Cell proliferation dynamics of somatic and germline tissues during zooidal life span in the colonial tunicate *Botryllus primigenus*

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ABSTRACT

Botryllus primigenus is a colonial tunicate in which three successive generations develop synchronously. In order to identify proliferation centers and possible adult stem cells during asexual reproduction, somatic and germline cells were labeled with 5-bromo-2'-deoxyuridine (BrdU). In the youngest generation, multipotent epithelial cells exhibited an average labeling index (LI) of 30% 24 h after BrdU injection. In the middle generation, the LI of organ rudiments decreased gradually and reached zero by the beginning of the eldest generation. Exceptionally, cells of specialized tissues such as the pharyngeal inner longitudinal vessel and the posterior end of the endostyle continued DNA synthesis and mitosis even in the eldest generation. Proliferating somatic and germline cells of younger generations expressed a *Botryllus myc* homolog (*BpMyc*), but adult tissues did not. This result strongly suggests that in *B. primigenus* undifferentiated progenitor cells are discernible from possible adult stem cells by the presence or absence of *BpMyc*.

INTRODUCTION

The regenerative potential of living organisms is guaranteed by cell activities such as proliferation, differentiation, and morphogenesis. An example of a group of cells that executes such activities is known as the regeneration blastema in planarians (Reddien and Sanchez Alvarado, 2004), annelids (Paulus and Muller, 2006), and amphibians (Brookes and Kumar, 2005). Blastema cells arise either from undifferentiated reserve cells or from dedifferentiated cells in preexisting tissues (Okada, 1991). The reserve cells are also known as stem cells, which include the multipotent adult progenitor cells (MAPCs) in mammals (Jiang et al., 2002) and the crypt cells in the vertebrate small intestine (Potten and Loeffler, 1987; Ishizuya-Oka, 2007). Another example of formative tissue is multipotent epithelium such as the peritoneum in bryozoans (Brien, 1968), the coelomic epithelium in sea cucumbers (Mashanov et al., 2005), and certain types of mesodermal and endodermal epithelia in colonial tunicates (Kawamura et al., 2008). These epithelial cells change the cell fate directly without cell dissociation or blastema formation. The molecular and cellular bases of the regenerative potential are major subjects of study in developmental biology and regenerative medicine.

In colonial tunicates belonging to the Stolidobranchiata, the atrial epithelium is developmentally multipotent (Abott, 1953; Kawamura and Nakauchi, 1986a; Casagrande et al., 1993; Kawamura and Fujiwara, 1994; Burighel et al., 1998). Its regenerative potential is utilized for asexual reproduction, referred to as pallear budding, in which the atrial epithelium

evaginates along with the epidermis to form the inner vesicle of a double-walled bud (Berrill, 1940, 1941a; Izzard, 1973). The inner vesicle plays a key role in renewing and remodeling the body architecture (Fig. 1) (for reviews, Berrill, 1950a; Manni et al., 2007; Kawamura et al., 2008). In *Polyandrocarpa misakiensis*, the cell proliferation kinetics of the multipotent epithelium has been examined only at the early stages of bud development (Kawamura and Nakauchi, 1986b; Kawamura et al., 1988). In *Botryllus primigenus*, the mitotic indices of multipotent cells have been superficially examined during the zooidal life span (Kawamura et al., 2006).

Species belonging to the genera *Botryllus* and *Botrylloides* form a flat, gelatinous colony, in which three successive asexual generations coexist (Fig. 1A) (Watanabe, 1953; Laird et al., 2005; Manni et al., 2007). The zooids are a few millimeters at most in length. The bud primordium (the youngest, 3rd generation) develops synchronously into the middle (2nd) generation that can produce buds. Only zooids of the 1st generation feed and degenerate under the synchronous time schedule (Lauzon et al., 2002). As developing zooids transit from one generation to the next every 4–6 days, the life span of zooids is less than 3 weeks. From the viewpoint of the colonial growth cycle in *Botryllus*, Watanabe (1953) proposed four phases of colony growth (A, B, C, and D) with reference to zooidal development and degeneration (Fig. 1B) (Kawamura and Sugino, 1999). Zooidal developmental stages have recently been revised in *B. schlosseri* (Manni et al., 2007). Zooids possess free undifferentiated cells known as hemoblasts in the hemocoel (Wright, 1981). All these features of the

developmental synchrony and short life span in *Botryllus* zooids are advantageous for studying the relationships among cell proliferation, cell differentiation, and multipotency.

This study was designed to disclose the cell proliferation dynamics of *B. primigenus* during the life span of the asexual zooids. Colonies were injected with a thymidine analog, 5-bromo-2'-deoxyuridine (BrdU), through the vascular ampullae and chased for 1–4 days. The quantitative data on labeled nuclei indicate, for the first time, possible adult stem cells in *B. primigenus*. They also suggest the mechanism by which blood cells in the test vessel are recruited. In order to evaluate the relationship between the proliferation and developmental multipotency of tunicate cells, we examined the expression of the *Botryllus myc* homolog. In mammals, *c-myc* is a proto-oncogene that encodes a nuclear protein (Reed et al., 1987). It plays a role in the regulation of proliferation and differentiation in embryonic and postembryonic progenitor cells (Zanet et al., 2005). In *Xenopus*, limb regeneration accompanies the enhanced expression of the *c-myc* gene (Geraudie et al., 1990). In the present study, using a *myc* homolog, we demonstrate that in *B. primigenus* the multipotent formative cells and tissues are discernible from adult proliferating cells.

RESULTS

Preliminary experiments for labeling and chasing

As pilot experiments, trypan blue solution (12 μ l) was injected via a single ampulla at the colonial periphery (Fig. 1C). The dye spread successfully into the colony, at first in a patch-like manner

(Fig. 1C), then becoming evenly distributed a few hours later. The colony appeared healthy and grew continuously without apparent colony-wide cell death. We therefore decided to inject BrdU solution (12 μ l) into one or two (12 μ l $\times$ 2) sites in the ampullae. Next, the concentration of BrdU to be injected was considered. A total of 7, 14, or 35 ng BrdU was administered to colonies (approximately 4 cm square). One fourth of the colony was extirpated and fixed at definite time periods. The signal intensity of 7 ng-injected colonies was somewhat weaker than that obtained using the other concentrations; however, the labeling indices for each concentration were similar to one another. In the following experiments, the data for the 14 ng-injected colonies were mainly used.

Another preliminary experiment was performed in order to examine the extent to which the labeling index (LI) altered during the time period of chase after BrdU injection. In the pharynx and some other tissues, the LI did not alter dramatically from 18 h to 96 h (Table 1). This result may indicate that in injected colonies the supply of free BrdU becomes exhausted within 24 h. Therefore, the data for 24 h-chased colonies were used exclusively in this study. Unexpectedly, we were unable to observe labeled nuclei at 6 h and 12 h after injection under the original experimental condition. We found later that successful results were obtained when the volume to be injected was reduced to 1 μ l without altering the total amount of BrdU (Fig. 2A). So, this condition of injection was used only for serial chase experiments. The detailed observations on long-term chase after labeling will be described later.

