RESEARCH REPORT

# A morphological and functional characterization of *Bombyx mori* larval midgut cells in culture

**G Cermenati, P Corti, S Caccia, B Giordana, M Casartelli**

*Dipartimento di Biologia, Università degli Studi di Milano, 20133 Milano, Italy*

## Abstract

Recent studies have shown that *Bombyx mori* larval midgut can transport proteins unaltered following the transcellular pathway by transcytosis. The numerous steps involved in this complex process are still unknown in the insect midgut, and a promising tool to elucidate this aspect is the availability of single midgut cells in culture suitable for transport experiments. Mature midgut cells in culture were obtained from stem cells isolated from *B. mori* larvae cultured in Grace's medium supplemented with 20-hydroxyecdysone (20-HE) and α-arylphorin. After three weeks, up to 60 % of the cultured cells were differentiated into columnar and goblet cells, the two predominant cell types in the midgut epithelium. These cells presented *in vitro* the same shape, morphology and polarity recorded *in vivo*, even if their dimensions were slightly reduced. Columnar cells displayed a well developed cytoskeletal arrangement, with actin filaments highly organized within the thick brush border and distributed in faint filaments in the cell cytoplasm. Microtubules formed a substantial net just beneath the brush border and ran longitudinally from the apical to the basal pole of the cell. Cultured cells homogenates displayed aminopeptidase N and alkaline phosphatase activity, proving that these two enzymes, involved *in vivo* in the intermediate and final digestion, are expressed also *in vitro*. The columnar cells differentiated in culture were able to internalize two model proteins with quite different transport rates.

Key words: *Bombyx mori* larval midgut; stem cells; columnar cells in culture; cytoskeletal scaffolding; digestive enzymes; protein uptake

## Introduction

The lepidopteran midgut is formed by a folded epithelial cells monolayer, separated from underlying muscles and tracheae by a thin basal membrane and composed by three main cell types, goblet, columnar, stem cells (Cioffi, 1979; Baldwin and Hakim, 1991), and by fewer endocrine cells. Goblet cells have a peculiar shape, with a basally located nucleus and a cavity lined by an apical plasma membrane forming numerous microvilli, where a V-H$^+$-ATPase pump (Wieczorek *et al.*, 1989) and a K$^+$/2H$^+$ antiport (Azuma *et al.*, 1995) are expressed. The combined activity of these two transporters generates the high electrical voltage, the active secretion of K$^+$ and the extreme luminal alkalinisation typical of the lepidopteran midgut epitehelium. Columnar cells have an almost cylindrical shape with a central nucleus, an apical thick brush border and deep infoldings of the basal plasma membrane (Cioffi, 1979; Baldwin and Hakim, 1991). This cell type is involved in nutrient digestion (Terra and Ferreira, 2005) and absorption (Giordana *et al.*, 1982, 1998). The small stem cells, roughly conical- or spindle-shaped with a large nucleus, are located at the base of the epithelium (Turbeck, 1974; Baldwin and Hakim, 1991): they are the only cell type that undergoes mitosis (Loeb and Hakim, 1996) and their proliferation initiates in proximity of the moults (Baldwin and Hakim, 1991). In the last decade primary cultures of mature cells from several lepidopteran species were obtained successfully from the isolated midgut stem cells maintained in a culture medium integrated with 20-hydroxyecdisone and α-arylphorin, a subunit isolated from a perivisceral fat body extract of *Manduca sexta* pupae (Sadrud-Din *et al.*, 1994,

*Corresponding author*
Morena Casartelli
Dipartimento di Biologia
Università degli Studi di Milano
via Celoria 26, 20133 Milano, Italy
E-mail: morena.casartelli@unimi.it

1996; Loeb and Hakim, 1996; Blackburn *et al.*, 2004). Differentiation to goblet and columnar cells requires the presence of factors released in the medium by the actively developing cell culture (Sadrud-Din *et al.*, 1996). More recently, some of the peptides that, acting like the mammalian growth factors, induce cell differentiation have been identified (Loeb *et al.*, 1999; Goto *et al.*, 2001; Loeb and Jaffe, 2002).

We have recently shown that *Bombyx mori* larval midgut isolated and perfused *in vitro* can transport two selected proteins unaltered from the lumen to the haemolymph side of the epithelium by transcytosis (Casartelli *et al.*, 2005, 2007). The availability of single mature columnar cells in culture may represent the best tool to study in detail the multiple steps involved in this complex pathway. We have therefore standardised a primary culture of larval midgut cells from *B. mori* and examined some morphological features of the isolated cells grown and differentiated in culture. We also investigated if they expressed some of the digestive enzymes detected *in vivo* and if they could internalize two different proteins, albumin labelled with fluorescein isothiocyanate (FITC-albumin) and green fluorescent protein (GFP).

## Materials and methods

*Experimental animals*
*Bombyx mori* eggs and the artificial diet (Cappellozza *et al.*, 2005) were provided by CRA-Institute for Sericulture (Padova, Italy). Larvae were reared under controlled conditions (25 ± 1 °C, 65-70 % RH, 12L:12D photoperiod).

