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MINIREVIEW

Autophagy studies in *Bombyx mori*

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Abstract

Autophagy, which is well conserved from yeast to mammals, plays essential roles in development and diseases. Using the domesticated silkworm, *Bombyx mori*, as a model insect, several reports on autophagy have been made recently. Autophagic features are observed in the midgut and fat body during the larval-pupal transition as well as the silk gland and ovarian nurse cells during the pupal stage. There are 14 autophagy related (*Atg*) genes, including at least two transcript variants of *Atg1*, predicated in *Bombyx*. Expression of most *Atg* genes is consistent with the autophagy process in the fat body during the larval-pupal transition, and reduction of *Atg1* expression by RNAi blocks this process. The molting hormone, 20-hydroxyecdysone (20E), and starvation induce autophagy in the fat body by upregulating *Atg* gene expression and blocking the PI3K-TORC1 pathway. Meanwhile, autophagy precedes apoptosis in the midgut and other larval tissues during the larval-pupal transition, while the detailed mechanism is not illustrated yet. We assume that there are at least four future directions about autophagy studies in *Bombyx* during the next years: (1) physiological functions of autophagy; (2) identification of new components involved in the autophagy process; (3) detailed molecular mechanism of autophagosome formation; (4) functional relationship between autophagy and apoptosis.

Key Words: autophagy; *Atg* genes; 20-hydroxyecdysone; *Bombyx mori*

Introduction

Macroautophagy (hereafter referred to as autophagy) is well conserved from yeast to mammals. Autophagy was first discovered in the mouse kidney with membrane-bound compartments termed “dense bodies”, which were subsequently shown to include lysosomal enzymes (Clark, 1957; Novikoff, 1959). Autophagy is responsible for bulk degradation of intracellular long-lived proteins, superfluous organelles, or clear of invading microorganisms, playing key roles in many physiological and developmental processes, such as cell survival, cell death, metabolism and innate immunity (Yang and Klionsky, 2010). Under certain circumstances, weak autophagy helps cell survival, whereas extensive autophagy leads to cell death (Shintani and Klionsky, 2004). Autophagy is involved in the utilization of intracellular lipids to maintain cellular energy homeostasis: during fasting condition, autophagy is triggered to enclose droplet parts for lipid degradation, while fed with high-fat diet, blocking of autophagy causes lipid accumulation (Singh *et al.,* 2009). Moreover, abnormal autophagy is related to many human diseases, such as neurodegenerative diseases (Alzheimer's and Huntington’s) and tumors (Yang and Klionsky, 2010). During the last decade, autophagy has been a hot topic in the biology field. The process of autophagy is marked by the formation of autophagosome and autolysosome. Autophagy is governed by a series of autophagy-related (Atg) protein complexes, and the core machinery of autophagosome formation involves at least four Atg protein complexes. Autophagosome initiation requires the Ulk1/Atg1-Atg13 protein kinase complex. Autophagosome nucleation involves the Beclin-1/Atg6-PIK3C3/Vps34-Atg14L complex. Autophagosome expansion and completion is governed by two ubiquitin-like conjugating systems: Atg5-Atg12-Atg16L1 and Atg8-PE conjugates (Boya *et al.,* 2013; Jin and Klionsky, 2013). The Atg12-Atg5-Atg16L1 complex is formed via Atg16

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homooligomerization and acts like a dimer at the outer membrane of the phagophore disassociated from the phagophore near the time of autophagosome completion. Unlike canonical ubiquitination, Atg12-Atg5 conjugation is irreversible (Mizushima *et al*., 2003). Atg8-PE conjugation localizes on both the outer and inner membrane of the phagophore, and Atg8-PE is the only Atg protein remained in mature autophagosome. Therefore, Atg8-PE is usually used as a marker of autophagosome formation (Levine and Klionsky, 2004). Finally, the mature autophagosomes fuse with lysosomes to form autolysosomes, in which bulk degradation of dysfunctional proteins, unnecessary organelles, and invading microorganisms is completed (Klionsky *et al.*, 2012).

Autophagy is triggered in response to various unfavorable conditions, such as starvation. Starvation triggers autophagy by inhibiting PI3K-Akt-TORC1 pathway combined with inducing expression of some *Atg* genes. Under favorable conditions, TORC1 phosphorylates and inactivates the Ulk1/Atg1-Atg13 protein kinase complex to inhibit autophagosome initiation (Levine and Klionsky, 2004). Under glucose deprivation, the energy sensor AMPK not only inhibits TORC1 activity, but also directly phosphorylates and activates the Ulk1/Atg1-Atg13 protein kinase complex to induce autophagosome formation (Lippai *et al.,* 2008; Egan *et al.,* 2011). Moreover, in response to amino acid starvation, the phosphorylation of beclin-1/Atg6 by ULK1/Atg1 is required for full autophagic induction (Kim *et al.,* 2013; Russell *et al.,* 2013). In insects, the molting hormone (20-hydroxyecdysone, 20E) signaling, including 20E-EcR/USP complex and its downstream transcriptional factors, induces autophagy by blocking PI3K-Akt-TORC1 pathway and upregulating *Atg* genes (Baehrecke, 2003; Tian *et al.,* 2013; Tracy and Baehrecke, 2013; Liu *et al.,* 2013, 2014; Yin and Thummel, 2005).