Labeled cells in the pharynx

The bud primordium consists of a

thickened disc of the atrial epithelium (Fig. 2B). This evaginates together with the epidermis to form the inner vesicle of a double-walled bud. The outer vesicle (epidermis) showed the relatively stable LI until the end of the 2nd generation (Table 2). The LI of the bud's inner vesicle was approximately 15% (Table 2; phase B of the 3rd generation), the value comparable with that of the parental atrial epithelium continuous with the inner vesicle (Table 2; phase A of the 2nd generation). The inner vesicle formed two diverticula (gut rudiment and neural complex) at developmental stages 4–5. Each rudiment showed the highest LI at the end of the 3rd generation (Table 2).

The remaining inner vesicle was subdivided into the branchial and peribranchial chambers at stage 6 (Fig. 2C). The pharyngeal wall consists of outer and inner epithelia, of which the LI was 15% at the beginning of 2nd generation; the index gradually fell until the emergence of the 1st generation (Table 2). In contrast, the atrial epithelium continuous with the outer pharyngeal epithelium rapidly lost the labeling activity at the middle stage of the 2nd generation (Table 2). The inner pharyngeal epithelium was further elaborated to form the prepharyngeal epithelium, inner longitudinal vessels, and the endostyle (Fig. 2D–F). The prepharyngeal epithelium was labeled not only in the 2nd generation but also in the 1st generation (Fig. 2E, I, Table 2). The inner longitudinal vessels of the pharynx retained an exceptionally high LI until the late stage of the 1st generation (Fig. 2J; Table 2). The endostyle incorporated BrdU most actively around phase B of the 2nd generation (Table 2). Thereafter, the signal disappeared from most areas of the endostyle (Fig. 2G); however, the posterior end retained a high LI score

until phase B of the 1st generation (Fig. 2H).

Labeled cells in the gut

After being separated from the pharyngeal rudiment, the gut rudiment incorporated BrdU most actively (LI = 62%) at the end of the 3rd generation (Fig. 3A; Table 2). It then subdivided into the esophagus, stomach, pyloric cecum, and intestine. The esophagus was labeled most actively during the early phases of the 2nd generation (Fig. 3B; Table 2). The labeling index then decreased. However, even in the 1st generation, the nuclear labeling was retained in specific areas of the folded epithelium (Fig. 3E). The stomach was labeled most actively at phase A of the 2nd generation (Fig. 3C, D; Table 2). From phase C of the 2nd generation onward, labeled nuclei were rarely observed; however, they still persisted at phase A of the 1st generation (Fig. 3F; Table 2). The labeling index of the intestine was high at phase A of the 2nd generation (Fig. 3C, D; Table 2). It decreased rapidly at phase B, and reached zero by the beginning of the 1st generation (Fig. 3G; Table 2). Likewise, the pyloric cecum incorporated BrdU only during the early phases of the 2nd generation (Fig. 3D, H).

Labeled cells in the neural complex

The neural complex is a diverticulum situated dorsally on the roof of the pharyngeal epithelium. It appeared at stages 4–5 of the 3rd generation (Fig. 4A, B). The tube retained a relatively high labeling index (20–30%) until phase B of the 2nd generation (Table 2). At this stage, the labeled nuclei were restricted to the bottom of the tube (Figs. 4C, 5E), whose opening to the pharyngeal chamber is termed the ciliated groove.

The dorsal ganglion appeared as a

cell aggregate at approximately the same time as the neural complex (Fig. 4A, B). Single cells exhibited a low labeling index throughout the formation of the brain (Table 2). At phase B of the 2nd generation, the nuclear labeling was still observable in a very few cells (Fig. 4D); however, thereafter it fell to zero (Fig. 4E; Table 2).

Labeled cells in the hemocoel

Labeled blood cells were notable in the ventral blood sinus around the endostyle (Fig. 5A, B). They were also observable around the digestive tract and within the pharynx (Fig. 5C, D). The LI was 10-15% throughout zooidal development, and it became highest (almost 20%) in the early phase of the 2nd generation (Table 3). Many blood cells gathering around the oral and atrial siphons were labeled with BrdU (Fig. 5E). In the test vessel, many labeled cells were observable. Most of these were the epidermal epithelium and a very small number of cells were blood cells (Fig. 5F arrowheads). The LI of blood cells proper was approximately 3 % (Table 1).

Labeled cells in the gonad

A loose cell mass first appeared in stages 4–5 of the 3rd generation. The LI was similar to that of single cells in the blood sinus (Table 3). It soon attained a value of approximately 50% in stage 6 of the 3rd generation (Fig. 6A; Table 3). This high LI value persisted until phase B of the 2nd generation. The loose cell mass was transferred to the 3rd generation, during which it grew into a compact cell mass (Fig. 6B; Table 3). When colonies became sexually mature, the compact cell mass remained and grew further in the 2nd generation to differentiate in situ into the testis (Fig. 6C). Initially, the labeled cells were distributed evenly in the testes;

however, by the end of the 2nd generation they were localized at the periphery (Fig. 6D). Labeled cells were never observable in the 1st generation (Table 3).

In the developing testis, labeled mitosis was first observed 6 h after injection (Fig. 6C', arrowhead). The ratio of labeled mitosis to labeled nuclei was approximately 15% in 6 h-chased specimens and 9% in 12 h-chased specimens (Table 1). It became approximately 30% 18 h after injection (Fig. 6C, arrowheads), and then it fell rapidly 24 h after injection (Table 1).

A loose cell mass also developed into the ovary. A few cells were associated with each other in the gonadal space (Fig. 6E). Usually, these were equally labeled with BrdU. At the previtellogenesis stage, only primary follicle cells that surrounded the oocyte incorporated BrdU (Fig. 6F). At the vitellogenesis stage, accessory cells in the ovary were no longer labeled with BrdU (Fig. 6G).

Long-term chase

As shown in the preliminary experiments, the LIs of the pharynx were not dramatically altered between 24 h chase and 96 h chase (Fig. 7A, B; Table 1). This was also the case in the esophagus (Fig. 7D, E) and stomach (Fig. 7F, G). In the epidermis and endostyle, the value was somewhat lowered at 96 h (Fig. 7A, C; Table 1). The LI of the compact cell mass increased by approximately 2-fold from 18 h to 24 h and then became stable (Table 1). Blood cells in the test vessel yielded exceptional data. Their labeling index was very low (approximately 3 %) at 18–24 h, but it increased by more than 10-fold at 96 h (Fig. 7H, I; Table 1).

