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The regenerative nidi of the locust midgut as a model to study epithelial cell differentiation from stem cells

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Summary

A better knowledge of the regulatory mechanisms involved in stem cell proliferation and/or differentiation could reveal new methods for the treatment of some diseases. Most previous studies in the field of stem cell biology have been carried out on cultured isolated cells. In the case of adult tissue stem cells, mesenchymal bone marrow derived cells have been most widely studied, while the undifferentiated stem cells present in the epithelial tissues are less known. In order to advance further our understanding of epithelial tissue stem cells, new *in vivo* models are required. The present study focuses on the dynamics of a new and simple model of intestinal epithelial regeneration found in the midgut of the migratory locust, *Locusta migratoria* (Linnaeus 1758). The locust midgut consists of three cell types: columnar cells, endocrine cells and undifferentiated regenerative clustered cells. The undifferentiated epithelial midgut cells give rise to two other cell types and are located in a nest of regenerative cells known as regenerative niche. We have performed single and continuous bromodeoxyuridine (BrdU) administration experiments to study regeneration niches and their cellular dynamics. Immunocytochemistry and immunofluorescence techniques were used to detect the incorporation of BrdU into regenerative niches and the presence of FMRFamide-like immunoreactivity, as a marker for endocrine cell differentiation. Some isolated label retaining cells (LRC) were observed at the niche base 10–15 days after the final BrdU administration. We propose that these cells are the stem cells of this epithelial tissue. We also calculated the length of the cell cycle phases for a subpopulation of transit amplifying cells within the regenerative niche: G1, 2.5±0.5 h; S, 5.5±0.5 h; G2, 0.75±0.25 h; M, 2.5±0.5 h. These amplifying cells will give rise to the columnar epithelial non-endocrine lineage. The differentiation of an endocrine cell from a niche stem cell occurs less frequently and thus leads to a lower proportion of endocrine cells as compared with epithelial columnar digestive cells (up to three endocrine cells per niche). Endocrine cell commitment seems to occur very early in the differentiation process within the niche, as double-labelled BrdU and FMRF endocrine cells have never been found. The only exception is the endocrine cells located in the ampullar region of the midgut, some of which show double immunostaining after long-term chronic BrdU injection. In summary, we have characterized a new and simple animal model of epithelial stem cell regeneration that may be useful for understanding the complex biological process that drives tissue renewal from undifferentiated and uncommitted progenitor cells.

Key words: stem cell, bromodeoxyuridine, BrdU, *Locusta migratoria*, insect, midgut, proliferation, FMRFa, immunodetection, endoreduplication, epithelial regeneration.

Introduction

Tissue homeostasis is a complex coordinated activity involving key regulatory processes including programmed cell death, cell proliferation and differentiation. Homeostasis is required for morphogenesis and the maintenance of the appropriate organ shape, size and function. It is based on a delicate balance between the rate of generation of new differentiated cells and that of cell loss (Brittan and Wright, 2002; Potten et al., 1997). The production of new differentiated cells usually depends on a stem cell with the capacity to divide several times, giving rise to one or more types of differentiated cells (Potten, 1998; Potten and Loeffler, 1990; Slorach et al., 1999). A stem cell generally divides into two daughter cells, one of which remains a stem cell while the other goes down the route of differentiation. Amplifying cells may give rise to an increasing number of differentiation-committed cells.

Stem cells, other than haematopoietic stem cells, have thus far been characterized from tissues in a limited number of adult animal organs. New models of pluripotent adult stem cells in animals are required to provide research tools to identify,

characterize and purify these cells. These new models for stem cell biology need to be studied both *in vitro* and *in vivo*. *In vitro* studies on isolated cells are simple and very informative but offer less accurate information on the complexity of the fine-tuned regulation of stem cells *in vivo*.

At present, several animal models are being studied to better understand the regulation of the proliferative capacity of stem cells in fully developed organs. Some models, such as haematopoietic stem cell models (Blau et al., 2001; Huang and Wang, 2001) or epidermis and gut epithelium models (Liang and Bickenbach, 2002; Marshman et al., 2002; Martin et al., 1998; Mills and Gordon, 2001; Slorach et al., 1999), show that some adult stem cells are found in a specific location of the tissue, known as the stem cell niche. The niche is formed by stem cells and neighbouring proliferating and differentiating cells. Neighbouring cells interact with the stem cells, keeping them in the niche, where a specialized microenvironment preserves their stem cell characteristics. Niches can be simple or complex. A simple niche contains only one type of stem cell, generally anchored to a neighbouring cell. Complex niches house two or more types of stem cells and require more complex regulation (Ohlstein et al., 2004). There are six models where stem cell niches have been described in more detail (Fuchs et al., 2004; Spradling et al., 2001). Three of them – the *Drosophila* ovary (Xie et al., 2005; Xie and Spradling, 2000), the vertebrate bone marrow (Calvi et al., 2003; Zhang et al., 2003) and the crypt of the mammalian intestinal villus (Brittan and Wright, 2002; Wong, 2004) – are considered simple niche models. The remaining three – the *Drosophila* testis (Xie et al., 2005; Yamashita et al., 2003), the subventricular zone of the vertebrate brain (Song et al., 2002) and the hair follicle (Tumbar et al., 2004) – constitute complex niches. In all these models, it has been proposed that the daughter stem cells start to differentiate when they detach from the neighbouring cells. Thus, the spatial distribution within the stem cell niche seems to play an important role as a mechanism of maintaining stem cell status and regulating cell differentiation (Ohlstein et al., 2004).