*Histological analysis of IV instar midgut epithelium during pre-moult, moult and V instar feeding period*
Silkworms at the above indicated stages of development were anaesthetized with $CO_2$. The midgut was explanted, deprived of the peritrophic membrane and Malpighian tubules and fixed in pycric acid, formaldehyde and glutaraldehyde (PAFG) at room temperature for 2 h and then at 4 °C over-night according to Ermak and Eakin (1976). The samples were then washed in 0.1 M cacodylate buffer (pH 7.4), postfixed in 1 % osmium tetroxide in the same buffer for 2 h, washed in distilled water and left for 2 h in 2 % uranyl acetate. After dehydratation in a graded ethanol series, samples were embedded in EPON resin and the polymerisation was performed at 60 °C for 48 h. Semithin sections were cut with a Reichert Ultracut E microtome and observed at the light microscope (AXIOVERT 200M equipped with AXIOCam HRm, Zeiss, Germany).

*Preparation of midgut cells cultures*
Larvae of *B. mori* at the end on the IV instar, just before the IV moult, were anaesthetized with $CO_2$ and surface-sterilized by consecutive immersions, lasting approximately 2 min each, in the following solutions: 10 % (v/v) detergent (Pharma Soap Medical); 2 % (v/v) p-hydroxybenzoic acid methyl ester (Sigma), prepared from a stock solution of 15 % p-hydroxybenzoic acid methyl ester (w/v) in 95 % ethanol; 0.1 % (v/v) sodium

hypochlorite. Silkworms were cut between the second and the third pair of thoracic legs and behind the third pair of abdominal appendages to exclude the foregut and the hindgut, and the peritrophic membrane along with the enclosed intestinal contents was removed. The central part of the larva was transferred to a Petri dish containing a sterile physiological solution composed of 47 mM KCl, 20.5 mM $MgCl_2$, 20 mM $MgSO_4$, 5.3 mM $K_2HPO_4$, 5.6 mM $KH_2PO_4$, 1 mM $CaCl_2$, 75 mM sucrose at pH 7, 0.2 % (v/v) gentamicin (50 mg/ml, Sigma), 0.01 % (v/v) Antibiotic-Antimycotic Solution 1X (Sigma). To this solution was added 0.003 ‰ (v/v) sodium hypochlorite. The ventral cuticle was cut longitudinally and the midgut, deprived of muscles and silk glands, was isolated. Dissected midguts from 8-10 animals were cut along the longitudinal axis and rinsed twice (10 min for each rinse) in the above mentioned sterile physiological solution added with 0.003 ‰ (v/v) sodium hypochlorite, then again twice (for 10 min each) in the sterile physiological solution. Midguts were pooled into a strainer (100 μm mesh size), placed in a Petri dish containing few ml of the latter solution and left under mild agitation for 1 h. In these conditions, the loosely attached stem cells migrated away from the tissue. The tissue within the sieve was discarded and the free cells in the filtrate were collected and pelleted by gentle centrifugation at 400xg for 5 min. Cells were then resuspended in growth medium, composed by a mixture of 67.4 % Grace's insect medium (GIBCO), 11.2 % 100 mM KOH, 6.7 % Fetal Bovine Serum (GIBCO), 0.5 % vitamins mix (composed by, in mg/100ml: 300 riboflavin, 150 pyridoxine hydrochloride, 150 thiamine hydrochloride, 150 folic acid, 600 nicotinic acid, 600 pantothenic acid, 12 biotin, 1.2 vitamin $B_{12}$), 0.018 ‰ Antibiotic-Antimycotic Solution 1X (Sigma), 0.1 % gentamicine (50 mg/ml, Sigma). Cultured cells were supplemented with $6\times10^{-8}$ M 20-hydroxyecdysone (Sigma) and 100 ng/ml α-arylphorin (purified according to Blackburn *et al.*, 2004 in Insect Bio-control Laboratory, USDA, Beltsville, MD, USA), kindly donated by Prof RS Hakim, Howard University, Washington, DC, USA. All the solutions used were routinely sterilized by filtration (Nalgene, 0.2 μm pore size) prior to use. Three ml of the cell suspension in growth medium were distributed in the wells (35 mm in diameter) of six well plates. Cultures were incubated at 25 °C. One ml of medium from each well was routinely replaced with 1 ml of fresh medium once a week.

*Viability of cells in culture, recognition and count of stem cells, differentiating cells and mature cells along six weeks*
Cell viability was checked with the Trypan blue test in the initial stem cell culture and every seven days: viable cells excluded the dye, whereas dead cells became blue. An aliquot of the cell culture was removed, the cells were centrifuged for 5 min at 400xg and then resuspended in a known amount of the physiological solution (see above). An aliquot of the suspension was mixed with 0.4 % (w/v) trypan blue (Sigma) (2:1). After 2 min, viable and dead cells were counted under the inverted microscope using a haemocytometer slide (Burker). Viable cells

were then classified in four different categories (stem, differentiating, columnar and goblet cells) on the basis of their morphological features and counted every seven days for six weeks. Differences between the four categories along this experimental period were tested by Student's *t* test.

## Immunodetection of microtubules in cultured columnar cells

Three weeks old cultured cells were pelletted at 400xg for 5 min and resuspended in the suitable volume of physiological solution (see above). Cells were then fixed and permeabilized for 5 min with ice-cold methanol. After 3 rinses in PBS, cells were incubated for 15 min in PBS containing 1 % bovine serum albumin (BSA) and for 1 h with the anti-α-tubulin mouse IgG (Sigma), diluted 1:500 in PBS with 1 % BSA. The cells were then washed 3 times in PBS plus 1 % BSA and incubated for 1 h with Alexa Fluor 488-conjugated anti-mouse IgG antibody raised in donkey (Molecular Probes) diluted 1:1000 in PBS plus 1 % BSA. After 3 rinses in PBS, the samples were mounted in DABCO (Sigma)-Mowiol (Calbiochem), covered with a cover-slip and examined with a CLSM TCS SP2 AOBS (Leica Microsystems Heidelberg GmbH, Germany) equipped with an argon ion laser (458, 476, 488, 496 or 514 nm excitation), two HeNe lasers (543, 594 and 633 nm excitation) and tunable emission wavelength collection. A 63X Leica oil immersion plan apo (NA1,4) objective and a 2X zoom were used for all the experiments.