Apoptosis is the major type of programmed cell death (PCD) in organisms, autophagy is considered as the second type of PCD, and the relationship between autophagy and apoptosis is complex. Under certain circumstances, autophagy prevents cell death from apoptosis, whereas extensive autophagy will cause cell death (Shintani and Klionsky, 2004). Dying cells often display accumulation of autophagosomes, and adopt a morphology called autophagic cell death. It is usually agreed that this case is cell death with autophagy rather than cell death by autophagy (Kroemer and Levine, 2008). In *Drosophila*, both autophagy and caspases function in parallel, contributing to autophagic cell death in the dying salivary gland during metamorphosis, but autophagy plays a more important role than caspases (Berry and Baehrecke, 2007; Scott *et al.,* 2007). Moreover, autophagy, but not caspases, governs cell death in the midgut during metamorphosis (Denton *et al*., 2009). A balancing crosstalk occurs between autophagy and caspase activity in the remodeling fat body, as the inhibition of autophagy induces caspase activity and the inhibition of apoptosis induces autophagy (Liu *et al*., 2013, 2014).

The domesticated silkworm, *Bombyx mori*, emerges as a model organism not only for lepidopterans but also for general biology (Xia *et al*., 2014). In view of the importance of autophagy, we here summarize the associated reports in *Bombyx* and wish to inspire *Bombyx* studies on autophagy in the future.

Autophagy detection in Bombyx

In insects, autophagy was first described in the larva of the butterfly, *Calpodes ethlius* by morphological observation (Locke and Collins, 1965). Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes were recently emphasized by the autophagy consortium. Methods corresponding to monitor the numbers or volume of autophagic compartments (*e.g.,* autophagosomes or autolysosomes) and to measure autophagic flux are summarized (Klionsky *et al.,* 2012). Morphological observation by transmission electron microscope (TEM) is still the most important method to detect autophagic compartments. By observation using TEM, the autophagic features, such as vacuoles, autophagosome, high-density bodies, and autolysosomes could be distinguished clearly in *Bombyx* organs during metamorphosis (Sumithra *et al*., 2010; Franzetti *et al.,* 2012; Tian *et al*., 2013). Atg8 is the most widely monitored Atg protein, and Atg8-PE is an efficient indicator of autophagy in mammals (Klionsky *et al.,* 2012). An antibody against *Bombyx* Atg8 was generated, which is able to detect the protein level of both Atg8 and Atg8-PE by western blotting (Franzetti *et al*., 2012; Tian *et al*., 2013) and the aggregated puncta of Atg8 by immunochemistry (Franzetti *et al*., 2012). The fusion of autophagosomes with lysosomes can be visualized by Lyso Tracker Red staining, which successfully represents the trend of autophagy in the remodeling fat body as detected by TEM (Tian *et al*., 2013). In addition, the activity of acid phosphatase can partially and indirectly reflect autophagy (Franzetti *et al*., 2012; Tian *et al.,* 2013).

When autophagy is referred in *Bombyx* and other lepidopterans, we suggest that at least two detection methods should be used simultaneously. For example, both TEM observation and Lyso Tracker Red staining are necessary and sufficient to detect autophagy in the *Bombyx* fat body.

Atg genes and Atg proteins in Bombyx

Fourteen yeast or mammalian homologous *Atg* genes (*Atg1*, *Atg2*, *Atg3*, *Atg4*, *Atg5*, *Atg6*, *Atg7*, *Atg8*, *Atg9*, *Atg11*, *Atg12*, *Atg13*, *Atg16* and *Atg18*) were predicated in *Bombyx*, most of which contain the conserved ATG protein domains (Zhang *et al*., 2009; Tian *et al*., 2013). Either Atg1 or Atg6 harbors a protein kinase domain at its N-terminus. Crystal structure of Atg8 reveals an ubiquitin-fold domain at its C-terminus, which is similar to Atg8 proteins identified from other organisms. In addition, there are two helices at the N-terminus of BmAtg8 (Hu *et al*., 2010). Until now, two transcript variants of *BmAtg1* were reported, which are evolutionary conserved with the orthologs from *Drosophila* (Casati *et al*., 2012). We found another *Atg1* transcript variant, which is shorter but more abundant than the previously reported ones (Li *et al.,* unpublished data).