In the present work, we introduce a new stem cell model based on the intestinal epithelium of *Locusta migratoria*. It is a potentially useful model to study stem cell biology based on a simple and accessible material: the midgut epithelium of the desert locust.

The midgut of *Locusta migratoria* is a simple columnar epithelium with three cell types: regenerative, differentiated columnar and endocrine. Differentiated columnar cells are very tall and have long microvilli at the luminal side. The columnar cells are metabolically very active, as they are involved in nutrient absorption, secretion of digestive enzymes and formation of the peritrophic membrane (Lehane and Billingsley, 1996).

The endocrine cells are spread along the epithelium and are interspersed with the columnar cells, just as they are in the mammalian intestinal diffuse endocrine system (Zudaire et al., 1998). These cells remain in contact with the basal lamina and reach the lumen through a long and thin cytoplasmatic prolongation. They are easily identifiable because they are loaded with cytoplasmic secretory granules. The granules store active peptides, which can be detected by specific immunocytochemical techniques. The endocrine cells are particularly abundant in the posterior region of the midgut and, in particular, in the ampullae through which the Malpighian tubules drain to the midgut (Zudaire et al., 1998).

The regenerative cells may give rise to both types of differentiated cells. Each regenerative unit is located basally and is made up by concentrically layered cells that remain in contact with the basal lamina and do not come in contact with the midgut lumen.

Our main hypothesis is that in the midgut epithelium of the locust a stem cell lies in the centre of the niche surrounded by amplification-differentiating cells. The aim of our study was to explore the existence of these two functionally different cell types by means of several experiments designed to determine the proliferative dynamics of the locust midgut epithelial cells and the differentiation activity within the niche.

To test our hypothesis we have used bromodeoxyuridine (BrdU). BrdU is an analogue for thymidine, which is incorporated into DNA during the S phase. Our previous unpublished data showed that, once injected, BrdU bioavailability in the haemolymph lasts for about 30 min. BrdU pulse–chase experiments were designed to follow the fate of the nascent midgut epithelial cells. In addition, data obtained by BrdU immunolocalisation were complemented by simultaneous detection of FMRFamide (FMRFa)-like immunoreactivity as a means of identifying endocrine cells.

Materials and methods

Insects and BrdU administration

Fifth-instar locusts (*Locusta migratoria*) of both sexes were kept at 30°C within a 12 h:12 h light:dark period. Each locust was housed in a plastic box (12 cm × 12 cm × 5 cm) with a plastic perch and was fed *ad libitum* with seedling and germ wheat. BrdU (B-5002; Sigma, Madrid, Spain) administration was performed 24 h after the insect's moult. BrdU (100 μg) was injected between the first and the second abdominal disk in a final volume of 10 μl using a Hamilton microsyringe (801; Hamilton, Bonaduz, Switzerland). Dental wax was used to avoid haemolymph leakage.

Dissection and processing of the samples

After severing the head and the tip of the abdomen, the entire gut was pulled out, opened along its length, and cleaned with Mordue's locust saline (Mordue, 1969). The tissue was fixed in Bouin's fluid for 24 h. Guts were thoroughly washed in 70% ethanol, embedded in paraffin and sectioned at 4 μm thickness.

Immunocytochemistry for BrdU

After deparaffination with xylene and rehydration, endogenous peroxidase was blocked with H_2O_2. Next, the sections were treated for one hour at room temperature with a commercial preparation of nuclease (RPN202; Amersham Pharmacia, Amersham, UK). After blocking non-specific

binding sites with 5% normal goat serum, the sections were incubated overnight with a primary monoclonal antibody (mouse anti-BrdU immunoglobulins; clone BU-1; RPN202; Amersham Pharmacia) diluted 1:200 in TBS. A secondary anti-mouse antibody with polymer-horseradish peroxidase labelling (Envision^{TM}; K4007; Dako, Cambridgeshire, UK) was used. The sections were counterstained with haematoxylin, dehydrated and mounted in DPX.

Double immunocytochemistry

For the simultaneous immunocytochemical localization of BrdU and FMRFa, pre-treatment was performed as described above. After blocking non-specific binding sites with a mix of 5% goat serum and 5% pig serum, sections were incubated simultaneously with the above-mentioned mouse anti-BrdU monoclonal antibody and a rabbit polyclonal antibody against FMRFa (IHC8755; Peninsula laboratories, Weil am Rhein, Germany), diluted at 1:200 and 1:500 in TBS, respectively. For FMRFa detection, sections were first incubated with a secondary anti-rabbit biotinylated antibody, followed by a streptavidin-alkaline phosphatase complex (S-2890; Sigma-Aldrich, UK) incubation. The samples were developed using a substrate chromogen solution for alkaline phosphatase, composed of 50 μl Hexazotized New Fucsin [a mixture of 25 μl of New Fuchsin (N-0638; Sigma) (at 4% in 2 mol l^{-1} HCl) and 25 μl of sodium nitrite (6549; Merck, Madrid, Spain) (at 4% in water)] and 1 ml of a Naftol phosphate solution [1 mg of Naftol AS-TR-PO4 (N-6000; Sigma) dissolved in 20 μl of *N*,*N*-dimethylformamide (141785, Panreac, Castellar del Vallés, Spain) and further dissolved in 1 ml Tris-HCl buffer]. The mixture was finally diluted in 3 ml of Tris-HCl buffer. Detection of BrdU primary antibody and counterstaining was performed as described above. Finally, sections were mounted in PBS-glycerol.