## Detection of actin filaments in cultured columnar cells

Three weeks old cultured cells were pelletted at 400xg for 5 min and resuspended in the appropriate volume of physiological solution (see above). Cells were then incubated for 10 min in 4 % paraformaldehyde in PBS, washed 3 times in PBS and permeabilized for 4 min with 0.1 % Triton-X100 in PBS. The cells were washed 3 times in PBS and then incubated for 20 min with 4.3 µg/ml TRITC-phalloidin. After 3 rinses in PBS, the samples where mounted in DABCO (Sigma)-Mowiol (Calbiochem), covered with a cover-slip and examined with a confocal microscope (see above).

## Enzymes assay

Three weeks old midgut cells in culture were pelletted by gentle centrifugation at 400xg for 5 min and resuspended in a small amount of physiological solution (see above). After three washes, the pellet was resuspended in a buffer solution (100 mM mannitol, 10 mM Hepes-Tris at pH 7.2) and lysated in the eppendorf vial with a motor for pellet pestle (Sigma). Protein concentration in the lysate was determined according to Bradford (1976) with BSA as standard. All enzymatic assays were conducted under conditions in which products formation depended linearly on enzyme concentration. Aminopeptidase N and alkaline phosphatase activities were determined at 25 °C by measuring the release of *p*-nitroaniline from L-leucine-*p*-nitroanilide in 40 mM Tris-HCl at pH 7.5 or of *p*-nitrophenol from *p*-nitrophenylphosphate in 1 M Tris-HCl at pH 8, respectively. Enzymes activities were determined in triplicate or quadruplicate in a Pharmacia Biotech Ultrospec 3000 spectrophotometer.

## Proteins internalization in cultured columnar cells

Three weeks old cultured cells were pelletted by gentle centrifugation at 400xg for 5 min and resuspended in a small volume of physiological solution (see above). Cells were incubated at 25 °C for 2 h in the presence of 1.4 µM FITC-albumin (Sigma) or 1.5 µM GFP (Vector ETC). At the end of the incubation, the cells were washed three times with the physiological solution, fixed for 10 min with 4 % paraformaldehyde in PBS and then rinsed three times in PBS. Cells were mounted in DABCO (Sigma)-Mowiol (Calbiochem) and examined with a confocal microscope (see above).

# Results

## Stem cells differentiation in culture and morphology of midgut cells in vivo and in vitro

To isolate the largest possible number of stem cells from *B. mori* midgut, we performed an histological analysis of the tissue in three different instances of the larval development. In Fig. 1 is shown the midgut epithelium dissected from larvae immediately before the IV moult (a), during the IV moult (b) or in the middle of the V instar during the feeding period (c). In the period just before the last larval-larval moult, the stem cells are located in numerous nidi at the base of the epithelium (Fig. 1a). During the moult (Fig. 1b), the stem cells proliferate and then differentiate, each of the newly developed cell inserting between the mature cells of the IV instar epithelium. In the V instar, during the feeding period (Fig. 1c), very few single stem cells are visible, far less numerous than in the pre-moult period. Therefore, the largest number of stem cells can be isolated from *B. mori* larval midguts in the period just preceding the IV moult.

To monitor cell development in culture with time, we followed the growth, differentiation and percentage distribution of each cell type for 42 days (Figs 2, 3), by identifying the different cell types every 7 days on the basis of their morphological features. Stem cells were round, with a diameter of 4-8 µm (Fig. 2a) and some of them could be observed in mitosis (Fig. 2b). Cells in an early stage of differentiation were round, with long tenuous membrane projections and numerous granules in the cytoplasm (Fig. 2c): as suggested by Sadrud-Din *et al.* (1996), these spherical cells with uniformly distributed microvilli appears to correspond to the initial phase of differentiation of columnar cells, that in a more advanced phase were triangular in shape (Fig. 2d), differing from adult ones for their small dimension (10-25 µm). Young columnar (Fig. 2e) and goblet (Fig. 2g) cells had the same shape of the respective mature cells (Figs 2f, h) but their dimensions were smaller (between 25-30 µm). Mature columnar cells were characterized by a well developed apical membrane with numerous microvilli, a centrally placed nucleus and a cylindrical or cubical shape (Fig. 2f), while mature goblet cells were flask-like and presented the typical wide cavity, the apical valve and a basally located
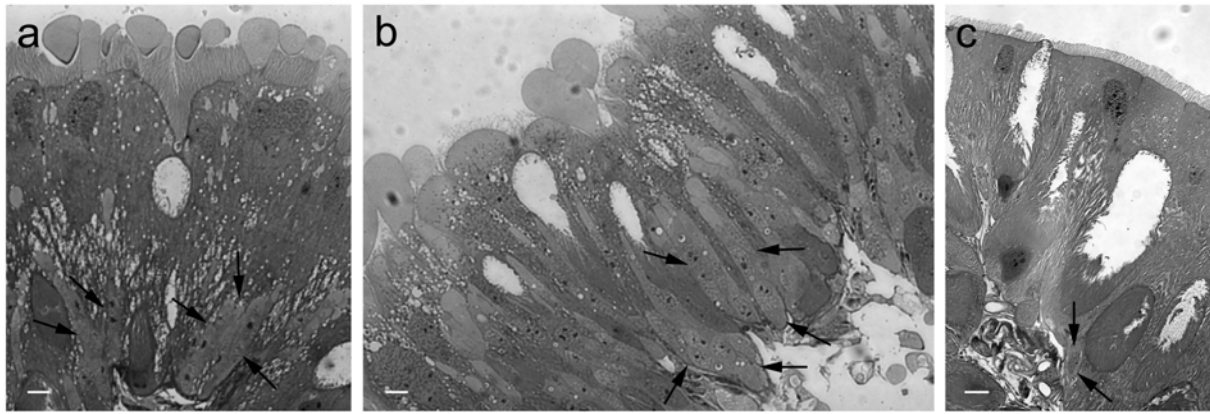
**Fig. 1** Semithin sections of the midgut epithelium of *Bombyx mori* larvae dissected immediately before the IV moult (a), during the moult (b) and in the V instar feeding period (c). Stem cells nidi (a), proliferating cells (b) and a single stem cell (c) are indicated by arrows. Bars = 10 µm.

