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Monitoring stem cell proliferation and differentiation in primary midgut cell cultures from *Heliothis virescens* larvae using flow cytometry

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ABSTRACT

In the midgut of *Heliothis virescens* larvae, proliferation and differentiation of stem cell populations allow for midgut growth and regeneration. Basic epithelial regenerative function can be assessed *in vitro* by purifying these two cell type populations, yet efficient high throughput methods to monitor midgut stem cell proliferation and differentiation are not available. We describe a flow cytometry method to differentiate stem from mature midgut cells and use it to monitor proliferation, differentiation and death in primary midgut stem cell cultures from *H. virescens* larvae. Our method is based on differential light scattering and vital stain fluorescence properties to distinguish between stem and mature midgut cells. Using this method, we monitored proliferation and differentiation of *H. virescens* midgut cells cultured in the presence of fetal bovine serum (FBS) or AlbuMAX II. Supplementation with FBS resulted in increased stem cell differentiation after 5 days of culture, while AlbuMAX II-supplemented medium promoted stem cell proliferation. These data demonstrate utility of our flow cytometry method for studying stem cell-based epithelial regeneration, and indicate that AlbuMAX II-supplemented medium may be used to maintain pluripotency in primary midgut stem cell cultures.

1. Introduction

In lepidopteran larvae, as in most animals, growth and regeneration of digestive epithelia are dependent on stem cell proliferation and differentiation (Hakim et al., 2001; Loeb and Hakim, 1996). Gut stem cells have unlimited self renewal capacity and through division they can generate two types of cells, one functional replicate and another that differentiates to a mature form (Sadrud-Din et al., 1996). This process allows the population of stem cells to remain constant while simultaneously providing new mature cells to maintain homeostasis (Hakim et al., 2010; Watt and Hogan, 2000).

Gut regeneration in lepidopteran larvae has been mostly studied in reference to its relevance to susceptibility to entomopathogens (Forcada et al., 1999; Hoover et al., 2000; Kirkpatrick et al., 1998; Martinez-Ramirez et al., 1999), or parasite infection and colonization (Kotsyfakis et al., 2005; Vlachou et al., 2005). Most of the information on this regenerative process is derived from *in vitro* studies using primary midgut cell cultures (Hakim et al., 2009, 2010). This primary cell culture system allows intrinsic studies on midgut epithelial regeneration (Goto et al., 2005; Hakim et al., 2001; Loeb, 2005, 2006) or pathogenesis (Loeb et al., 2000; Loeb et al., 2001a, 2001b), circumventing potential background from other tissues.

In vertebrate systems, discrimination of mature and stem cells from *in vitro* cultures by flow cytometry is typically accomplished using proliferation markers or cell immunophenotyping (Kang and Sanchez Alvarado, 2009; Keeney and Sutherland, 2000; Preffer and Dombkowski, 2009). Similarly, in invertebrate systems midgut stem cells have been detected by monitoring DNA replication using bromodeoxyuridine (BrdU) incorporation (Illa-Bochaca and Montuenga, 2006; Park and Takeda, 2008; Parthasarathy and Palli, 2008; Tettamanti et al., 2007, 2008). However, BrdU staining includes cell fixation and permeation, which limits the possibility of further experimentation using live cells. In contrast, labeling of stem cells with cell type-specific markers may be accomplished without affecting cell viability (MacArthur et al., 2006; Shen et al., 2008; Weir et al., 2008). In lepidopteran models, there has been a lack of information on stem cell-specific antigens, which has prevented the use of immunophenotyping and multiparameter flow cytometry. Alternative methods to discriminate mature cells from stem cells using flow cytometry have been reported (Goodell et al., 1996), although they have not been attempted on insect cell cultures.

Due to lack of stem cell-specific biomarkers for primary midgut cell cultures in lepidopteran systems, there are no available methods to allow quantitative high throughput detection of

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lepidopteran midgut stem cells *in vitro*. In these primary cultures, mature and stem cells have been discriminated based on gross morphological attributes (Hakim et al., 2009). To address this need, the goal of our project was to develop a method that would allow quantitative discrimination of mature and stem cells using primary midgut cell cultures from *H. virescens* larvae as model, and to use this method in monitoring midgut stem cell proliferation and differentiation. Using differential light scattering and vital staining properties for each cell type, we developed a flow cytometry method able to discriminate mature cells from stem cells in primary cultures from *H. virescens* midgut larvae. Furthermore, we report successful use of this method to monitor stem cell proliferation and differentiation after incubation with supplemented media, which allowed identification of media additives that maintain pluripotency and viability of the stem cells.

2. Materials and methods

2.1. Insects

H. virescens eggs were obtained from laboratory colonies of Benzon Research (Carlisle, PA), or were kindly supplied by Dr. Fred Gould (North Carolina State University). No differences between larvae hatched from eggs of either source were observed in our experiments. Upon hatching, larvae were reared on artificial diet (BioServ, NJ) at 28 °C on a 18 L:6 D photoperiod. Early fourth instar larvae, as determined by time since hatching and size, were anesthetized on ice for 10 min and midguts carefully dissected under sterile conditions and used for preparation of primary cell cultures as described below.