Whole mount immunofluorescence

The dissected midgut was fixed for 24 h in 4% formaldehyde. After being washed with 70% ethanol and saline buffer, the whole-mount samples (≈100 μm thickness) were incubated in a 1% Triton solution (T-8532; Sigma-Aldrich). Next, the midgut portion was simultaneously incubated overnight at 4°C with both primary antibodies, monoclonal (rat anti-BrdU immunoglobulins; ab6326; Abcam, Cambridge, UK) and polyclonal (rabbit anti-FMRFa immunoglobulins; IHC8755; Peninsula Laboratories) diluted at a ratio of 1:200 and 1:500, respectively. Secondary antibodies, Alexa 488 goat anti-rat and Alexa 555 donkey anti-rabbit (A-31572 and A-11006, respectively; Molecular Probes, Breda, The Netherlands), were used for detection. Samples were incubated in the mixture of secondary antibodies for 30 min at room temperature. Finally, sections were mounted in an antifading medium (S-2828; Molecular Probes).

Confocal image acquisition and 3-D reconstruction

Ten images, corresponding to different niches, were acquired from each sample with a Leica TCS-SP2 AOBS confocal microscope. Confocal stacks were reconstructed in 3-D with Amira software (Mercury computer systems, Berlin, Germany). All the confocal and 3-D reconstructions were processed according to previously published procedures (Wouterlood, 2006).

Image acquisition and distance measurement in sections from paraffin embedded material

Two images were taken from each immunostained section with an optic Zeiss 800 E microscope at 200× magnification. For each image, two reference distances were measured using the analySIS software (Soft Imaging System GmbH, Münster, Germany): the average distance between the longitudinal muscular layer (LML) and the apical part of the niche ('niche distance') and the distance between the LML and the apical part of the farthest immunostained nuclei ('labelled distance'). Both distances were represented graphically against experimental time.

Experimental procedure

Single BrdU administration

To determine which midgut cells incorporate BrdU and to establish their fate, we performed a pulse–chase experiment consisting of a single BrdU administration and tracking of the labelled cells for 8 days.

Twenty insects (two insects per day plus two negative BrdU controls) were collected 24 h after the moult into the sixth instar. Dissection was performed at 1 and 4 h after BrdU administration and then every 24 h from Day 1 to Day 8. Niche distance and labelled distance measurements were taken for all the insects. In addition, we carried out a separate quantitative analysis to distinguish three different types of niche. Niches were characterized by measuring the nuclear diameter of BrdU-labelled cells only in zones where the epithelium was sectioned in a perfectly longitudinal way. Small nuclei were less than or equal to 5–6 μm in diameter, while medium-sized nuclei were between 8 and 12 μm diameters. Niches with one or more small positive nuclei (SN), niches with only medium-sized positive nuclei (MN) and negative niches with no BrdU-stained cells (NN) were quantified separately. Data were analysed with a chi-square test.

Single BrdU administration for confocal microscopy 3-D reconstruction and cell recount

The aim of this experiment was to estimate the total number of BrdU-labelled cells per niche and to study their spatial distribution pattern. A total of 15 insects was collected and dissected at different time intervals, i.e. at 4 h (Day 0) and on Days 1, 2 and 3 after BrdU administration, and were then prepared for confocal microscopy. Labelled BrdU nuclei and immunostained FMRFa-like cells were counted in each 3-D reconstructed niche. Data were analysed with Kruskal–Wallis and Mann–Whitney *U* statistical tests.

Continuous BrdU administration

We performed continuous BrdU administration experiments in order to study the dynamics of cycling cells within the niche

and to ascertain whether these cells are synchronized in their cycle. Two groups of 14 insects were injected over a period of 8 days. In both groups, BrdU administration was performed 24 h after the moult and then every 24 h for Group 1 and every 48 h for Group 2 until dissection day.

Cell cycle experiment

A separate experiment was performed to study the duration of the cell cycle and each of the cell cycle phases. 144 insects were collected 24 h after the moult and were injected with BrdU. After BrdU administration, the locusts were dissected at 30 min, at 1 h and then every hour until 24 h had elapsed. To determine the cell cycle phases we used the method of Willems (Willems, 1972). This method extrapolates cell cycle duration from the graphical representation of the BrdU labelled/unlabelled mitosis ratio against time. The total cell cycle (Tc) duration is equivalent to the distance between the first and second labelled mitosis peaks.

Label-retaining cell experiment

According to previous studies (Cairns, 2002; Potten et al., 2002) label-retaining cells (LRC) are very likely to be stem cells. To study the presence of LRC, we administered a daily BrdU dose to a sample of 27 locusts for five days. Locusts were dissected at days 10, 12, 14, 16 and 18 after the last BrdU administration, and the presence of LRCs was assessed.

Results

Single BrdU administration experiments

BrdU immunoreactivity appeared as a dark granulated material in the nuclei of midgut epithelial cells. The number and distribution of BrdU-positive nuclei and the intensity of staining varied depending on the time lapsed from the injection to the dissection of the midgut.

In the first 48 h, all the BrdU labelled cells were located in the niche area and no immunoreactive nuclei were observed in the differentiated columnar cells (Fig. 1A). Not all cells within a given niche were immunostained for BrdU. Between 48 and 72 h after a single BrdU administration, a fraction of labelled cells was observed just above the niche area. A group of positive small to medium-sized nuclei remained in the niche. From Day 4 to Day 8, the proportion of BrdU-immunoreactive nuclei in the niche markedly decreased, while an increasing fraction of differentiated columnar cells nuclei were immunostained in the epithelium (Fig. 1B). The distances between the epithelium base and the front wave of labelled cells were measured and represented graphically (Fig. 2). The distance between the center of the regenerative niche and these labelled cells increased progressively with time. The statistical correlation between distance and experimental time is significant ($P<0.05$), which suggests that cells are progressively moving away from the center of the niche to the taller epithelial region in contact with the lumen.