nucleus (Fig. 2h). Both these cells showed most of the apparent morphological features seen *in vivo* but they were never as tall as those of the original epithelium (60 to 80 µm, Fig. 1).

As shown in Fig. 3, in the filtrate collected after a mild agitation of the pre-moult midgut tissue, the stem cells represented the 87.4 ± 3.6 % (3 determinations) of the total viable cells present in the medium. After six days in culture, the reduction of stem cells was accompanied by an increase of differentiating, columnar and goblet cells. At the end of the following 7 days, the percentage of stem cells were further decreased, while differentiating cells were the most represented cell type. From the third week on, less than 10 % were stem cells and the remaining cells were represented by percentage values not statistically different (ranging between 27.6 ± 3.4 % (3 determinations) and 41.4 ± 5.3 % (3 determinations)) of columnar, goblet and differentiating cells.

All along the experimental period here considered, viable cells were 79.2 ± 5.1 % (21 determinations) of the total cells present in the culture.

The columnar cells used for the experiments reported in this paper came from three weeks-old cultures, although the cells maintained the same functional properties till day 42 (data not shown).

*Organisation of the cytoskeleton in cultured columnar cells*

We examined the distribution of microtubules and actin filaments in columnar cells. As shown in figure 4 b, a large number of microtubules ran parallel to the apical cell surface, forming a dense network just below the apical microvilli. Deeper down the cell, numerous bundles of microtubules are oriented longitudinally along the basal-apical axis of the cell.

Phalloidin stained conspicuously actin filaments within the brush border microvilli of columnar cells both in the initial phase of differentiation (Fig. 5a) and in the mature phase (Fig. 5b). In the latter figure a number of filaments running from the basolateral

membrane deep into the cytoplasm were also detectable.

*Enzymes activity*

Columnar cells *in vivo* are responsible for the production of the different classes of enzymes involved in the digestion of ingested nutrients (Terra and Ferreira, 2005). We investigated if the activity of two enzymes currently used as marker enzymes of the apical membrane of midgut columnar cells, i.e. leucine-aminopeptidase N (APN) and alkaline phosphatase (AlP), could be detected in the lysate of three weeks old cell cultures. Although after 20 days in culture columnar cells represented only the 28.9 ± 2.7 % (3 determinations) of all the cells in culture (Fig. 3), an activity of both enzymes could be measured: APN and AlP specific activities (mU/mg of protein) were 890 ± 88 (5 determinations) and 131 ± 12 (4 determinations) respectively.