Mitotic figures in developing buds and

zooids

In order to confirm the results of nuclear labeling with BrdU, mitotic activities were examined in colchicines-treated colonies. In the 3rd generation (stage 6), the outer and inner pharyngeal epithelia were thickened evenly (Fig. 8A), and mitotic figures were observable abundantly (Fig. 8B arrowheads). The gut rudiment and the atrial epithelium also divided actively (Fig. 8C arrowheads). In the 2nd generation (stage 7), thickened epithelia became gradually limited (Fig. 8D); stigmatal anlage (Fig. 8E), endostyle (Fig. 8F), inner longitudinal vessel, gut rudiment, and prepharyngeal epithelium (not shown). Mitotic activities were also limited to these tissues (Fig. 8E,F arrowheads). At the end of the 2nd generation (phase C-D, stage 8), mitotic figures were further limited to, for example, the posterior area of the endostyle (Fig. 8G). The stomach showed no longer mitotic activities (not shown).

In the 1st generation (stage 9), a very limited number of dividing cells were still observable in specialized tissues such as the posterior end of the endostyle (Fig. 8H arrowheads) and the inner longitudinal vessel (Fig. 8I arrowheads). The esophagus, intestine, and pyloric ducts no longer showed the mitotic activity (not shown). In order to obtain further evidence, tissues of the 1st generation were immunostained with the anti-PCNA antibody. The thickened epithelium of the inner longitudinal vessel was stained (Fig. 8J).

Gene expression in proliferating multipotent cells

The Myc homolog of *B. primigenus* (*BpMyc*, accession number AB362816) was expressed in the multipotent bud primordium of the 3rd

generation (Fig. 9A, B, C). Signals persisted in the developing pharynx of the 2nd generation (Fig. 9D). Especially, thickened epithelia of the endostyle, outer pharyngeal epithelium, and inner longitudinal vessels emitted the strong signal. The sense probe did not stain the bud primordium nor developing tissues at all (Fig. 9E).

Signals of the developing digestive tract became weak at the early phase of the 2nd generation (Fig. 9F). In the 1st generation, no signals were detected in the pharynx, inner longitudinal vessels, esophagus, and stomach (Fig. 9H, I).

Strong signals were detected from loose cell masses (Fig. 9G) and compact cell masses (Fig. 9B) in the germline tissues. In developing testis (Fig. 9G) and oocytes (not shown), signals became very weak.

DISCUSSION

Consideration of the preliminary experiments

In this study, as a pilot experiment, sterile seawater (12 or 24 μ l) of cell culture grade was injected into a colony at one or two sites through the vascular ampullae; these colonies appeared healthy after injection and grew continuously without apparent colony-wide cell death. Using 14 pg BrdU per colony, the nuclei were labeled satisfactorily at 18–24 h after injection; however, we were unable to detect any labeled nuclei in colonies at 6 h and 12 h after BrdU injection. These results suggest that the injection might cause some temporary damages to the colonies. Several possible causes were considered, and finally we found that in order to obtain data earlier than 18 h, a total volume to be injected should be reduced to 1 μ l without altering the amount of

BrdU.

Proliferation dynamics of epithelial multipotent cells

This study has demonstrated that in *B. primigenus* the bud primordium established from the atrial epithelium shows a relatively low LI comparable with that of the atrial epithelium. Then, the value rises in organ rudiments, and finally the LI of most organs fell to nearly zero at phase C or D of the 2nd generation (Table 2, Fig. 10). Recently, we investigated the kinetics of the mitotic index (MI) during the zooidal life span in *B. primigenus* (Kawamura et al., 2006). All tissues and organs had ceased mitosis by the end of the 2nd generation, although the ganglion cells had precociously ceased to divide. The results of the present study are roughly consistent with those for the MI. In *P. misakiensis*, the dorsal strand cells derived from the neural complex exhibited an ultrastructurally undifferentiated configuration even in adult zooids (Koyama, 2002). Consistent with this finding, we observed labeled nuclei at the bottom of the neural complex tube of the 2nd generation in *B. primigenus*. The present study also demonstrated that a small number of cells in the 1st generation could still incorporate BrdU in some organs such as the pharynx and digestive tract.

In polystyelid tunicates, including *Polyandrocarpa*, *Metandrocarpa*, and *Polyzoa*, the atrial epithelium is able to form a bud primordium anywhere along the zooidal body axis (Abbott, 1953; Fujimoto and Watanabe, 1976; Watanabe and Newberry, 1976; Kawamura and Watanabe, 1982). In these animals, therefore, whole cells of the atrial epithelium should be considered multipotent. In contrast, in botryllid

tunicates such as *Botryllus* and *Symplesma*, the bud primordium is restricted to a predictable region(s) of the atrial epithelium (Berrill, 1940, 1941a, b; Sabbadin, 1955; Sugimoto and Nakauchi, 1974). With respect to this observation, the present study demonstrated that in *B. primigenus* the LI of the atrial epithelium distant from the bud primordium decreased rapidly during the 2nd generation. It may be true that in botryllid tunicates the atrial epithelium loses both the proliferative potential and regenerative multipotency as soon as it develops the bud primordium.

Proliferation dynamics of coelomic cells

In *B. primigenus*, coelomic cells in the zooidal blood sinus exhibited a relatively stable LI from the 3rd to the 1st generation, of which the early 2nd generation exhibited the highest labeling activity (Fig. 10). Labeled cells were notable around the viscera, particularly the endostyle, stomach, and pharynx. In *Styela clava*, the hematopoietic nodule has been found in the blood sinus around the endostyle (Ermak, 1976). Although we do not know whether budding tunicates contain hematopoietic tissues, it is evident that coelomic cells proliferate actively around the viscera.

The LI of coelomic cells in the test vessel was very low, suggesting that blood cells outside the zooids rarely enter the cell division cycle. In contrast, blood cells chased for 96 h exhibited a high LI (55.4%). This increase in LI should not be attributed to the uptake of remaining free BrdU, if any, in the body fluid during the long period of chase, since in other tissues the LI did not increase markedly after a prolonged chase. A probable explanation is that in *B. primigenus* a majority of the labeled free cells arise in the zooidal blood sinus and thereafter

enter the common vascular system.

During asexual reproduction, coelomic undifferentiated cells, the hemoblasts, differentiate into various tissues such as muscle cells, pyloric gland, germ cells, and multipotent epithelium (Mukai and Watanabe, 1976; Nunzi et al., 1979; Kawamura and Nakauchi, 1986a; Sunanaga et al., 2006, 2007; Sugino et al., 2007). In *Symplegma reptans*, hemoblasts cluster around the siphon anlagen of developing buds. When they become associated with the epidermis, the nucleus becomes indented and myofilaments appear in the cytoplasm facing the epidermis (Sugino et al., 2007). Similarly, in *B. primigenus*, coelomic free cells were associated with the siphon anlagen (Fig. 5E). These possible muscle precursor cells still possessed proliferation activity at phase B of the 2nd generation. This may explain how the multinucleation is established in the smooth body muscle cells of tunicates (Shinohara and Konishi, 1982).