Induction of autophagy in Bombyx

20E and starvation are the two important stimuli of autophagy in insects. In the anterior silk gland in *Bombyx*, autophagy emerges right after the appearance of EcR-B1 protein as well as the transcripts of *EcR*, *E74A* and *Br-C*, suggesting that 20E signaling induces autophagy in this organ (Goncu and Parlak, 2009; Li *et al*., 2010). As revealed by a series of cellular, biochemical, molecular, and genetic studies in the fat body, 20E induces autophagy by upregulating *Atg* gene expression and blocking the PI3K-TORC1 pathway (Tian *et al*., 2013). Starvation can trigger autophagy in the absence of 20E in *Drosophila* (Chang and Neufeld, 2010; Jin and Klionsky, 2013). In the *Bombyx* fat body, starvation not only blocks TORC1 activity, which phosphorylates and inactivates the Atg1-Atg13 complex in feeding condition, but also upregulates some *Atg* genes (Tian *et al.,* 2013). Nevertheless, the induction of *Atg1* expression in the mdigut is much less significant than that in the fat body (Casati *et al*., 2012). Notably, 20E reduces food consumption and causes starvation-like condition during molting and pupation in *Bombyx*, providing a correlation between 20E and starvation in the induction of autophagy (Wang *et al*., 2010). The observations on autophagy during the *Bombyx* metamorphosis suggest that autophagy may play a role in preventing the onset of cell death under certain nutrient-deprivation conditions (Romanelli *et al*., 2014).

In addition, Infected by a tachnid parasitoid, *Exorista bombycis* , cell death is induced in the *Bombyx* larval integumental epithelial cells with existence of Atg5 protein and upreguation of *Atg5* expression, which are associated with signs of autophagy (Anitha *et al*., 2014). This report may inspire the studies on how autophagy is involved in immune responses after parasitoid or pathogen infection.

Induction of autophagy in *Bombyx* is summarized in Figure 1.

Autophagic cell death and apoptosis in Bombyx

The relationship between autophagy and apoptosis is context-specific in *Drosophila* (Liu et *al.,* 2013). During *Bombyx* metamorphosis, it appears that autophagy precedes apoptosis in general, and that autophagy might contribute to cell death in a variety of larval tissues, *e.g.,* in the midgut and fat body during the larval-pupal transition as well as in the anterior silk gland and ovarian nurse cells during the pupal stage.

In the remodeling midgut, autophagy precedes apoptosis and gradually synergizes together to mediate its demise (Franzetti *et al*., 2012). Autophagic features were observed in the disintegrated perivisceral fat body cells, and considered to attribute to autophagic cell death (Sumithra *et al*., 2010). In the peripheral fat body tissues isolated from the 5th abdominal segment, there was co-existence of autophagy and caspase activity during the larval-pupal transition (Tian *et al.,* 2012, 2013). In the anterior silk gland, autophagy appears earlier than apoptosis, and they might interact with each other during its degeneration process (Goncu and Parlak, 2008; Li *et al*., 2010). During the middle stage of vitellogenesis, paraptosis precedes both apoptosis and autophagy, which in later stage operate synergistically to result in a more efficient elimination of the degenerated nurse cells (Mpakou *et al*., 2006, 2008).

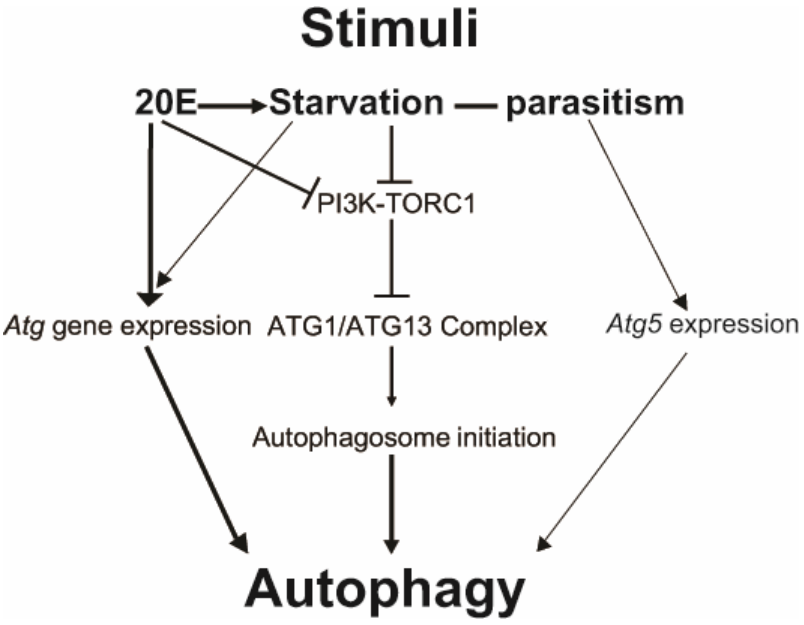

Fig. 1 Induction of autophagy in *B. mori.* The upstream stimuli of autophagy in *Bombyx* include 20E, starvation and parasitism. 20E induces autophagy mainly by upregulating *Atg* gene expression and the inhibition of TORC1 activity. 20E reduces food consumption and causes starvation-like condition. Starvation leads to autophagy mainly by the inhibition of TORC1 activity and partially by the induction of *Atg* gene expression. Parasitism might cause autophagy by the induction of *Atg5* expression.

