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Midgut epithelium in molting silkworm: A fine balance among cell growth, differentiation, and survival

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ABSTRACT

The midgut of insects has attracted great attention as a system for studying intestinal stem cells (ISCs) as well as cell death-related processes, such as apoptosis and autophagy. Among insects, Lepidoptera represent a good model to analyze these cells and processes. In particular, larva–larva molting is an interesting developmental phase since the larva must deal with nutrient starvation and its organs are subjected to rearrangements due to proliferation and differentiation events. Several studies have analyzed ISCs *in vitro* and characterized key factors involved in their division and differentiation during molt. However, *in vivo* studies performed during larva–larva transition on these cells, and on the whole midgut epithelium, are fragmentary.

In the present study, we analyzed the larval midgut epithelium of the silkworm, *Bombyx mori*, during larva–larva molting, focusing our attention on ISCs. Moreover, we investigated the metabolic changes that occur in the epithelium and evaluated the intervention of autophagy.

Our data on ISCs proliferation and differentiation, autophagy activation, and metabolic and functional activities of the midgut cells shed light on the complexity of this organ during the molting phase.
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1. Introduction

In insects, postembryonic development is characterized by alternating between feeding and molting stages, the latter culminating in ecdysis. During this process, the insect loosens the connections between its living tissues and the extracellular cuticle, escapes from it, takes up water or air to expand the new, flexible exoskeleton, and then quickly hardens it for purposes of defense and locomotion (Wigglesworth, 1972). This is a life-threatening process since a failure in the sequence of events, which are triggered by hormones and involve the coordinated expression of a wide array of genes, can result in the insect's death (Riddiford et al., 2003; Zhao et al., 2006). Together with the growth that occurs during the intermolt period, the gain in size during the intramolt phase (molt increment) also contributes to the growth of the insect body (Gullan and Cranston, 2014). In insects, different types of stem cells are recruited during molting to promote the growth and remodeling of the larval organs (Corley and Lavine, 2006). In this context, the larval midgut of holometabolous insects is no exception.

The alimentary canal of the larva is subdivided into three major regions, namely, the foregut, the midgut, and the hindgut, which have different functional roles in feeding and digestion. The foregut is primarily responsible for food ingestion and storage, the hindgut is implicated in the osmotic regulation of internal fluids, while the midgut is involved in nutrient digestion and absorption (Dow, 1986). In lepidopteran larvae, the midgut consists of a

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monolayered epithelium essentially formed by columnar cells, which produce digestive enzymes and absorb nutrients, and goblet cells, which are involved in ion transport (Terra and Ferreira, 2005). Two other cell types are found in the midgut, namely, endocrine and intestinal stem cells (ISCs). Endocrine cells are sparse in the epithelium and produce the gastrointestinal hormones needed to control the production and secretion of digestive enzymes and for proliferation and differentiation of ISCs (Sehnal and Zitnan, 1996; Rost-Roszkowska et al., 2008). ISCs reside on the basal lamina that supports the epithelium, among columnar and goblet cells. ISCs repair the damaged midgut by replacement in order to maintain the functional integrity of the tissue (Smagghe et al., 2005; Hakim et al., 2010). In addition to the repair function, ISCs proliferate extensively at each larva–larva molt and then intercalate between the mature cells, differentiating into columnar and goblet cells (Baldwin and Hakim, 1991b; Baldwin et al., 1996). A third role of ISCs is to generate a new functional midgut at larva–pupa molting, which is maintained up to the adult stage (Tettamanti et al., 2007a; Franzetti et al., 2015). In the last few decades, studies on cultured stem cells derived from the midgut of different lepidopteran species have provided insights into the factors that control proliferation and differentiation of these cells (Smagghe et al., 2003; Blackburn et al., 2004; Loeb et al., 2004; Smagghe et al., 2005). This work yielded important information about the setup of midgut primary cultures (Casartelli et al., 2007; Hakim et al., 2009) and their use as a tool to study the physiological properties of midgut cells *in vitro* (Casartelli et al., 2008).