In *B. primigenus*, germline hemoblasts formed a loose cell aggregate in the gonadal space at stages 4–5 of the 3rd generation. Their LI was initially almost 20%, a value comparable to that of coelomic cells in the blood sinus (Table 3). However, it increased rapidly to 50% (Table 3, Fig. 10). In general, this high rate of cell proliferation is characteristic of progenitor cells derived from stem cells (Hall and Watt, 1989).

In the developing testes, labeled mitosis was first observed 6 h after injection, suggesting that in primordial male germ cells the time length of G₂ + M phases of cell cycling was 6 h or less. The ratio of labeled mitosis to labeled nuclei varied from 15% (6 h later) to 30% (18 h later) through 9% (12 h later), and then it fell rapidly 24 h after injection. These results suggested that although the

primary peak of labeled mitosis was somewhat obscure, the cell cycle time of primordial male germ cells might be 12 h or more.

In the juvenile ovaries of *B. schlosseri*, the oocyte and primary follicle cells are ultrastructurally discernible from each other (Manni et al., 1994). In the present study, a few cells in the ovary were evenly labeled, which suggests that after one or more cell divisions the cells differentiate into either oocyte or primary follicle cells.

Possible adult stem cells

In mammals and other vertebrates, developmentally restricted stem cells regulate tissue homeostasis in adult organisms (Hall and Watt, 1989). For example, crypt cells in the digestive tract continue to proliferate and differentiate into functional enterocytes (Potten and Loeffler, 1987; Ishizuya-Oka, 2007). Consequently, they compensate for cell decay occurring in the small intestine due to events such as cell ageing and microbial infection. This highly organized stem cell system also operates in some invertebrate organs, such as the gonads (Yamashita et al., 2003) and intestine (Ohlstein et al., 2007) of *Drosophila*.

As previously mentioned, the life span of each *B. primigenus* zooid is very short. The zooidal MI falls to almost zero by the end of the 2nd generation (Kawamura et al., 2006). We therefore did not expect the existence of adult stem cells in *Botryllus* colonies. Surprisingly, results of BrdU incorporation in the present study suggested that, in *B. primigenus*, continuously proliferating cells indeed exist in several types of adult tissues such as the inner longitudinal vessels of the pharynx. In order to confirm these results, mitotic figures

were examined in colchicine-treated colonies. In the 3rd generation, dividing cells were evenly distributed in the thickened inner epithelium, but they became restricted to specialized tissues during zooidal development. The data were consistent with those of BrdU incorporation. In the 1st generation, the inner longitudinal vessels, pre-pharyngeal epithelium, and the posterior end of the endostyle still showed mitotic activities. However, the esophagus did not have any mitotic figures in contrast with the result of nuclear labeling. Results of anti-PCNA immunohistochemistry did not support the idea that the esophagus of the 1st generation contains proliferative cells (Fig. 8V). Therefore, it is safe at present to exclude the esophagus from the candidate for adult stem cells.

No literature is currently available regarding adult stem cells in both solitary and colonial tunicates. When we consider the long-term cell proliferation of the endostyle, the asexual reproduction of pelagic tunicates may be instructive. In *Pyrosoma*, oozoids and blastozooids form the primary and secondary stolons, respectively. Both stolons are ventroposterior outgrowths arising from the epidermis and the posterior extension of the endostyle (Berrill, 1950a). In *Doliolum*, oozoids form a ventral stolon, the inner tube of which is derived from the posterior end of the endostyle. The ventral stolon gives rise to numerous pro-buds by self-division or strobilation. The pro-buds then migrate toward the dorsal side where they develop into functional blastozooids (Berrill, 1950b). In *Salpa* also, the posterior end of the endostyle extends to form the inner, formative tissue of the stolon. It is therefore evident that in pelagic tunicates the endostyle plays an important role in asexual reproduction. The permanent cell

growth of the endostyle in *B. primigenus* may reflect the evolutionary significance of the endostyle in stolon formation in pelagic tunicates.

Molecular features of proliferating cells in *Botryllus*

As shown in this paper, both epithelial and coelomic multipotent cells in *B. primigenus* begin with the relatively low LI, and have the highest LI at the boundary between the 2nd and 3rd generations (Fig. 10). Thereafter, the LI decreases gradually during the 2nd generation and drops to zero by the beginning of the 1st generation with some exceptional tissues. Interestingly, this change of the LI is very similar to the fluctuation of telomerase activity in *B. schlosseri* (Laird and Weissman, 2004). In sexually immature colonies of *B. schlosseri*, the peak of the activity is located at the boundary between 2nd and 3rd generations. In sexually mature colonies, it shifts to phase A of the 2nd generation. Epithelial tissues and gonads in developing buds as well as embryonic tissues show the highest telomerase activity, while the activity is undetectable from blood cells collected probably from the terminal test vessel (Laird and Weissman, 2004). In this connection, it is interesting that in *B. primigenus* peripheral blood cells show a very low LI.

The expression pattern of *c-myc* homolog is also interesting in relation to cell proliferation and multipotency. In mammals, *c-myc* is a proto-oncogene that encodes the transcription factor that forms Myc/Max dimers (Pulverer et al., 2000). In mice, it plays a role in the proliferation and differentiation of progenitor cells derived from epidermal stem cells (Arnold and Watt, 2001; Zanet et al., 2005). *c-myc* is essential for the

regeneration of rat liver (Mauleon et al., 2004; Sanders and Gruppuso, 2006). In *B. primigenus* the expression pattern of *BpMyc* overlapped roughly with the cell proliferation pattern. The bud primordium that occupies the center for cell proliferation emitted the most intense *BpMyc* signal. A strong signal was also observable in loose and compact cell masses in the gonads. Both the bud primordium and germline progenitor cells are known to have an undifferentiated configuration (Manni et al., 1994; Sunanaga et al., 2006). It is therefore an interesting question as to whether *BpMyc* is related to cell proliferation or to the undifferentiated state of cells.

In *B. primigenus*, the pharyngeal rudiment and gut rudiment were *BpMyc*-positive in the 2nd generation. The stigmatal anlagen is a thickened disc of the outer pharyngeal epithelium that is relatively undifferentiated (Casagrande et al., 1993). In contrast, no tissues in the 1st generation were stained with an antisense probe of *BpMyc*, although they contained adult proliferative cells such as the pharyngeal inner longitudinal vessel and plicated portions of the esophagus. These results strongly suggest that in *B. primigenus* undifferentiated cells but not proliferative cells express *BpMyc*. In *Xenopus*, *c-myc* is induced in forelimb regenerates (Geraudie et al., 1990; Lemaitre et al., 1992). The gene expression is strongest in the deepest (cell proliferation) area of the epidermis and moderate in the blastemal mesenchyme, in which dedifferentiated cells are intermingled (Geraudie et al., 1990). Our results show for the first time that in colonial tunicates *BpMyc* is a new marker of undifferentiated or dedifferentiated cells.

