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Ecdysteroid promotes cell cycle progression in the *Bombyx* wing disc through activation of c-Myc

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ABSTRACT

Developmental switching from growth to metamorphosis in imaginal primordia is an essential process of adult body planning in holometabolous insects. Although it is disciplined by a sequential action of the ecdysteroid, molecular mechanisms linking to cell proliferation are poorly understood. In the present study, we investigated the expression control of cell cycle–related genes by the ecdysteroid using the wing disc of the final-instar larvae of the silkworm, *Bombyx mori*. We found that the expression level of *c-myc* was remarkably elevated in the post-feeding cell proliferation phase, which coincided with a small increase in ecdysteroid titer. An *in vitro* wing disc culture showed that supplementation of the moderate level of the ecdysteroid upregulated *c-myc* expression within an hour and subsequently increased the expression of cell cycle core regulators, including A-, B-, D-, and E-type *cyclin* genes, *Cdc25* and *E2F1*. We demonstrated that *c-myc* upregulation by the ecdysteroid was not inhibited in the presence of a protein synthesis inhibitor, suggesting a possibility that the ecdysteroid directly stimulates *c-myc* expression. Finally, results from the administration of a c-Myc inhibitor demonstrated that c-Myc plays an essential role in 20E-inducible cell proliferation. These findings suggested a novel pathway for ecdysteroid-inducible cell proliferation in insects, and it is likely to be conserved between insects and mammals in terms of steroid hormone regulation.

1. Introduction

Developmental switching from growth to metamorphosis is an essential process in determining appropriate body size and shape in holometabolous insects (Mirth and Riddiford, 2007; Andersen et al., 2013; Ninov and Martin-Blanco, 2014; Nijhout and Callier, 2015). These developmental transitions are strictly controlled by endocrine systems. The steroid hormone ecdysteroid is the master regulator of insect ecdysis and metamorphosis and has been a focus in extensive investigations (Riddiford et al., 2003; King-Jones and Thummel, 2005; Cranna and Quinn, 2009; Yamanaka et al., 2013).

The molecular pathway of the ecdysteroid signaling cascade is best documented in regard to metamorphosis (White et al., 1997; Riddiford et al., 2003). An active form of ecdysone, 20-hydroxyecdysone (20E), binds with the heterodimer of two nuclear receptors, the ecdysone receptor (EcR) and the ultraspiracle (USP). This complex promotes the expression of primary-response transcription factors by interacting with an ecdysone response element (EcRE) in the promoter region (e.g., Burtis et al., 1990; Segraves and Hogness, 1990; White et al., 1997). The rise of these transcription factors hierarchically induces the expression of a series of genes executing metamorphosis, e.g., degeneration of larval organs (Jiang et al., 2000), cuticular formation (Wang et al., 2009a, 2010) and tanning (Hiruma and Riddiford, 2009), and induction of ecdysing behaviors (Zitnan et al., 2007).

Ecdysteroid control of cell proliferation in imaginal primordia plays a central role in appropriate adult organ construction (Cranna and Quinn, 2009; Quinn et al., 2012). Some reports have highlighted the importance of a small increase in 20E titer prior to the main prepupal or pupal surge. A moderate 20E increase of less than one-tenth of the main peak level promotes cell proliferation in undifferentiated imaginal primordia (Yaginuma et al., 1988; Kawasaki, 1995a; Champlin and Truman, 1998a). Such responses are essential for the differentiation stimulated by the subsequent

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main 20E surge and could affect the size and morphology of adult organs (Champlin and Truman, 1998b; Nijhout and Grunert, 2010). Nevertheless, investigations of the molecular signaling pathway that links a 20E cue and cell cycle regulation are quite limited.

The only example that unveils the signaling pathway has been demonstrated in the wing disc of *Drosophila melanogaster* (Mitchell et al., 2008, 2013). In this system, the ecdysteroid stimulates the expression of a transcription factor Crol, which subsequently enhances the expression of dMyc (the *Drosophila* orthologue of a proto-oncogene c-Myc), Stg/Cdc25, and CycB by suppressing the Wg/Wnt pathway. The arising question is whether Crol-mediated induction of c-Myc and CycB is a common pathway for ecdysteroid control of cell proliferation in imaginal primordia among insects. Responses to ecdysteroid signaling vary with different tissues (Quinn et al., 2012), developmental stages (Koyama et al., 2004; Fujiwara and Ogai, 2001), and concentrations (Yaginuma et al., 1988; Kawasaki, 1995a; Champlin and Truman, 1998a, b). Moreover, a mammalian steroid hormone promotes cell proliferation through a different c-Myc activation pathway (Han et al., 2006; Wang et al., 2011).