A Pearson's chi-square test showed significant ($P<0.01$) differences in each niche type according to the presence of small or medium-sized positive nuclei (SN, MN, NN) throughout the 8 days of experimentation. The percentage of SNs was maintained for periods of 48–72 h. Every 48–72 h, the SN proportion was reduced by ~50%. Throughout the 8 days, the SN proportion decreased three times, from an initial value of 36% to 22%, then to 12% and finally to 5% on Day 8 (Fig. 3). MN frequency was approximately 35–40% after 48 h, from Days 2 to 3 it decreased to 15–20% and was maintained until Day 8 at this level. The frequency of NN increased from 20% to 80% across the 8 days of experimentation, parallel to the decrease of the other niche types.

Confocal microscopy-based 3-D visualization of BrdU-labelled niches provided additional morphological information. Labelled nuclei were usually observed in groups of 5–8 (Fig. 4A). The intensity and intranuclear distribution pattern of the BrdU label changed from Day 0, in which nuclei showed a pattern of compact and intense labelling, to Days 1–3, when a more sparse and granular pattern was found in most nuclei. The total number of labelled BrdU nuclei was counted from 3-D reconstructed niches during the first 72 h. A Mann–Whitney *U* statistical test was performed in order to compare the mean number of positive BrdU cells per niche in each day. A statistically significant increase in the number of BrdU-labelled cells occurred from Day 0 (4 h after injection) to Day 1 (Fig. 5). The number of positive cells in Day 1 was almost double that of Day 0 and did not change significantly in the following 48 h.

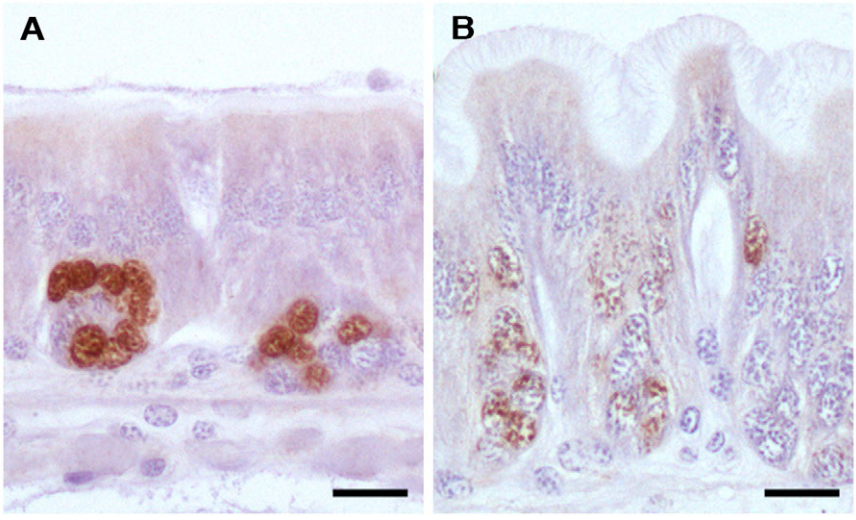

Fig. 1. Single BrdU administration experiment. Insects dissected 4 h (A) and 120 h (B) after the BrdU administration. Scale bar, 20 μm.

Continuous BrdU administration experiments

These experiments were performed in order to analyse whether the cells within the niche are synchronized. As expected, the insects

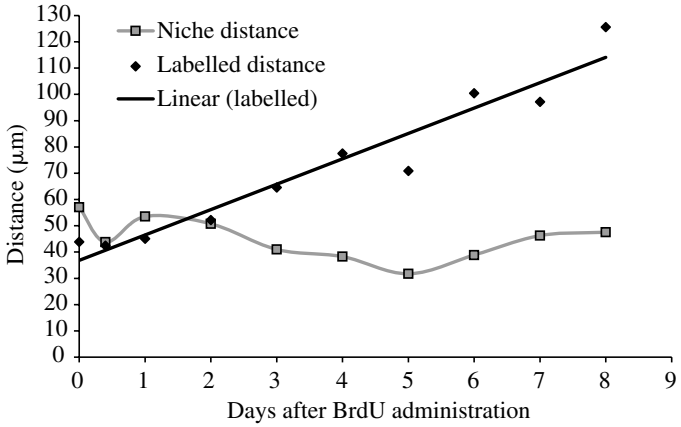

Fig. 2. Representation of niche height and distance of labelled nuclei to the base of the epithelium distances plotted against time (N=20). Labelled distance adjusts significantly to a lineal regression (R=0.93) with the following formula y=0.39x+37.3.

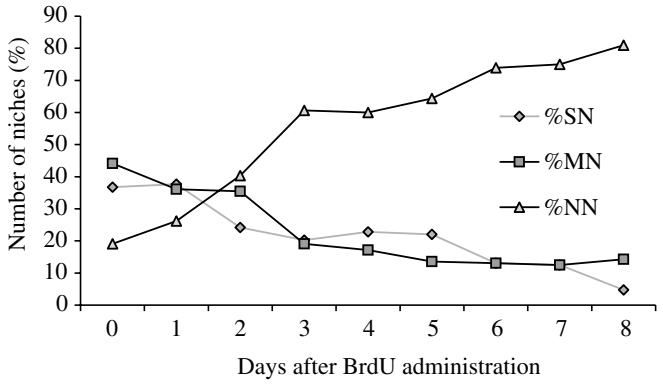

Fig. 3. Frequencies of the three different niche types (SN, MN and NN) over 8 days of experimentation in the single BrdU administration experiment (N=20). SN, niches with small positive nuclei; MN, niches with only medium-sized positive nuclei; NN, negative niches with no BrdU-stained cells.

that received a BrdU dose every 24 h for 3–8 days (Group 1) showed 95% of the niche cells labelled (Fig. 6A). However, the insects belonging to Group 2, which received an intermittent BrdU dose every 48 h, showed clusters of unlabelled cells within the niche (Fig. 6B).