EXPERIMENTAL PROCEDURES

Animals Colonies of *Botryllus primigenus* were collected at Uranouchi Inlet of Kochi Prefecture. They were attached to glass plates and reared in culture boxes settled near the Usa Marine Biological Institute, Kochi University. Before experiments, colonies of approximately 4 cm square were prepared. Each colony contained 40-50 adult zooids.

BrdU solution The stock solution (10 mM) of 5-bromo-2'-deoxyuridine (BrdU) was diluted 100-fold or 500-fold with sterile seawater of cell culture grade (Kawamura et al., 2006). Phenol red was added to the injection solution at the final concentration of 0.005% in order to visually ascertain dye and BrdU in the circulatory blood following microinjection.

Microinjection The BrdU solution was loaded in the glass needle settled in the Micro Capillary System (MN-151, MMO-220A, Narishige, Japan). Total volume (12 µl or 24 µl) was injected into a colony of approximately 4 cm square via a single ampulla or two ampullae distant from each other. Otherwise, one µl solution was injected. The final amount of BrdU administered was 7 ng, 14 ng, or 35 ng per colony. The colony was cultured at 20 °C in the laboratory before fixation.

Fixation and embedding One forth colonial pieces were extirpated from the BrdU-injected parent colony 6 h, 12 h, 18 h, and 24 h or 1, 2, 3, and 4 days after injection. They were allowed to adhere to cover glasses for 15 minutes in a moist chamber. Then, they were immersed and fixed for 1 h on ice in 70% ethanol containing 50 mM glycine buffer (pH

2.0). Specimens were rinsed several times with 70% ethanol and stocked at -20 °C in 70% ethanol.

Specimens were cut into adequate size (approximately 2 x 2 mm square), dehydrated with ethanol, and immersed overnight in plastic resin (Technovit 8100, Heraeus Kulzer Co., Germany). They were then embedded in gelatin capsule and sectioned at 2-3 µm thickness. One or two colonies were sacrificed at each colonial phase (Fig. 1), and finally data from single colonies were adopted in this study.

Immunostaining Sections were pretreated for 10 min with the blocking solution containing 0.5 % skim milk on ice. Mouse anti-BrdU monoclonal antibody (5-bromo- 2'-deoxy-uridine labeling and detection kit II, Boehringer Mannheim, Germany) was diluted 100-fold with the incubation buffer, reacted with sections for 1 h at room temperature, and washed twice for each 10 min with phosphate-buffered saline containing 0.1% tween 20 (PBST). Horse anti-mouse IgG labeled with alkaline phosphatase (AP-2000, Vector, USA) was diluted 200-fold with PBST. Reaction was made for 30 min at room temperature. After thorough washing with PBST, specimens were stained for 15-30 min with color development solution for AP (0.3 mg NBT, 0.15 mg 5-bromo-4-chloro-3-indolyl phosphate, 100 mM NaCl and 50 mM MgCl₂ in 100 mM Tris-HCl, pH 9.5). Ten sections from single colonies were examined to calculate average number of labeled nuclei at definite stages of zooidal development.

Unlabeled sections were used for anti-PCNA immunohistochemistry. The monoclonal antibody was purchased from Novus (NB500-106). Other procedures

were similar to those described above. Horse anti-mouse IgG labeled with horseradish peroxidase (PI-2000, Vector, USA) were used as secondary antibody. Sections were colored by Trueblue (KPL, USA).

Labeling index A few colonies were taken from each colonial phase for nuclear labeling with BrdU. After immunostaining of serial sections, labeled and unlabeled nuclei were counted in 10 sections of respective zooids and buds of the same developmental stage. Average values of labeling index and their standard deviations taken from single colonies were adopted in this study.

Mitotic figures Colonies were treated with 1 mM colchicine (Nakarai Tesque Co., Japan) for 24 h before fixation, as described previously (Kawamura and Nakauchi, 1986b). They were fixed in Zamboni's fixative, dehydrated, and embedded in JB-4 plastic resin (Polyscience Inc. USA), and serially sectioned at 2 µm thickness. Sections were stained with 1 % Toluidine blue in phosphate buffer (pH 7.0).

Isolation of BpMyc cDNA cDNA fragments encoding *Botryllus myc* homologue (*BpMyc*) were amplified by polymerase chain reaction (PCR) from *B. primigenus* cDNA pool, as described previously (Sunanaga et al., 2006). The following degenerate primers (Myc 1 and Myc 2) were designed by Ms. Kyoko Mori for conserved Myc boxes I and II: Myc 1, 5'-GA(AG)GA(TCAG)AT(TCA)TGGA A(AG)AA(AG)TT-3'; Myc 2, 5'-CCACAT(AG)CA(AG)TC(TC)T(TG) (TAG)AT-3'. To isolate longer cDNA fragments, another degenerate primer

(Myc 3) was designed by Mr. Takaomi Isozaki for the basic helix loop helix motif located at the C-terminal region of Myc proteins: Myc 3, 5'-TGIC(TG)(TC)TC(TCAG)A(AG)(TCAG)A(TC)(AG)TT(AG)TG-3'. A specific forward primer, Myc 4 was 5'-GCACCGATGCCACTCTATCT-3'. PCR products were subcloned in a TA cloning vector (pGEM-T, Promega, Madison, WI, USA). The nucleotide sequences of cDNA inserts were determined with ABI PRISM3100-Avant genetic analyzer system (Applied Biosystems, Foster, CA, USA). For cycle sequence reaction, BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems) was used.

Whole mount in situ hybridization
Specimens were fixed in 4% paraformaldehyde in phosphate-buffer-saline (PBS) at 4°C for 10 hours. The fixed specimens were rinsed in PBST (PBS containing 0.1% Tween20), and were dehydrated in graded series of methanol and stored in 75% methanol at -20°C. In situ hybridization was performed according to Sunanaga et al. (2006). In brief, a digoxigenin-labelled antisense RNA probe was used for hybridization. To prevent the nonspecific hybridization, dehydrated specimens were treated with xylene at room temperature for 30 min before the proteinase K treatment. After the hybridization they were treated with the anti-dig antibody labeled with AP (Roche). To avoid the nonspecific staining, they were pretreated with the blocking solution containing 2 % skim milk on ice for 15 to 18 hours before antibody treatment. Specimens stained were embedded in JB-4, as described above.