The present study aimed to clear the molecular pathway of cell cycle regulation by the ecdysteroid at developmental switching by using the wing disc of *Bombyx mori*. During the final larval stage, the *Bombyx* wing disc undergoes a clear developmental change: after achieving the highest body weight, wing disc growth becomes liberated from the larval body, and the disc exponentially expands with an increase in the cell number at the post-feeding stage (Fig. 1; Kurushima and Ohtaki, 1975). It is suggested that a moderate increase in ecdysteroid titer governs this developmental transition of the cell cycle status (Kawasaki, 1995a, 1998). Therefore, we investigated the molecular pathway involved in ecdysteroid-inducible cell-cycle regulation by pursuing expression profiles of canonical cell cycle genes and their regulatory factors. Our results suggested that, unlike the known pathway in *Drosophila*, the moderate level of ecdysteroids provokes the expression of cell cycle–related genes via direct activation of the c-Myc gene.

2. Materials and methods

2.1. Insects and wing disc culture

The present study used an F1 hybrid race of laboratory-maintained strains (N124 and C124) of *B. mori*. Larvae were reared on mulberry leaves or an artificial diet (Nihon Nosan Kogyo) at 25 °C under a 12 h light and 12 h dark photoperiod.

Larvae five days after the fourth molting were used for *in vitro* culture experiments. Forewing discs were dissected in phosphate-buffered saline (PBS, pH 7.4). Tissue fragments attached to the forewing discs, except for tracheal pads, were removed as completely as possible, and the discs were rinsed in PBS three times and then in Grace's insect culture medium (Invitrogen) once. Four discs were incubated in a culture dish containing 1 ml of Grace's medium at 25 °C as described previously (Kawasaki, 1989). After a preculture in a hormone-free medium for 24 h, 20E (Sigma) was added at a final concentration of 0.5–2000 ng/ml and kept for up to another 24 h. In some experiments, the c-Myc inhibitor, 10058-F4 (Sigma), was added at a final concentration of 100 μM (Huang et al., 2006). In another experiment, wing discs were soaked in a medium containing cycloheximide (Sigma) at a final concentration of 50 μg/ml for 1 h before 20E was supplied.

2.2. Identification of cell cycle–related genes in B. mori

Bombyx orthologues of cell cycle control genes, including *cycA*, *cycB*, *cycD*, *cycE*, *Cdc25*, *E2F1*, and *E2F4*, were identified using the silkworm genome database (http://sgp.dna.affrc.go.jp/) and the National Center for Biotechnology Information (NCBI) BLAST similarity search program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). *Bombyx* homologues of *Drosophila dmyc* (FBgn0262656), *wg* (FBgn0004009), and *crol* (FBgn0020309) were also determined

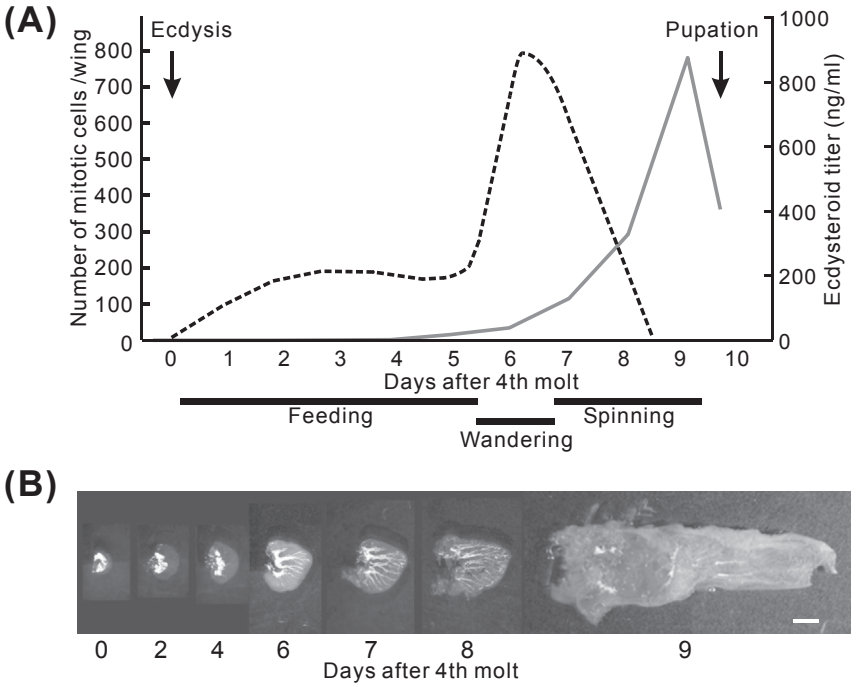

Fig. 1. Developmental profiles of cell proliferation in wing discs during the last larval stage in *Bombyx mori*. (A) Schematic representation of time-course changes in the number of mitotic cells (black broken line, derived from Kurushima and Ohtaki (1975) and Kawasaki (1998)) and ecdysteroid titer (gray line, derived from Sakurai et al., 1998). (B) Photographs of developing wing discs. The scale bar indicates 1 mm.