*Proteins absorption in cultured columnar cells*

We have shown that FITC-albumin is transported across the lepidopteran larval midgut *in vitro* by transcytosis (Casartelli *et al.*, 2005), entering the cell through the apical membrane and being released in the haemocoel across the basolateral membrane. At variance, the green fluorescent protein GFP fed *in vivo* to the hemipteran *Lygus hesperus* reached without degradation the haemocoel following apparently a paracellular pathway across the junctional complex (Habibi *et al.*, 2002). We investigated if mature columnar cells in culture were able to internalize these two proteins, by incubating the cells for 2 h in the presence of 1.4 µM FITC-albumin or 1.5 µM GFP. The fluorescent proteins taken up by columnar cells after 2 h of incubation were detected by confocal laser microscopy: while columnar cells internalized abundantly FITC-albumin (Fig. 6a), GFP could not be detected inside the cells (Fig. 6b). Intracellular FITC-albumin was never diffused uniformly into the cytoplasm but was always localized in vesicular structures clearly visible as spots.
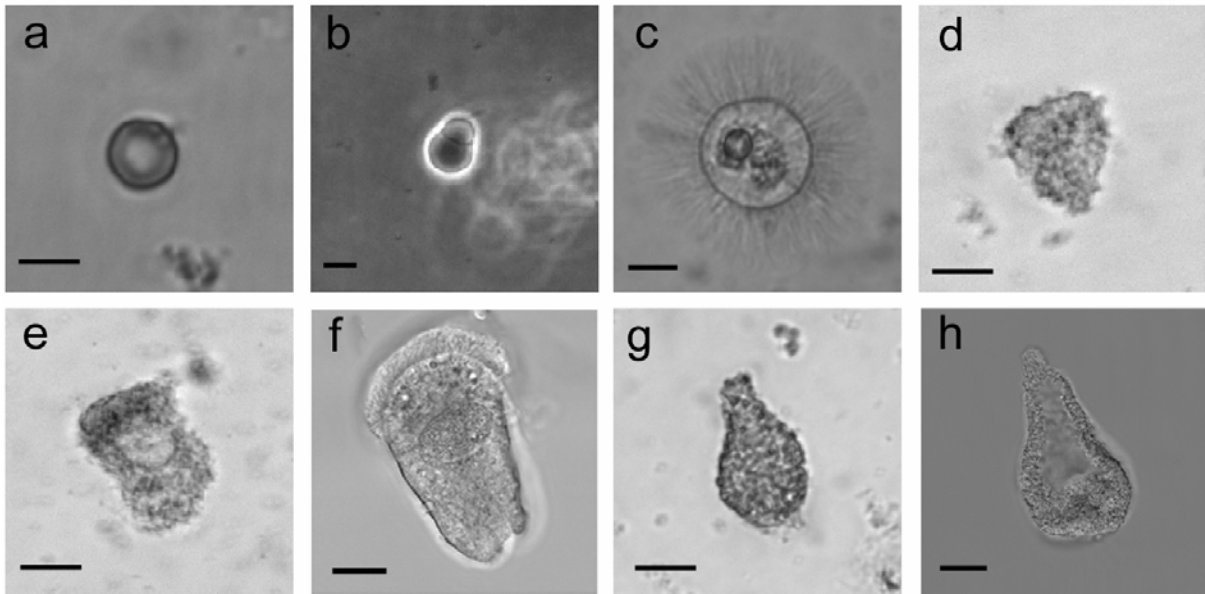
**Fig. 2** Morphology of cultured midgut cells. Brightfield images (acquired with confocal laser scanning microscope) of fixed cells: stem cell (a); stem cell in mitosis (b); differentiating cells (c, d); columnar (e) and goblet (g) cells in an early phase of differentiation; and fully differentiated (f, h). Bars = 5 μm (a, b); 10 μm (c-h).

## Discussion

Detailed information on the mechanisms involved in peptide and protein absorption by the insect gut can have a considerable impact on the development of new delivery strategies for orally administered insecticidal proteins targeting haemocoelic receptors. Proteins are absorbed by the insect midgut *in vivo* (Fishman *et al.*, 1984; Modespacher *et al.*, 1986; Zlotkin *et al.*, 1992; Ben-Yakir and Shochat, 1996; Powell *et al.*, 1998; Habibi *et al.*, 2002; Kurahashi *et al.*, 2005), and recent studies performed *in vitro* in *B. mori* larval midgut showed that two selected proteins crossed unaltered the intestinal barrier by transcytosis (Casartelli *et al.*, 2005, 2007). We considered that midgut columnar cells in primary culture could be a powerful tool to identify the different steps involved in this composite transcellular pathway.

Sadrud-Din *et al.* (1994, 1996) obtained primary cultures of midgut cells from stem cells isolated from *M. sexta* larval epithelium, identifying the conditions *in vitro* in which the stem cells could survive, multiply and differentiate. Stem cells from the insect midgut can be easily removed from the tissue because they are not linked to the other cells by junctions and their proliferation *in vitro* can be induced by ecdysone or 20-hydroxyecdysone (Smagghe *et al.*, 2005) and by fat body extracts or its derivates (Hakim *et al.*, 2007), while differentiation is stimulated by various factors produced by the mature and differentiating cells in the culture (Loeb *et al.*, 2004). Following the same method, we isolated the stem cells from the midguts of pre-moult IV instar *B. mori* larvae and obtained their proliferation and differentiation in culture. We analysed the evolution of the culture by counting the total number of cells, testing their viability and determining the different cell types among the living cells every seven days for 6 weeks (Fig. 3). The initial culture was almost exclusively composed of stem cells (87.4 ± 3.6 %, 3 determinations) but six days later all the cell types shown in figure 2 were already present. The progressive drop in stem cells observed in the following 21 days was compensated by the parallel increase in differentiating cells and in young and mature columnar and goblet cells. From the third week on, these three cell types represented about 30 % each of total living cells. The morphology of the different kind of cells shown in figure 2 is in large agreement with that reported by Sadrud-Din *et al.* (1996). It is worth noting that, following a drastic decrease after 27 days, stem cells
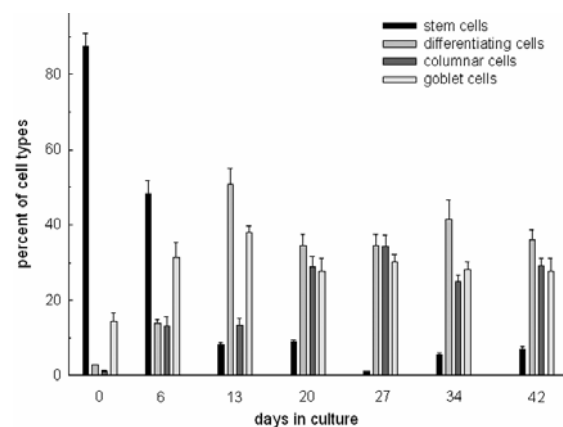