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REFERENCES

- Abbott DP. 1953. Asexual reproduction in the colonial ascidian *Metandrocarpa taylori* Huntsman. Univ. Calif. Publ. Zool. 61: 1-78.
- Arnold I. Watt FM. 2001. c-Myc activation in transgenic mouse epidermis results in mobilization of stem cells and differentiation of their progeny. Curr. Biol. 11: 558-568.
- Berrill NJ. 1940. The development of a colonial organism: *Symplegma viride*. Biol. Bull. 79: 272-281.
- Berrill NJ. 1941a. The development of the bud in *Botryllus*. Biol. Bull. 80: 169-184.
- Berrill NJ. 1941b. Size and morphogenesis in the bud of *Botryllus*. Biol. Bull. 80: 185-193.
- Berrill N.J. 1950a. The tunicata with an account of the British Species. London, Ray Society, 354 p.
- Berrill NJ. 1950b. Budding in *Pyrosoma*. J. Morphol. 87: 537-552.
- Brien P. 1968. Blastogenesis and morphogenesis. In: Abercrombie M, Brachet J, King TJ, editors. Advances in Morphogenesis. Vol 7. New York: Academic Press. p.151-203.
- Brockes JP. Kumar A. 2005. Appendage regeneration in adult vertebrates and implications for regenerative medicine. Science 310: 1919-1923.
- Burighel P. Lane N.J. Zaniolo G. Manni L. 1998. Neurogenic role of the neural gland in the development of the ascidian, *Botryllus schlosseri* (Tunicata, Urochordata). J. Comp. Neurol. 394: 230-241.
- Casagrande L. Martinucci G.B. Burighel P. 1993. Origin and differentiation of branchial stigmata in the compound ascidian *Botryllus schlosseri* (Tunicata). Anim. Biol. 2, 111-121.
- Ermak TH. 1976. The hematogenic tissue of tunicates. In: Wright RK, Cooper EL, editors. The Phylogeny of Thymus and Bone Marrow-Bursa Cells. Amsterdam: Elsevier. P. 45-56.
- Fujimoto K. Watanabe H. 1976. Studies on the sexual reproduction in the polystyelid ascidian, *Polyzoa vesiculiphora*. J. Morphol. 150: 607-622.
- Geraudie J. Hourdry J. Vríz S. Singer M. Mechali M. 1990. Enhanced c-myc gene expression during forelimb regenerative outgrowth in the young *Xenopus laevis*. Proc. Natl. Acad. Sci. USA 87: 3797-3801.
- Hall PA. Watt FM. 1989. Stem cells: the generation and maintenance of cellular diversity. Development 106: 619-633.
- Ishizuya-Oka A. 2007. Regeneration of the amphibian intestinal epithelium under the control of stem cell niche. Dev. Growth Differ. 49: 99-107.
- Izzard C.S. 1973. Development of polarity and bilateral symmetry in the palleal bud of *Botryllus schlosseri* (Pallas). J. Morphol. 139: 1-26.
- Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE et al. 2002. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418: 41-49.
- Kawamura K. Sugino Y. Sunanaga T. Fujiwara S. 2008. Multipotent epithelial cells in the process of regeneration and asexual reproduction in colonial tunicates. Dev. Growth Differ. 50: 1-10.
- Kawamura K. Fujiwara S. 1994. Transdifferentiation of pigmented multipotent epithelium during morphallactic development of budding tunicates. Int. J. Dev. Biol. 38: 369-377.
- Kawamura K. Nakauchi M. 1986a. Development of spatial organization in palleal buds of the compound ascidian,

- Symplegma reptans*. Biol. Bull. 171: 580-597.
- Kawamura K. Nakauchi M. 1986b. Mitosis and body patterning during morphallactic development of palleal buds in ascidians. Develop. Biol. 116: 39-50.
- Kawamura K. Ohkawa S. Michibata H. Nakauchi M. 1988. Autoradiographic studies on cell population kinetics in epithelial and haemopoietic stem cell lines in the process of palleal budding of ascidians. Mem. Fac. Sci. Kochi Univ. Ser. D 9: 1-12.
- Kawamura K. Sugino YM. 1999. Cell adhesion in the process of asexual reproduction of tunicates. Micro. Res. Tech. 44: 269-278.
- Kawamura K. Takeoka S. Takahashi S. Sunanaga T. 2006. *In vitro* culture of mesenchymal lineage cells established from the colonial tunicate *Botryllus primigenus*. Zoolog Sci 23: 245-254.
- Kawamura K. Watanabe H. 1982. Pattern development in palleal buds of botryllid ascidians: Relation between parental and its buds in their body axes. J. Morphol. 173: 293-304.
- Koyama H. 2002. The dorsal strand of *Polyandrocarpa misakiensis* (Protochordata: Asciacea): a light and electron microscopic study. Acta. Zool. 83: 231-243.
- Laird DJ. Weissman IL. 2004. Telomerase maintained in self-renewing tissues during serial regeneration of the urochordate *Botryllus schlosseri*. Develop. Biol. 273: 185-194.
- Laird DJ. De Tomaso AW. Weissman IL. 2005. Stem cells are units of natural selection in a colonial ascidian. Cell 123: 1351-1360.
- Lauzon RJ. Ishizuka KJ. Weissman IL. 2002. Cyclical generation and degeneration of organs in a colonial urochordate involves crosstalk between old and new: A model for development and regeneration. Develop. Biol. 249: 333-48.
- Lemaitre J-M. Mechali M. Geraudie J. 1992. Nerve-dependent expression of c-myc protein during forelimb regeneration of *Xenopus laevis* froglets. Int. J. Dev. Biol. 36: 483-489.
- Manni L. Zaniolo G. Burighel P. 1994. Ultrastructural study of oogenesis in the compound ascidian *Botryllus schlosseri* (Tunicate). Acta Zool. 75: 101-113.
- Manni L. Zaniolo G. Cima F. Burighel P. Ballarin L. 2007. *Botryllus schlosseri*: a model ascidian for the study of asexual reproduction. Dev. Dyn. 236: 335-352.
- Mashanov V.S. Dolmatov I.Y. Heinzeller T. 2005. Transdifferentiation in holothurian gut regeneration. Biol. Bull. 209: 184-193.
- Mauleon I. Lombard MN. Munoz-Alonso MJ. Canelles M. Leon J. 2004. Kinetics of myc-max-mad gene expression during hepatocyte proliferation in vivo: Differential regulation of mad family and stress-mediated induction of c-myc. Mol. Carcinog. 39: 85-90.
- Mukai H. Watanabe H. 1976. Studies on the formation of germ cells in a compound ascidian *Botryllus primigenus* Oka. J. Morphol. 148: 337-362.
- Nunzi MG. Burighel P. Schiaffino S. 1979. Muscle cell differentiation in the ascidian heart. Develop. Biol. 68: 371-380.
- Ohlstein B. Spradling A. 2007. Multipotent *Drosophila* intestinal stem cells specify daughter cell fates by differential Notch signaling. Science 315: 988-992.
- Okada TS. 1991. Transdifferentiation.