In *Bombyx* SPC Bm36 cells, amino acid starvation rapidly induces autophagy, inhibition of autophagy at the early stage of starvation results in necrotic-like cell death. In addition, parasitism causes autophagy preceding apoptosis in *Bombyx* integumental epithelium (Anitha *et al*., 2014). These data indicate that autophagy prevents *Bombyx* cells and other Lepidoptera insect cells from death at an early stage of non-favorable conditions (Wu *et al*., 2011; Romanelli *et al*., 2014). The underlying molecular mechanism of the functional relationship between autophagy and apoptosis requires further investigation in detail.

Future directions

Despite several reports on autophagy have been made in *Bombyx* during the last years, autophagy is still a new research area in this model insect. We assume that there are at least four future directions about autophagy studies in *Bombyx* during the next years.

1. Physiological functions of autophagy

Previous studies suggest that autophagy plays essential roles in cell survival and cell death in *Bombyx* and other insects. Most likely, during *Bombyx* metamorphosis, weak autophagy helps cell survival at the onset of cell death, whereas extensive autophagy exaggerates and eventually causes cell death. Autophagy is suspected to recycle of cell components derived from larval midgut degeneration (Franzetti *et al.*, 2012, 2015). Moreover, it has been long thought that autophagy is involved in the mobilization of stored nutrients in the larval fat body, but little evidence has been provided and no detailed studies has been reported. We suppose that autophagy might play essential roles in regulating cell fate and metabolism during *Bombyx* metamorphosis. With the rapid development of powerful genetic tools, including systematic RNAi, baculovirus-mediated overexpression, piggyBac-mediated gene function, and TALEN- or Cas9-mediated genome editing, we should be able to deeply investigate the physiological functions of autophagy in *Bombyx* within the coming years.

2. Identification of new components involved in the autophagy process

In recent decades, autophagy is well documented in yeasts, but many questions remain in high organisms. Although many *Atg* genes are conserved from yeast to insects to humans (Chang and Neufeld, 2010; Malagoli *et al*., 2010), the molecular mechanism how Atg proteins collaborate to form autophagosome and to originate autophagosome membrane are largely unknown in higher eukaryotes, and these questions are worthy of further investigation in *Bombyx*. So far, only 14 homologous *Atg* genes have been predicted in *Bombyx*, we suspect that many more *Atg* genes in this organism are still unknown. Using those known Atg proteins as baits to prey their interacted proteins via immunoprecipitation and yeast-two-hybrid, we should be able to identify some new components involved in autophagy in *Bombyx*. This study will be very meaningful from both functional and evolutionary views.

3. Detailed molecular mechanism of autophagosome formation

Once the new components in the four Atg protein complexes have been identified, we should have a better idea about the detailed molecular mechanism of autophagosome formation in *Bombyx*, at least from a comparative prospective. It will be very interesting to examine how the Atg protein complexes are modified at the post-transcriptional levels. A detailed bioinformatic analysis revealed that the phosphorylation sites identified from mammalian ULK1 are not conserved in the insect Atg1 proteins (Li *et al*., unpublished data). We are currently investigating phosphorylation modifications of the *Bombyx* Atg1-Atg13 protein kinase complex by AMPK and TORC1, which might be different from the *Drosophila* Atg1-Atg13 protein kinase complex as well. A comparative analysis of the phosphorylation sties in the Atg1-Atg13 complex will shed light on the evolution of autophagosome formation from yeast to insects to humans.

4. Functional relationship between autophagy and apoptosis

The functional relationship between autophagy and apoptosis is complex, and it is context-specific in *Drosophila*. Although it is known that autophagy precedes apoptosis in *Bombyx*, the detailed molecular mechanism has not been understood yet. In mammalian cells, Atg4, Atg5, and Beclin-1/Atg6 act as the molecular switches between autophagy and apoptosis (Betin and Lane, 2009; Yousefi *et al*., 2006; Wirawan *et al.,* 2012), whether those homologous Atg proteins (and other unknown proteins) play similar roles in regulating autophagy-mediated apoptosis should be examined in *Bombyx*.

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