2.2. Establishment of primary mature and stem midgut cell cultures

All dissections and transfers were done in the sterile environment of a biosafety cabinet. Fourth instar larvae were anesthetized on ice for 15 min, and then surface sterilized for 30 s in a cleaning solution (10% Palmolive detergent plus 0.1% Clorox) before dissecting the midgut. After dissection, midguts were cleaned for food, peritrophic matrix, and malphigian tubules using forceps, and then briefly washed in sterile Ringer's (Barbosa, 1974) containing 0.5% (v/v) gentamicin (Invitrogen, CA), 0.1% Clorox, and 1x antibiotic/antimycotic (Invitrogen, CA). Incubation media was prepared by mixing in a 3:1 ratio supplemented Grace's Insect Medium (containing lactalbumin hydrolysate and yeastolate; Invitrogen, CA) containing 1x antibiotic/antimycotic and 0.1% gentamicin with sterile Ringer's. Five to six clean midguts were cut in sections with micro-scissors and incubated in 2 ml of incubation media for 90 min at room temperature. After this incubation, midgut tissue was homogenized carefully by pipetting and sieved through 70 μm cell strainers (BD Biosciences, NJ) into a sterile 50 ml conical tube. Tubes were centrifuged (400*g* for 5 min at 4 °C) and the supernatant discarded. The pellet containing midgut mature and stem cells was suspended in 1 ml of incubation media. Stem cells were separated from mature cells using a density gradient as described elsewhere (Loeb and Hakim, 1999). Briefly, samples were overlaid on 3 ml of Ficoll-Paque (GE Life Sciences, NJ) in a 15 ml culture tube and centrifuged (600*g* for 15 min at 4 °C). After centrifugation, stem cells were collected from the top 0.99 ml, the immediate 2.75 ml containing debris were discarded, and the bottom 0.25 ml containing the pellet was collected for mature cells. Ficoll-Paque was eliminated from stem and mature cell samples by washing twice with incubation media (600*g* for 5 min at 4 °C). Final stem and mature cell pellets were suspended in 0.35 or 1 ml, respectively, of incubation media. Stem and mature cell samples that were prepared simultaneously were pooled and the number of cells counted using a hemocytometer. Using this procedure, we repeatedly obtained approximately 8×10^5 stem cells and 1×10^7 mature cells from 30 larvae. Stem cells were diluted to 4×10^5 cells/mL and mature cells were diluted to 4×10^5 cells/mL with incubation media (as described above) and kept in a sterile incubator at 26 °C.

2.3. Vital staining of primary cell cultures

We used calcein acetoxymethyl ester (calcein AM) to fluorescently stain viable cells in mature and stem cell cultures, as described in the LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen, CA). Using this procedure, activation of calcein AM by intracellular esterases fluorescently labels viable cells in a culture. Calcein AM was briefly acclimated to room temperature, and then diluted to a 20 μM working solution with DMSO. Viable cell staining was done by incubating calcein AM solution with cell cultures (2 μl of working solution per ml of cell culture) for 20 min at room temperature protected from light.

2.4. Flow cytometry

Primary cell cultures were collected in culture tubes and used for flow cytometry analysis using an LSR II flow cytometer (BD Bioscience, CA). Photomultiplier tube voltages were set to 620 eV for forward scatter channel (FSC), 228 eV for side scatter channel (SSC), and 368 eV for the FL1 channel. The threshold was set at 60,200 on the FSC channel to exclude debris. During analysis, distinct cell populations emerged based upon SSC in the *y*-axis and green fluorescence FL1 (calcein fluorescence) in the *x*-axis. Data from gating 5000 cells for each sample were analyzed using the DiVa software (BD Bioscience, CA). Using freshly prepared primary midgut stem and mature cell cultures, two gates to differentiate stem from mature cells were created based on the data from SSC-FL1 dot plots. Primary cell cultures treated with 50% ethanol (v/v) were used to define a third gate containing dead cells. Data were analyzed and graphed using CyflogicTM software (http://www.cyflogic.com; CyFlo Ltd, Finland). Statistical significance was tested using the SigmaPlot v11.0 software (Systat Software Inc., IL). When testing for differences in cell numbers between two samples we used the Student's *t*-test method ($P < 0.05$), while comparisons between multiple samples were tested for significance with analysis of variance (ANOVA) using the Holm–Sidak and Dunn's pairwise multiple comparison procedures ($P < 0.05$).

2.5. Primary cell culture treatments

To determine the effect of media additives on midgut cell cultures, stem or mature cell cultures (4×10^5 cells/mL) were diluted to 2×10^5 cells/mL in (1 ml final volume) incubation media alone, or containing either 40 g/L of AlbuMAX II (Invitrogen, CA) or 10% FBS (Biowhittaker, MD). Cells were incubated in wells of a 24-well cell culture-treated plate at 26 °C for five days, as this was the tested time frame resulting in more clear differences in cell number and type between control and experimental treatments, and with lower overall cell mortality (data not shown). Differentiation and proliferation in each sample were measured after the five-day incubation using staining and flow cytometry as detailed.

3. Results

3.1. Morphology of primary midgut stem and mature cell cultures

Cells in the three main developmental stages (stem, differentiating, and mature) in the midgut of *H. virescens* larvae can be easily identified in primary cultures based on the gross morphology