- Flexibility in Cell Differentiation. New York: Oxford University Press, 256 p.
- Paulus T. Muller MC. 2006. Cell proliferation dynamics and morphological differentiation during regeneration in *Dorvillea bermudensis* (Polychaeta, Dorvilleidae). *J. Morphol.* 267: 393-403.
- Potten CS. Loeffler M. 1987. A comprehensive model of the crypts of the small intestine of the mouse provides insight into the mechanisms of cell migration and proliferation hierarchy. *J. Theor. Biol.* 127: 381-391.
- Pulverer B. Sommer A. McArthur GA. Eisenman RN. Luscher B. 2000. Analysis of Myc/Max/Mad network members in adipogenesis: Inhibition of the proliferative burst and differentiation by ectopically expressed Mad1. *J. Cell Physiol.* 183: 399-410.
- Reddien PW. Sanchez Alvarado A. 2004. Fundamentals of planarian regeneration. *Annu. Rev. Cell Dev. Biol.* 20: 725-757.
- Reed JC. Alpers JD. Scherle PA. Hoover RG. Nowell PC. Prystowsky MB. 1987. Proto-oncogene expression in cloned T lymphocytes: migrations and growth factors induce different patterns of expression. *Oncogene* 1: 223-228.
- Sabbadin A. 1955. Osservazioni sulle sviluppo, l'accrescimento e la riproduzione di *Botryllus schlosseri* (Pallas), in condizioni di laboratorio. *Boll. Zool.* 22: 243-263.
- Sanders JA. Gruppuso PA. 2006. Coordinated regulation of c-Myc and Max in rat liver development. *Am. J. Physiol. Gastrointest. Liver Physiol.* 290: 145-155.
- Shinohara Y. Konishi K. 1982. Ultrastructure of the body-wall muscle of the ascidian *Halocynthia roretzi*: Smooth muscle cell with multiple nuclei. *J. Exp. Zool.* 221: 137-142.
- Sugimoto K. Nakauchi M. 1974. Budding, sexual reproduction and degeneration in the colonial ascidian, *Symplegma reptans*. *Biol. Bull.* 147: 213-226.
- Sugino YM, Matsumura M, Kawamura K. 2007. Body muscle cell differentiation from coelomic stem cells in colonial tunicates. *Zoolog. Sci.* 24: 542-546.
- Sunanaga T. Saito Y. Kawamura K. 2006. Postembryonic epigenesis of *Vasa*-positive germ cells from aggregated hemoblasts in the colonial ascidian, *Botryllus primigenus*. *Dev. Growth Differ.* 48: 87-100.
- Sunanaga T. Watanabe A. Kawamura K. 2007. Involvement of *vasa* homolog in germline recruitment from coelomic stem cells in budding tunicates. *Dev. Genes Evol.* 217: 1-11.
- Watanabe H. 1953. Studies on the regulation in fused colonies in *Botryllus primigenus* (Ascidiae Compositae). *Sci Rep Tokyo Bunrika Daigaku sec B* 7: 183-198.
- Watanabe H. Newberry AT. 1976. Budding by oozoids in the polystyelid ascidian *Metandrocarpa taylori* Huntsman. *J. Morphol.* 148: 161-176.
- Yamashita YM. Jones DL. Fuller MT. 2003. Orientation of asymmetric stem cell division by the APC tumor suppressor and centrosome. *Science* 301: 1547-1550.
- Zanet J. Pibre S. Jacquet C. Ramirez A. Moreno de Alboran I. Gandarillas A. 2005. Endogenous Myc controls mammalian epidermal cell size, hyperproliferation, endoreplication and stem cell amplification. *118: 1693-1704.*

FIGURE LEGENDS

Fig. 1 Photographs and Diagrams of buds, zooids, and colonies in *B. primigenus*. (A) Colonial margin where the eldest (1st) and middle (2nd) generations are seen. Bar, 0.5 mm. (B) Zooidal developmental stages and colonial growth phase. According to Manni et al. (2007), developmental stages of buds and zooids were designated as numerals. The concept of ‘unit’ and ‘phase’ came from Watanabe (1953). A unit consists of three successive generations, each developing synchronously. The youngest (3rd) generation in a unit transits through colonial growth phases A, B, C, and D, and then it attains to phase A of the 2nd generation. After the second cycle of phases, developing zooids become the 1st generation simultaneously. (C) A colony injected with trypan blue through a single ampulla (arrow). Bar, 0.5 mm. a, ampulla; e, eldest generation; m, middle generation.

Fig. 2 Nuclei labeled with BrdU during the development of pharynx. (A) Pharyngeal rudiment of 2nd generation (stage 8), 6 h after BrdU injection at phase B. Bar, 40 µm. (B) Bud primordium, stage 1, phase A. Arrowheads show the labeled nuclei. Bar, 20 µm. (C) Developing 3rd generation, stage 6, phase D. Bar, 100 µm. (D) Developing 2nd generation, stage 7, phase A. Bar, 50 µm. (E) Dorsal area of a 2nd generation, stage 7, phase A. Bar, 50 µm. (F) Antero-lateral portion of a 2nd generation, stage 8, phase B. Bar, 25 µm. (G) Anterior portion of the endostyle, 1st generation, stage 9, phase A. Bar, 50 µm. (H) Posterior portion of the endostyle, 1st generation, stage 9, phase A. Labeled nuclei were crowded at the posterior end of the endostyle. Bar, 25 µm. (I) Prepharyngeal epithelium, 1st

generation, stage 9, phase A. Bar, 50 µm. (J) Pharyngeal inner longitudinal vessel, 1st generation, stage 9, phase B. Bar, 50 µm. ae, atrial epithelium; b, bud primordium; bc, branchial chamber; en, endostyle; ep, epidermis; ilv, inner longitudinal vessel; ipe, inner pharyngeal epithelium; m, muscle; ope, outer pharyngeal epithelium; p, pharynx; pc, peribranchial chamber; pe, prepharyngeal epithelium; sti, stigmata.

Fig. 3 Labeled nuclei in the developing digestive tract. (A) Gut rudiment, 3rd generation of stage 6, phase D. Bar, 50 µm. (B) Developing esophagus, 2nd generation of stage 7, phase A. Bar, 25 µm. (C) Posterior half of the 2nd generation, stage 7, phase A. Bar, 50 µm. (D) Higher magnification of the digestive tract, 2nd generation of stage 7, phase A. Bar, 25 µm. (E) Esophagus, 1st generation of stage 9, phase A. Arrowheads show labeled nuclei. Bar, 50 µm. (F) Stomach, 1st generation of stage 9, phase A. Arrowhead shows a single labeled nucleus. Bar, 50 µm. (G) Intestine, 1st generation of stage 9, phase A. Bar, 50 µm. (H) Pyloric caecum, 1st generation of stage 9, phase A. Bar, 50 µm. ae, atrial epithelium; bc, branchial chamber; ep, epidermis; es, esophagus; gr, gut rudiment; in, intestine; pc, peribranchial chamber; pyc, pyloric caecum; sto, stomach.