Cell cycle duration

The duration of cell cycle phases was determined by graphical estimation. Using Willems method (Willems, 1972), we represented the ratio (percentage) of BrdU-labelled *vs* total (labelled + unlabelled) mitosis plotted against the time elapsed from BrdU injection to dissection. The complete cell cycle duration (Tc) was estimated to be 10 h, This time was calculated from the graph by measuring the distance between the two peaks of high percentage BrdU-labelled mitosis (Fig. 7).

Label-retaining cell experiments

Ten days after the last daily BrdU administration, almost 80–90% of the epithelial cells, including niche cells and columnar cells outside the niches, were labelled. Over the following days, labelled cells were progressively eliminated and replaced by a new generation of non-labelled cells. The high sustained proliferation rate of the cells within the niches allowed for a complete removal of BrdU-labelled cells within the epithelium at around Day 14 after the last BrdU administration, with the exception of some niches that presented a single long-term label-retaining cell (LRC) (Fig. 8). The LRCs were observed in a small number of niches with a random distribution along the midgut epithelium. A quantification of LRCs per millimetre was performed on the dissected insects on Days 14, 16 and 18. No significant differences (P>0.05) were observed between values on Days 14, 16 and 18, so data were pooled together (Table 1). We observed a mean of 0.15 LRCs per mm of sectioned midgut, which means that approximately 1.5% of the niches sectioned showed a long-term LRC. We also analysed the proportion of BrdU-positive cells with a small nucleus in the dissected insects at Days 10 and 12. We termed these cells ‘short-term

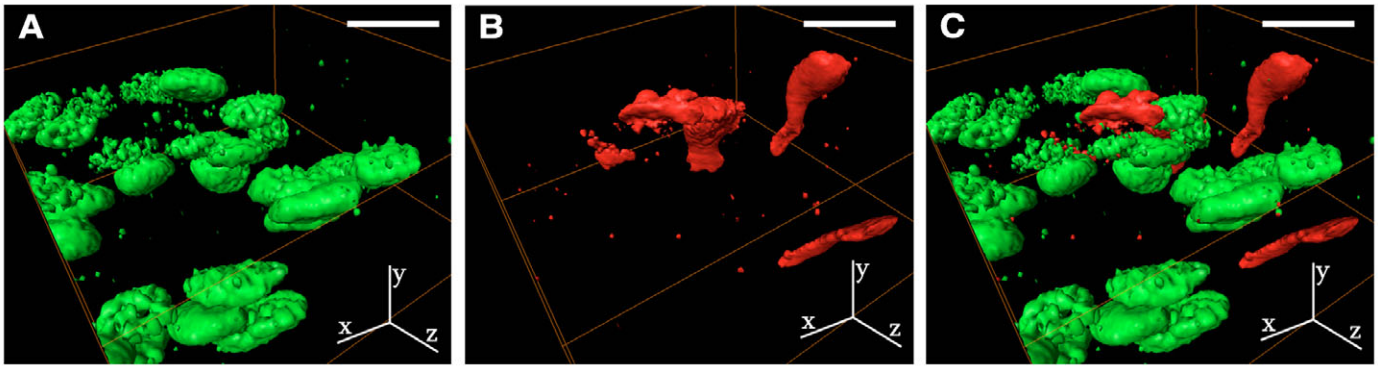

Fig. 4. Isometric vision of 3-D reconstructed image from confocal stack images. (A) BrdU-labelled nuclei (green) located inside the niche area. (B) FMRFamide-like cytoplasmatic detection of endocrine cells (red). (C) Superimposition of A and B; no colocalization was observed. Scale bar, 10 μm.

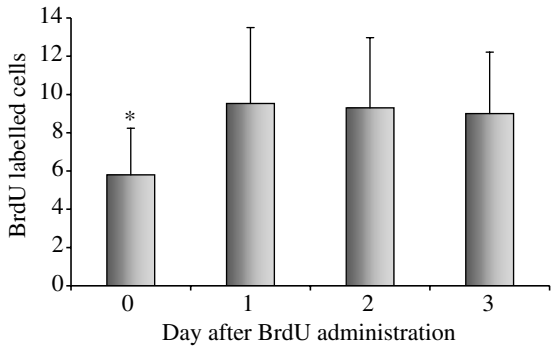

Fig. 5. Number of BrdU-labelled cells in the niche after a single BrdU administration. Significant differences (P<0.05) were only observed when comparing Day 0 with the other three days (N=15). This suggests that endoreduplication may occur in the BrdU-labelled cells.

LRC cells'. No significant differences (P>0.05) were observed in the proportion of short-term LRC cells between Days 10 and 12. Approximately 10.3% of the niches sectioned showed at least one short-term LRC cell.

