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...
homooligomerization and acts like a dimer at the outer membrane of the phagophore dissociated 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 Thrummel, 2005).

Apoptosis is the major type of programmed cell death (PCD) in organisms, autophagy was 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 caspase 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 mid gut 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 immunocytochemistry (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, 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).

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**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., 2008; 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 BmAgt1 were reported, which are evolutionarily 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 Parlaı̈k, 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 midgut 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 contribute 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 Parlaı̈k, 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).

Stimuli

20E → Starvation — parasitism

\[ \text{PI3K-TORC1} \]

Atg gene expression \hspace{1cm} ATG1/ATG13 Complex \hspace{1cm} Atg5 expression

Autophagosome initiation

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 sites 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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**References**


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Egan DF, Kim J, Shaw RJ, Guan KL. The autophagy initiating kinase ULK1 is regulated via opposing phosphorylation by AMPK and mTOR. Autophagy 7: 643-644, 2011.