**Fig. 3** Percentage of the various viable cell types in culture in the different days since stem cells isolation. Each bar represents the mean ± SE of three different determinations.
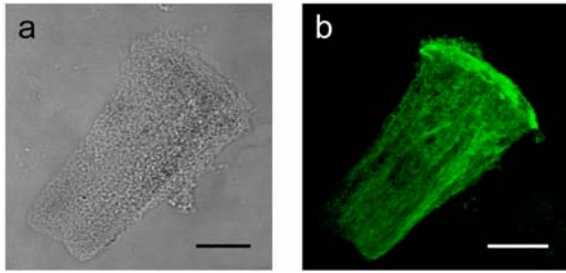
**Fig. 4** Brightfield (a) and confocal laser scanning micrographs (maximum projection) (b) of a typical cultured columnar cell in which is visible microtubules organisation. Immunolocalisation of microtubules was performed with an anti-α-tubulin primary antibody visualised with an Alexa Fluor 488-conjugated secondary antibody. Bars =10 μm.

number increases progressively in the subsequent two weeks, suggesting an enhanced production by the cell culture of specific proliferation factors, once reached a steady state. Although our present report of the cell culture properties is referred only to six weeks (Fig. 3), the culture maintains almost unaltered the characteristics shown here for up to three months (data not shown).

The integrity of the cytoskeleton is fundamental for the maintenance of cell shape and polarity and for the correct localization of membrane proteins in the apical and basal domains of the cell surface. Efficient targeting of vesicles loaded with the specific proteins, from the Golgi apparatus to the correct membrane domain, requires an intact microtubule organization (reviewed by Yeaman *et al.*, 1999). It has been shown in enterocytes and other polarized epithelia that disruption of microtubule architecture by colchicine or nocodazole leads to a preferential alteration of the delivery to the apical rather than to the basolatelal membrane. In rat intestinal epithelium, the microtubules organizing center(s), identified with anti γ-tubulin antibodies, is/are located as a band close to the sub-apical space near the terminal web, from which the fast growing positive ends of microtubules grow towards the basolateral membrane, forming bundles of apical-basal filaments along the cell axis, while the negative ends are apically located (Waschke and Drenckhahn, 2000). In the subapical-space microtubules still run in parallel and only few are oriented obliquely or perpendicularly, but they never cross the terminal web (Waschke and Drenckhahn, 2000). Microtubules distribution from the basal to the apical pole in *B. mori* midgut cells largely follows this scheme but, at variance with mammalian cells, in the insect they form a well developed felt just under the brush border (Fig. 4b). This particular structure could be related to the lack in insect columnar intestinal cells of a terminal web organized as that of mammals (Hull and Staehelin, 1979; Bonfanti *et al*, 1992; Gibson and Perrimon, 2003). As a matter of fact, in Caco-2 cells monolayer, an intestinal model epithelium, in which a complete terminal web presumably does never fully

differentiate, microtubules in the apical cytoplasm have a network-like arrangement (Waschke and Drenckhahn, 2000).

The disposition of actin cytoskeleton in the mature cell shown in Fig. 5b follows that classically described for polarized columnar cells (Yeaman *et al.*, 1999): it is highly organized within the apical microvilli and some filaments running from the basolateral membrane deep into the cytoplasm were also visible. Well structured actin filaments were also observable in columnar cells in an earlier phase of differentiation (Fig. 5a). As reported in the Results, some of the differentiating cells were round shaped with microvilli uniformly distributed all over the surface (Fig. 2c). Sadrud-Din *et al.* (1996) suggested that these cells corresponded to the initial phase of differentiation of columnar cells. The cell reported in Fig. 5a, a representative of columnar cells in a more advanced stage, suggests that the differentiation of the basolateral membrane, at least from a morphological point of view, is a delayed process.

The midgut epithelium performs *in vivo* the intermediate and final digestion of nutrients (Terra and Ferreira, 2005). Two enzymes involved in this process highly abundant in the midgut epithelium are aminopeptidase N (APN) and alkaline phosphatase (AlP). Different isoforms of APN were characterized in the insect midgut (Terra and Ferreira, 2005), where they play a critical role in the final digestion of polypeptides by catalyzing the hydrolysis of amino acid residues at the N-terminal end. Alkaline phosphatases, also present in the midgut epithelium as different isoforms (Ferreira and
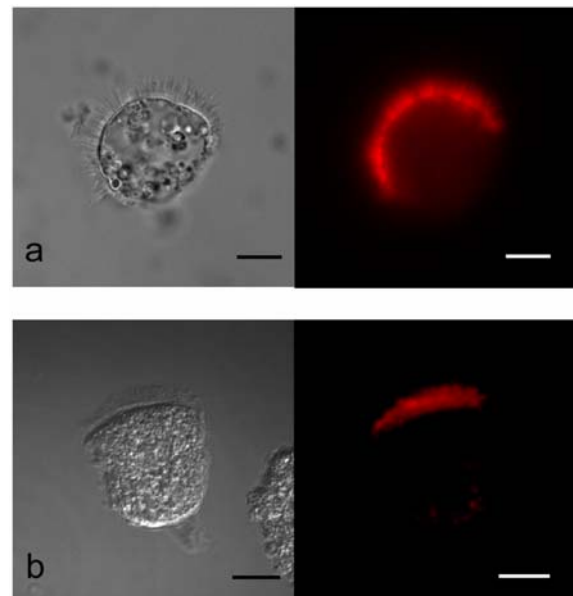


