 1 (Ago1) are two major enzymes in miRNA biogenesis and function (Azzam et al., 2012; Bryant et al., 2010; Tanaka and Piulachs, 2012); however, the function of Ago1 in panoistic ovaries is unknown. Here we used the migratory locust and we report for the first time that miRNAs are expressed in response to JH during vitellogenesis, and that Ago1 and Ago1-dependent miRNAs likely play a critical role in locust *Vg* expression, oocyte maturation and ovarian growth.

2. Materials and methods

2.1. Animals

Migratory locusts were reared in the gregarious phase under 14L:10D photoperiods and at 30 ± 2 °C. The diet included a continuous supply of dry regimen (wheat bran mixed with skim milk powder) with wheat seedlings provided once daily. JH-deprived female adult locusts were obtained by inactivation of the corpora allata with 500 μg of allatocide ethoxyprecocene (precocene) (Sigma–Aldrich) per locust within 12 h after eclosion. To replace JH activity, the active JH analog, s-(+)-methoprene (Sigma–Aldrich) was topically applied to the locusts (150 μg per locust in acetone) 10 d after precocene treatment.

2.2. RNA isolation and QRT-PCR

The fat body and ovary of each locust were collected and stored individually in liquid nitrogen. For gene expression, total RNA was extracted using TRIzol (Invitrogen) following the manufacturer's instruction. cDNA was reverse-transcribed with oligo(dT) primer and M-MLV reverse transcriptase (Promega). Quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) was performed using Mx3005P detection system (Agilent) at 95 °C for 2 min, and then 40 cycles at 95 °C for 20 s followed by 58 °C for 20 s and 68 °C for 1 min with β-actin as the endogenous control. cDNA equivalent to 15 or 20 ng of total RNA, 0.2 μM primer pairs and SYBR Green Real Master Mix (Tiangen) were used. For miRNA, small RNA was isolated using miRcute small RNA isolation kit (Tiangen) and cDNA was prepared by using microRNA first strand cDNA synthesis kit (Tiangen). QRT-PCR for miRNA was performed on Mx3005P detection system using miRcute miRNA qPCR kit (Tiangen) with cDNA equivalent to 5 ng of small RNA at 94 °C for 2 min, and 40 cycles at 94 °C for 20 s, 60 °C for 34 s with small nuclear RNA U6 as the reference. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels. Primers used for QRT-PCR are listed in Supplementary Table S1.

2.3. miRNA identification

miRNAs were sequenced and quantified by using Illumina sequencing technology (HiSeq™, 2000) from three small RNA libraries of JH-deprived fat body, and methoprene-induced fat bodies for 24 h and 48 h following precocene treatment. Empty adaptors, low quality sequences, rRNAs, tRNAs, and snoRNAs were eliminated from the raw sequences. miRNA were subsequently identified by referring to the genome database of *L. migratoria* (unpublished data). To recognize conserved miRNAs, similarity alignment against miRBase 19 was used. Candidate miRNAs with two mismatches to known conserved miRNA were considered as potential locust-specific miRNAs (Lm-miRNA).

2.4. RNA interference (RNAi)

Double strand RNAs (dsRNAs) for locust Ago1 (*LmAgo1*, accession number KF006338), Dicer 1 (*LmDcr1*, accession number JQ900305) and green fluorescent protein (GFP) were synthesized using T7 RiboMAX Express system (Promega) according to the manufacturer's manual. Primers for dsRNA synthesis were summarized in Table 1. For each gene, 32–35 female adult locusts were injected with 18 μg dsRNA (5–6 μg/μl) within 12 h after eclosion and boosted at 5 days after eclosion. dsRNA of GFP, which has no endogenously mRNA target in the migratory locust, was used as the mock control.

2.5. Tissue images and cell staining

Images of ovary and ovariole were captured with a Canon EOS550D camera and Olympus CKX41 microscope, respectively. The length of ovarioles was measured using the Leica M205C microscope. For actin and nuclei staining of oocyte follicular epithelium cells, ovaries were dissected in locust physiology saline (LPS) containing 150 mM NaCl, 10 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 4 mM $NaHCO_3$, 5 mM HEPES (pH 7.2), 90 mM Sucrose and 5 mM trehalose (da Silva and Lange, 2006). Sheath-free ovarioles were fixed in the 4% paraformaldehyde in PBS at room temperature for 20 min. After washing three times with PBS, ovarioles were permeabilized in LPS

Table 1
Primers used for *LmAgo1* and *LmDcr1* dsRNA synthesis.

Genes and primer	Nucleotide sequence (5′ to 3′)
LmAgo1	
dsLmAgo1_F	GTTCCGAGTATTAGGCCAA
dsLmAgo1_R	TTCCAGCAGGTATGTTTCC
dsLmAgo1_F-T7	TAATACGACTCACTATAGGGTTCCGAGTATTAGGCCAA
dsLmAgo1_R-T7	TAATACGACTCACTATAGGTTCCAGCAGGTATGTTTCC
LmDcr1	
dsLmDcr1_F	ACAAATCTTTACTCCTGTCGCT
dsLmDcr1_R	TTTCTTCTCCAAATCCTTCATAC
dsLmDcr1_F-T7	TAATACGACTCACTATAGGACAAATCTTTACTCCTGTCGCT
dsLmDcr1_R-T7	TAATACGACTCACTATAGGTTTCTTCTCCAAATCCTTCATAC

Underlined sequences indicate the T7 adaptor. F = forward primer; R = reverse primer.

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