Endocrine cell detection

FMRFa immunostaining was used to detect the endocrine cells that differentiate within the niche. FMRFa-like immunostaining was found in the cells of the diffuse endocrine system and was clearly cytoplasmic. The endocrine cells are in contact with the basal lamina and reach the midgut lumen through a slender cytoplasmic extension. 3-D reconstruction of the central midgut region (Fig. 4B,C) showed that 67% of the niches contain a single FMRFa-like immunoreactive cell, whilst 24% have two cells and 5% have three cells. The remaining 4% correspond to niches without FMRFa-like positive cells. Double immunodetection for BrdU and FMRFa was performed to determine the proliferative status of the endocrine lineage of the niche in several experimental situations. The nuclei of the endocrine cells are considerably smaller than the oval-shaped nuclei of the most abundant enterocytes and are usually located in the periphery of the niches. We performed a thorough search for double immunostained cells on the ventricle and ampulla midgut regions using double immunostaining. In the ventricle zone, no BrdU-positive endocrine cells were found in any of the experiments performed, including the continuous labelling experiments (Fig. 9A). However, in Group 1, treated during 8 days of continuous BrdU administration, 10% of endocrine cells of the ampullae showed BrdU and FMRFa-like colocalisation (Fig. 9B,C).

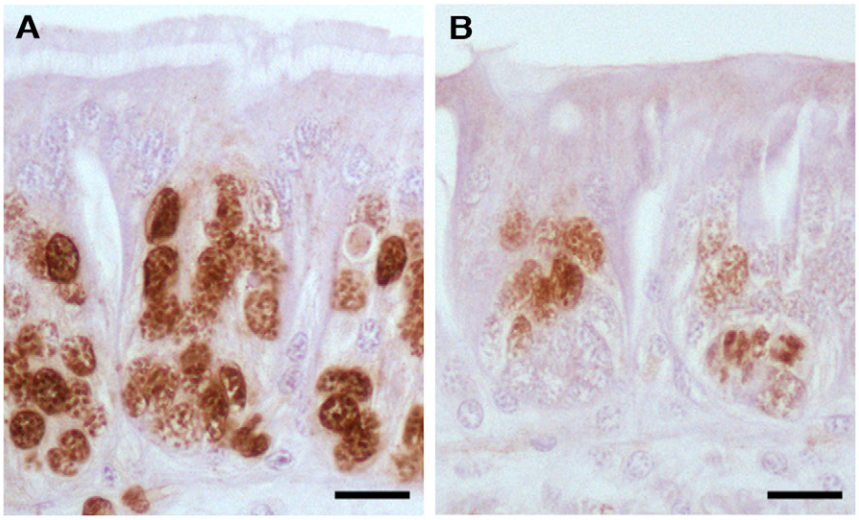

Fig. 6. Continuous BrdU administration experiments. Insects corresponding to Group 1 (A) and Group 2 (B), which received a BrdU administration every 24 h or 48 h, respectively. Scale bar, 20 μm.

Discussion

Our experimental results allowed us to assess cell population dynamics within the locust midgut niche. This model shares similarities with other simple niche models that have been studied (Fuchs et al., 2004; Ohlstein et al., 2004). As expected, the locust midgut niche is similar to the intestinal villus crypt while nonetheless representing a considerably simpler model. The stem cell of the mammalian intestinal villus crypt will renew the entire epithelium, giving rise to four types of differentiated cells, columnar, goblet, endocrine and Paneth cells (Marshman et al., 2002), while the stem cell of the locust midgut niche gives rise only to columnar and endocrine cells needed to renew the intestinal epithelium. In contrast to the mammalian tissue, in the locust midgut the regenerative cells are concentrated in a well-defined and easily identifiable region of the epithelium. They are potentially more accessible for isolation.

In other described regenerative niches, proliferating cells are also located in a circumscribed location, but the limits of the niche are not always evident (Chepko and Dickson, 2003; Ohlstein et al., 2004; Spradling et al., 2001). The results of our single BrdU administration experiments show that, immediately after injection, all the BrdU-labelled cells in the locust midgut are exclusively located in the niche area. Our previous data confirm these results as positive cells for PCNA (proliferating cell nuclear antigen), which are exclusively localized in the niche area and colocalize with BrdU-labelled cells (Zudaire et al., 2004).

When the stem cell of the intestinal villus crypt divides asymmetrically (Bach et al., 2000; Watt and Hogan, 2000), it produces a stem cell and a committed daughter cell that enters into a dividing transient state. These cells are known as transit amplifying cells (Slorach et al., 1999). In the mammalian intestine epithelium, transit amplifying cells proliferate and produce cells that differentiate