**Fig. 5** Brightfield and confocal laser scanning micrographs (maximum projection) of typical cultured columnar cells, in the initial phase of differentiation (a) and in the mature phase (b), in which is visible microfilaments organisation. Actin filaments were visualised with TRITC-phalloidin. Bars = 10 μm.
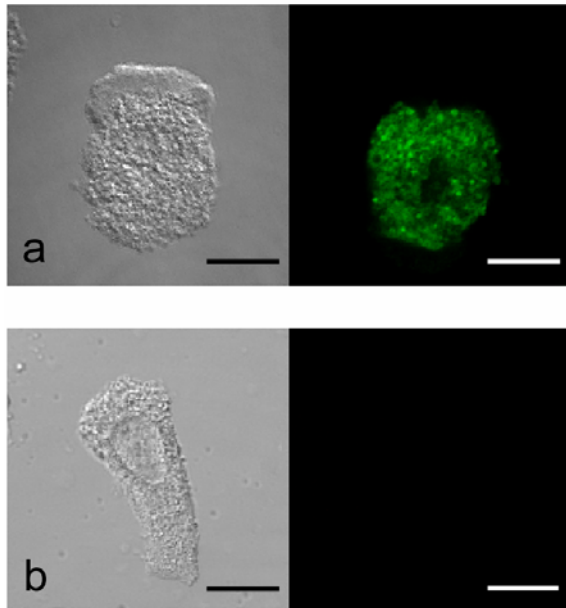
**Fig. 6** Brightfield and confocal laser scanning micrographs of a single optical section in a middle cell focal plane (where the nucleus was clearly evident) of cultured midgut columnar cells incubated for 2 h in the presence of 1.4 μM FITC-albumin (a) or 1.5 μM GFP (b). In the figure typical columnar cells are reported. Bars = 10 μm.

Terra, 2005), are responsible for the removal of the phosphate groups from various phosphorylated substrates, an important step to allow the subsequent absorption of the molecule. Both enzymes are expressed by columnar cells in culture, since their activity could be measured in the cell lysate.

Finally, we tested the ability to take up proteins from the medium by mature columnar cells. FITC-albumin, a protein actively absorbed by transcytosis by *B. mori* midgut epithelium *in vitro* (Casartelli *et al.*, 2005), was already detected inside the cell after 20 min (not shown) and was uniformly distributed as numerous spots after 2 h (Fig. 6a). Conversely, GFP, a protein that was supposed to reach the haemocel in the hemipteran *Lygus hesperus* following the paracellular pathway (Habibi *et al.*, 2002), was not found inside columnar cells after 2 h (Fig. 6b), intracellular traces of the protein becoming apparent only after 16 h (not shown). It is therefore feasible that the appearance of this protein in the haemocoel *in vivo* could be due to its passive leakage through the paracellular route rather than by an active process like transcytosis.

In conclusion, *B. mori* columnar cells, differentiated from stem cells *in vitro,* maintained in culture the typical morphological features of the epithelium *in vivo* and were able to perform at least some of the normal absorptive and digestive functions. Of particular interest for us was the ability of the cells to act selectively as regards the uptake of two model proteins. The very rapid internalization by the cell of FITC-albumin designates this protein as an excellent tool to assess the specific mechanisms involved in endocytosis and to finely characterize the multiple sequence of intracellular events involved in transcytosis.

**References**

Azuma M, Harvey WR, Wieczorek H. Stoichiometry of K$^+$/H$^+$ antiport helps to explain extracellular pH 11 in a model epithelium. FEBS Lett. 361: 153-156, 1995.

Baldwin KM, Hakim RS. Growth and differentiation of the larval midgut epithelium during molting in the moth *Manduca sexta.* Tissue Cell 23: 411-422, 1991.

Bonfanti P, Colombo A, Heintzelman MB, Mooseker MS, Camatini M. The molecular architecture of an insect midgut brush border cytoskeleton. Eur. J. Cell Biol. 57: 298-307, 1992.

Ben-Yakir D, Shochat C. The fate of immunoglobulin G fed to larvae of *Ostrinia nubilalis.* Entomol. Exp. Appl. 81: 1-5, 1996.

Blackburn MB, Loeb MJ, Clark E, Jaffe H. Stimulation of midgut stem cell proliferation by *Manduca sexta* α-Arylphorin. Arch. Insect Biochem. Physiol. 55: 26-32, 2004.

Bradford MM. A rapid sensitive method for the quantification of protein utilizing the principle of dye-protein binding. Anal. Biochem. 72: 248-254, 1976.

Cappellozza L, Cappellozza S, Saviane A, Sbrenna G. Artificial diet rearing system for silkworm *Bombyx mori* (Lepidoptera Bombycidae): effect of vitamin C deprivation on larval growth and cocoon production. Appl. Entomol. Zool. 40: 405-412, 2005.

Casartelli M, Corti P, Leonardi MG, Fiandra L, Burlini N, Pennacchio F, *et al.* Absorption of albumin by the midgut of a lepidopteran larva. J. Insect Physiol. 51: 933-940, 2005.

Casartelli M, Corti P, Cermenati G, Grimaldi A, Fiandra L, Santo N, *et al.* Absorption of horseradish peroxidase in *Bombyx mori* larval midgut. J. Insect Physiol. 53: 517-525, 2007.

Cioffi M. The morphology and fine structure of the larval midgut of a moth (*Manduca sexta*) in relation to ion transport. Tissue Cell 11: 467-469, 1979.

Ermak TH, Eakin RM. Fine structure of the cerebral and pygidial ocelli in *Chone ecaudata (Polychaeta: Sabellidae).* J. Ultrastruct. Res. 54: 243–260, 1976.