While the *in vitro* investigation reported above has helped us to better understand some of the regulatory mechanisms involved in the proliferation and differentiation of ISCs, *in vivo* studies are scarce. In particular, although the behavior of these stem cells during the larva–pupa transition has been characterized in detail (Uwo et al., 2002; Tettamanti et al., 2007a, 2008; Franzetti et al., 2012; Franzetti et al., 2015), only little information is available on ISCs during the larva–larva molting phase and, more in general, on the events occurring in the larval midgut epithelium during this larval developmental process (Baldwin and Hakim, 1991a, 1991b; Baldwin et al., 1993). Moreover, it is worthy of note that a proteomic study performed in *Helicoverpa armigera* molting larvae did not identify any obvious, differentially expressed protein spots in the midgut, thus suggesting that minor metabolic changes occur in this organ during larva–larva molt (Zhao et al., 2006). This evidence undoubtedly adds further complexity to the analysis of the whole organ. This lack of, or only fragmentary knowledge could hinder the full exploitation of lepidopteran midgut as a model system to study stem cell biology. Furthermore, full characterization of the events that occur in the midgut during larval–larval molting would provide additional advantages. In particular, during this developmental phase, the insect stops feeding, thus offering an opportunity to study the metabolic processes that are set in motion in this organ by physiological starvation.

In the present work (i) we studied the morphology and functional activity of the midgut epithelium of the silkworm, *Bombyx mori*, during larva–larva molting (IV to V larval instar), focusing our attention on ISCs; (ii) we analyzed the metabolic changes that occur in this organ; and (iii) we evaluated the activation of autophagy, a self-eating process that can be used by eukaryotic cells to cope with nutrient deprivation (He and Klionsky, 2009).

To our knowledge, this study represents the first morphological and functional characterization of the changes that occur in the midgut of a lepidopteron during larva–larva molt. Our results not only provide important insights into the field of stem cell biology, but also represent a useful reference for further studies on molt processes.

2. Materials and methods

2.1. Experimental animals

B. mori (polyhybrid strain (126 × 57) (70 × 90)) larvae were provided by CREA – Honey Bee and Silkworm Research Unit (Padova, Italy). The larvae were fed on artificial diet (Cappellozza et al., 2005) and reared at 25 ± 0.5 °C under a 12:12 h light:dark period and 70% relative humidity. The silkworm life cycle includes five larval instars separated by molts. At the end of the larval period, the animal undergoes a larval–pupal molt and initiates metamorphosis. Each larval stage lasts about four days, each larva–larva molting approximately 24 h. Since the processes that regulate the growth of organs at each larva–larva molting are conserved (Baldwin et al., 1996), we examined larva–larva molting from fourth to fifth instar in the present study. Here, owing to the considerable dimensions of the larvae, the midgut could be appropriately manipulated.

Larvae at the following stages were selected and used for the analyses:

- L4D3 and L4D4: third and fourth day of the fourth larval instar;
- Stage I: early molting larvae (0–12 h). Initiation of molting phase was determined by assessing the presence of visible signs of molting (initial detachment of the head capsule from the body cuticle);
- Stage II: late molting larvae (12–24 h). End of molting was assessed by the complete loss of the exuvia;
- L5D1: first day of the fifth larval instar.

2.2. Light microscopy and transmission electron microscopy (TEM)

The midgut was isolated from larvae anesthetized with CO_2 and immediately fixed in 4% glutaraldehyde (in 0.1 M Na-cacodylate buffer, pH 7.4) overnight at 4 °C. After postfixation in 1% osmium tetroxide for 1 h, samples were dehydrated in an ethanol series and embedded in resin (Epon/Araldite 812 mixture). Semithin sections were stained with crystal violet and basic fuchsin and observed by using a Nikon Eclipse Ni-U microscope equipped with a DS-5M-L1 digital camera system (Nikon, Tokyo, Japan). Ultrathin sections were stained with uranyl acetate and lead citrate and examined by using a Jeol JEM-1010 electron microscope (Jeol, Tokyo, Japan) equipped with an Olympus Morada digital camera (Olympus, Münster, Germany).

2.3. Morphometric analysis

In order to count the number of ISCs in the midgut epithelium, images of midgut samples from larvae at the end of fourth instar (L4D4) and Stage I, randomly taken at optical microscope (50 × magnification), were morphometrically analyzed. Two hundred images in total (five larvae; ten sections per larva; four images per section) were evaluated for each developmental stage. These sections were at a minimum distance of 50 μm from each other, to avoid counting the same cells twice. For each image, a 100-μm line segment was selected on the basal lamina of the midgut and the number of ISCs localized on this portion of the epithelial layer was counted by using ImageJ software (NIH, Bethesda, USA). Data were analyzed using the Student's *t* test. Results are expressed as the number of ISCs per 100 μm epithelium.

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