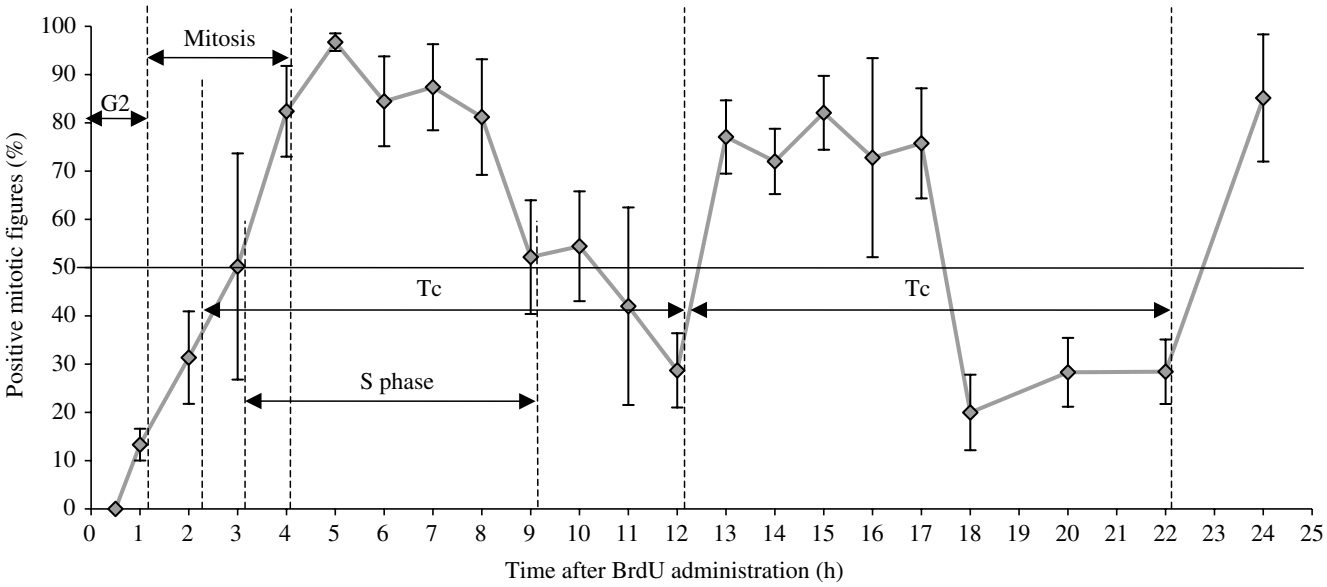

Fig. 7. Percentage of labelled mitosis against time (*N*=144). The cell cycle phase duration (Tc) was estimated following the method described by Willems (Willems, 1972). Percentage of positive mitotic figures refers to the ratio between BrdU labelled and total (labelled + unlabelled) mitosis.

and migrate towards the villus, where they perform their function before being shed to the lumen (Marshman et al., 2002). According to published reports, the cells produced in the mammalian crypt need approximately 3–8 days to reach the tip of the villus (Marshman et al., 2002; Potten, 1998). In a similar way, the locust midgut niche stem cell divides and gives rise to transit amplifying cells. Our data show that BrdU-labelled cells start to leave the niche between 24 and 48 h after BrdU administration. Differentiated cells maintain their migration through the gut epithelium towards the lumen for 5–8 days. Columnar cells are shed out of the lumen 8–10 days after they differentiate. These data are very similar to those observed by Endo in the cockroach midgut (Endo, 1984).

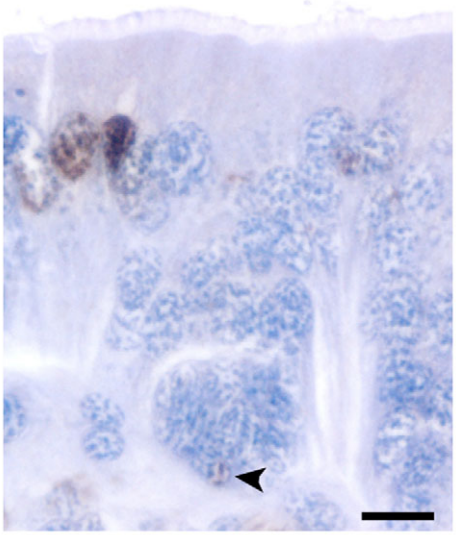

Fig. 8. Insect dissected 14 days after the last BrdU administration. The arrowhead shows a long-term label-retaining cell located at the base of the niche. Scale bar, 10 μm.

Transit amplifying cells have a higher probability to be labeled by BrdU than do stem cells due to their rate of proliferation. When we administered BrdU every 24 h, all the niches were almost completely labelled. Our results showing positive and negative cell clusters suggest the existence of synchronised groups of amplification cells within a given niche. According to the data obtained by 3-D reconstruction, 5–8 amplification cells could be found per niche at the BrdU administration time. This number doubled after 24 h of administration and did not change significantly in the following 2 days. These data suggest that not all the labeled cells that incorporate BrdU (and thus are synthesizing DNA at Day 0) enter into mitosis. According to previous studies (Zudaire et al., 2004), differentiated columnar cells have multiple DNA copies in their nuclei. To produce polyploid or polytenic cells, the cell must undergo multiple S phases without entering into mitosis, a process termed endoreduplication (Edgar and Orr-Weaver, 2001). The non-dividing labeled cells that we have found in this experiment could be endoreduplicating cells.

The cell cycle duration experiment showed a cell cycle time of ~10 h. This short cell cycle is characteristic of amplification cells, compared with the longer cell cycle expected for stem cells. The mouse intestinal villus crypt also presents two different cell populations: transient amplifying cells and stem cells (Slorach et al., 1999). The average cell cycle time for amplification cells in the mammalian intestinal villus is about 12–13 h, while the stem cell cycle lasts around 24 h. (Bach et al., 2000; Potten, 1998). Thus, in both models

Table 1. *Quantification of LRC (N=21) and short-term LRC (N=10) per mm of sectioned midgut, and estimation of the percentage of niches showing LRC or short-term LRC*

	Value ± s.d.	LRC/niche	Short-term LRC/niche
LRC/mm			
Day 14	0.18±0.06		
Day 16	0.19±0.07		
Day 18	0.10±0.06		
Mean	0.15±0.07		
Short-term LRC/mm			
Day 10	1.12±0.09		
Day 12	0.89±0.11		
Mean1.03±0.15			
Mean niches/mm	10.30±1.96	1.5%	10.3%

(mammals and locust), amplification cells divide approximately twice a day.