Fishman L, Primor N, Zlotkin E. The selective permeability of fleshfly midgut to an orally toxic cobra venom cardiotoxin. J. Exp. Biol. 108: 441-451, 1984.

Gibson MC, Perrimon N. Apicobasal polarization: epithelial form and function. Curr. Opinion Cell Biol. 15: 747-752, 2003.

Goto S, Takeda M, Loeb MJ, Hakim RS. Immunohistochemical detection of a putative insect cytochine, midgut differentiation factor 1 (MDF-1) in midgut columnar cells of *Heliothis virescens*. Invert. Reprod. Devel. 40: 117-124, 2001.

Giordana B, Sacchi VF, Hanozet GM. Intestinal amino acid absorption in lepidopteran larvae. Biochim. Biophys. Acta 692: 81-88, 1982.

Giordana B, Leonardi MG, Casartelli M, Consonni P, Parenti P. K$^+$-neutral amino acid symport of *Bombyx mori* larval midgut: a system operative in extreme conditions. Am. J. Physiol. 274: R1361-R1371, 1998.

Habibi J, Brandt SL, Coudron TA, Wagner RM, Wright MK, Backus EA, Huesing JE. Uptake, flow and digestion od casein and green fluorescent protein in the digestive system of *Lygus hesperus* Knight. Arch. Insect Biochem. Physiol. 50: 62-74, 2002.

Hakim RS, Blackburn MB, Corti P, Gelman DB, Goodman C, Elsen K, *et al.* Growth and mitogenic effects of arylphorin in vivo and in vitro. Arch. Insect Biochem. Physiol.64, 63-73, 2007.

Hull BE, Staehelin LA. The terminal web. A reevaluation of its structure and function. J. Cell Biol. 81: 67-82, 1979.

Kurahashi H, Atiwetin P, Nagaoka S, Miyata S, Kitajima S, Sugimura Y. Absorption of mulberry root urease to the hemolymph of the silkworm *Bombyx mori*. J. Insect Physiol. 51: 1055-1061, 2005.

Loeb MJ, Hakim RS. Insect midgut epithelium *in vitro*: an insect stem cell system. J. Insect Physiol. 42: 1103-1111, 1996.

Loeb MJ, Jaffe H, Gelman DB, Hakim RS. Two polypeptide factors that promote differentiation of insect midgut stem cells *in vitro*. Arch. Insect Biochem. Physiol. 40: 129-140, 1999.

Loeb MJ, Jaffe H. Peptides that elicit midgut stem cell differentiation isolated from chymotryptic digests of hemolymph from *Lymantria dispar* pupae. Arch. Insect Biochem. Physiol. 50: 85-96, 2002.

Loeb MJ, Coronel N, Natsukawa D, Takeda M. Implications for the functions of the four known midgut differentiation factors: an immunohistologic study of *Heliothis virescens*

midgut. Arch. Insect Biochem. Physiol. 56: 7-20, 2004.

Modespacher UP, Rudin W, Jenni L, Hecker H. Transport of peroxidase through the midgut epithelium of *Glossina m. morsitans* (Diptera, Glossinidae). Tissue Cell. 18: 429-236, 1986.

Powell KS, Spence J, Bharathi M, Gatehouse JH, Gatehouse AMR. Immunohistochemical and developmental studies to elucidate the mechanism of action of the snowdrop lectin on the rice brown planthopper, *Nilaparvata lugens* (Stal). J. Insect Physiol. 44: 529-539, 1998.

Sadrud-Din SY, Hakim RS, Loeb MJ. Proliferation and differentiation of midgut cells from *Manduca sexta*, *in vitro*. Invert. Reprod. Dev. 26: 197-204, 1994.

Sadrud-Din SY, Loeb MJ, Hakim RS. *In vitro* differentiation of isolated stem cells from the midgut of *Manduca sexta* larvae. J. Exp. Biol. 199: 319-325, 1996.

Smagghe G, Vanhassel W, Moeremans C, De Wilde D, Goto S, Loeb MJ, *et al.* Stimulation of midgut stem cell proliferation and differentiation by insect hormones and peptides. Ann. N.Y. Acad. Sci. 1040: 472-475, 2005.

Terra WR, Ferreira C. Biochemistry of digestion. In: Gilbert LI, Iatrou K, Gill SS (eds), Comprehensive molecular insect science, Oxford, Elsevier, 4: 171-224, 2005.

Turbeck BO. A study of the concentrally laminated concretions, 'spherites', in the regenerative cells of the midgut of lepidopterous larvae. Tissue Cell 6: 627-640, 1974.

Waschke J, Drenckhahn D. Uniform apicobasal polarity of microtubules and apical location of γ-tubulin in polarized intestinal epithelium in situ. Eur. J. Cell Biol. 79: 317-326, 2000.

Wieczorek H, Weerth S, Schindlbeck M, Klein U. A vacuolar-type proton pump in a vesicle fraction enriched with potassium transporting plasma membranes from tobacco hornworm midgut. J. Biol. Chem. 264: 11143-11148, 1989.

Yeaman C, Grindstaff KK, Nelson WJ. New perspectives on mechanisms involved in generating epithelial cell polarity. Physiol. Rev. 79: 73-98, 1999.

Zlotkin E, Fishman L, Shapiro JP. Oral toxicity to fleshflies of a neurotoxic polypeptide. Arch. Insect Biochem. Physiol. 21: 41-52, 1992.