Lack of specific intestinal stem cell markers is a major problem for studying the biology of intestinal epithelial differentiation from stem cells (Leedham et al., 2005; Marshman et al., 2002; Potten, 1998). However, the labelling retaining property is a potential surrogate marker for a stem cell (Braun et al., 2003; Braun and Watt, 2004). Long-term LRCs were round, with a nucleus smaller than that of amplifying or columnar cells and were localized basally in the niche. Insects dissected on Days 14–18 showed only 1.5% of the niches having long-term LRC. However, insects dissected on Days 10 and 12 showed 10% of the niches with short-term LRC. Results obtained in the quantification of three niche types showed that the proportion of SN with small short-term LRCs decreases every 48–72 h. From this, we can estimate that the cell cycle duration of these short-term LRCs is 2–3 days and thus it is slower than that of amplification cells and faster than that of long-term LRCs.

Studies of intestinal epithelium in mice have shown that endocrine cells share their clonal origin with other gut cells, suggesting a common stem cell origin (Thompson et al., 1990). The locust midgut endocrine cells also seem to be derived from the common precursor cells present in the niches, at least according to their spatial localization within the niche and their very close relationship with the rest of the epithelial cells. We did not observe colocalization among FMRFa-like immunolabelled cells and BrdU-labelled nuclei in the midgut ventricle. However, 10% of the endocrine cells of the Malpighian tubules ampullae draining to the midgut incorporated BrdU after 24 h administration. A similar lack of tritiated thymidine labelling was observed in the endocrine cells of *Periplaneta americana* midgut, suggesting a slower cell cycle for these cells (Endo, 1984). Our results suggest that new endocrine cells are indeed being generated in the ampullar region at a slow rate. Apparently, these cells do not proliferate or differentiate from stem cell-derived precursors in the ventricle, or, if they do, the process is extremely slow and not detectable under our experimental conditions. These regional differences in the generation of new endocrine cells are very intriguing and point towards a very fine regulation of endocrine differentiation by the microenvironment or by cell–cell interactions. The niches located in the ampullar region are bathed by the same haemolymph as those of the ventricle, suggesting that the regulation of endocrine cell differentiation within the niches is dependent upon local rather than systemic stimuli. The ampullar endocrine cells are very abundant and may be involved in the control of excretory and digestive physiology (Montuenga et al., 1996; Zudaire et al., 1998). The number of endocrine cells per niche did not change

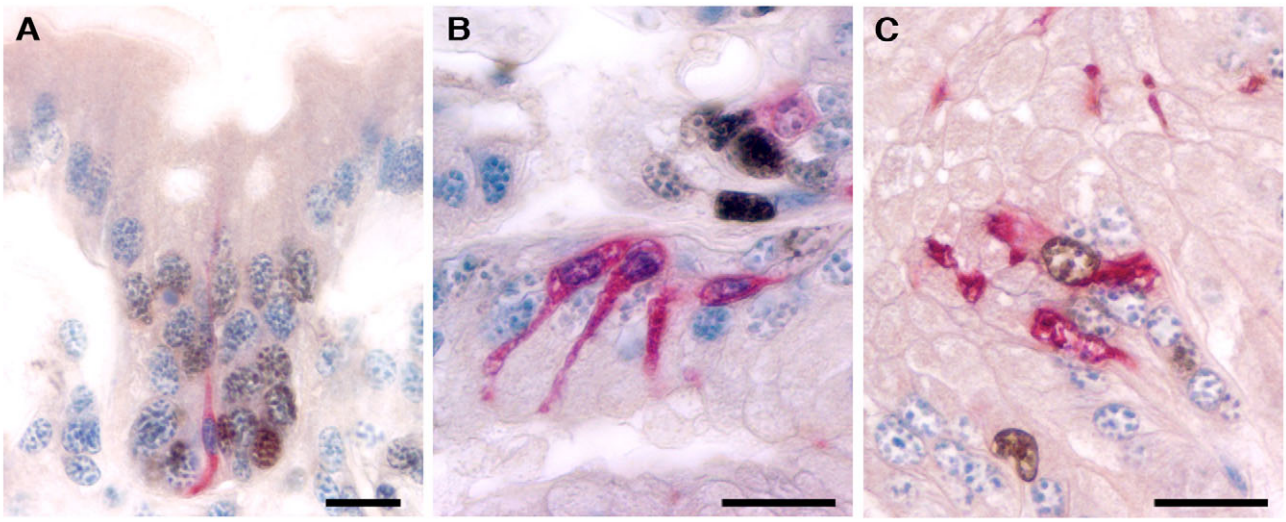

Fig. 9. Double immunocytochemistry, BrdU-labelled nuclei in brown and endocrine (FMRFamide-like) cells with pink cytoplasm. No colocalization was observed in the ventricle midgut area (A). However, BrdU-labelled nuclei can be clearly observed in some of the ampullar endocrine cells (B,C), some of which have double immunostaining for BrdU and FMRFa (C). Scale bar, 20 μm.

throughout the days of experimentation. Endocrine cells where not shed to the lumen as columnar enterocytes. Moreover, our unpublished data show that a good percentage of endocrine cells are already present in the epithelium of all the previous larval stages, with a particular increase in endocrine cell frequency in the 4th instar insects. All these data suggest that endocrine cells represent a very stable population with a very low proliferation rate, except in the ampullar region, where a higher turnover and differentiation of endocrine cells seems to occur.

In conclusion, we have described here a simple *in vivo* insect model of epithelial regeneration that may help in understanding the complex regulatory mechanisms of cell proliferation, differentiation and tissue homeostasis.

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