Fig. 4 Labeled nuclei in the developing neural complex. (A) Longitudinal section of the neural complex, 3rd generation of stage 6, phase D. Phase contrast microscopy. More labeled nuclei were located around the opening of the tube into the branchial chamber (arrowheads). Bar, 25 µm. (B) Transverse section of the neural complex, 3rd generation of stage 6, phase D. Arrows and arrowheads show

the labeled nuclei in the dorsal ganglion and the tube, respectively. Bar, 25 μm . (C) Dorsal area of a 2nd generation, stage 8, phase B. Bar, 50 μm . (D) The dorsal ganglion, 2nd generation of stage 8, phase B. Note that only two nuclei were labeled in the ganglion (arrowhead). Bar, 25 μm . (E) The dorsal ganglion of 1st generation, stage 9, phase B. Bar, 25 μm . bc, branchial chamber; bs, branchial siphon; cg, ciliated groove; dg, dorsal ganglion; ep, epidermis; nc, neural complex; pe, prepharyngeal epithelium.

Fig. 5 Labeled nuclei in coelomic cells. (A) Ventral blood sinus around the endostyle, 1st generation of stage 9, phase B. Bar, 50 μm . (B) Higher magnification of (A). Arrowhead shows a labeled coelomic cell. Bar, 25 μm . (C) Blood sinus in the pharynx, 1st generation of stage 9, phase A. Bar, 20 μm . (D) Blood sinus in the digestive tract, 1st generation of stage 9, phase A. Bar, 100 μm . (E) Blood sinus around the developing branchial siphon, 2nd generation of stage 8, phase B. Arrowheads show labeled coelomic cells associated with the siphon anlagen. Arrow shows a labeled cell at the bottom of the ciliated groove. Bar, 25 μm . (F) Blood cells in the test vessel, phase A. Only four blood cells were labeled (arrowheads). Other labeled cells were the epidermal wall of the test vessel. Bar, 50 μm . bs, branchial siphon; cg, ciliated groove; en, endostyle; in, intestine; pe, prepharyngeal epithelium; pyc, pyloric caecum; sto, stomach.

Fig. 6 Labeled nuclei in the gonad. (A) Loose cell mass, 3rd generation of stage 6, phase D. Arrowheads show the labeled nuclei of aggregated hemoblasts. Bar, 25 μm . (B) Compact cell mass, 2nd generation of stage 7, phase A. Bar, 25 μm . (C) Developing testis, 2nd generation

of stage 8, phase B. Arrowheads show labeled mitosis. Bar, 40 μm . (C') Developing testis, 6 h after BrdU injection. Arrowhead shows labeled mitosis. Bar, 40 μm . (D) Mature testis, 2nd generation of stage 8, phase D. Bar, 100 μm . (E) Paired cells (arrowheads) destined to the oogonium and the primary follicle cells, 2nd generation of stage 7, phase A. Bar, 25 μm . (F) Developing oocytes, 2nd generation of stage 8, phase B. Bar, 25 μm . (G) Testis and ovary, 2nd generation of stage 8, phase B. Bar, 100 μm . ae, atrial epithelium; ccm, compact cell mass; lcm, loose cell mass; oc, oocyte; te, testis.

Fig. 7 Long-term chase after labeling in various tissues. (A,D,F,H) 24 h chase. (B,C,E,G,I) 96 h chase. Arrowheads show labeled nuclei. (A,B,C) Injection was performed at colonial phase B. Bars, 50 μm . (D,E) Esophagus. Injection was performed at colonial phase D. Bar, 100 μm . (F,G) Stomach, injected at colonial phase C. Bars, 100 μm . (H,I) Test vessel injected at colonial phase A. Note that in (H) most of labeled cells are on the wall of vessel, whereas in (I) many blood cells are labeled. Bars, 50 μm . cc, coelomic (blood) cell; en, endostyle; ep, epidermis; es, esophagus; ilv, inner longitudinal vessel; tv, test vessel.

Fig. 8 Mitotic activities in developing tissues treated with 1 mM colchicine for 24 h before fixation. Arrowheads show mitotic figures. (A) The 3rd generation of stage 6, phase D. Bar, 40 μm . (B) Higher magnification of the boxed area in (A). Bar, 10 μm . (C) Dividing cells of the gut rudiment and atrial epithelium, 3rd generation of stage 6, phase D. Bar, 20 μm . (D) The 2nd generation of stage 7, phase A. Bar, 40 μm . (E) Higher magnification of the boxed area in (D).

Bar, 10 μ m. (F)The endostyle of stage 7, phase A. Bar, 10 μ m. (G)Posterior region of the endostyle, 2nd generation of stage 8, phase C. Bar, 40 μ m. (H)Posterior end of the endostyle, 1st generation of stage 9, phase A. Bar, 20 μ m. (I)Inner longitudinal vessel, 1st generation of phase A, stage 9. Bar, 20 μ m. (J)Inner longitudinal vessel stained with anti-PCNA antibody, phase A, stage 9. Bar, 20 μ m. ae, atrial epithelium; bc, branchial chamber; en, endostyle; ep, epidermis; gr gut rudiment; ipe, inner pharyngeal epithelium; oc, oocyte; ope, outer pharyngeal epithelium; pc, peribranchial chamber.

Fig. 9 *BpMyc* gene expression during zooidal development in *B. primigenus*. (A)Bud primordium (3rd generation, stage 1) and the 2nd generation (stage 7) at phase A. Bar, 50 μ m. (B)Bud hemisphere (3rd generation, stage 2) and the 2nd generation (stage 8) at phase B. Bar, 50 μ m. (C)Bud vesicle (3rd generation, stage 3) at phase B. Bar, 50 μ m. (D)Pharynx of the 2nd generation, stage 8, phase B. Outer and inner pharyngeal epithelia and inner longitudinal vessels were stained. Bar, 50 μ m. (E)Negative control using sense probe. Developing zooid (stage 7) and bud primordium (stage 1) at phase A. Bar, 40 μ m. (F)Digestive tract of the 2nd generation, stage 8, phase B. Both developing stomach and intestine were stained faintly. Bar, 50 μ m. (G)Gonad of the 2nd generation, phase B. Loose cell masses were stained heavily. Bar, 25 μ m. (H)Pharyngeal inner longitudinal vessel of the 1st generation, stage 9, phase B. Bar, 50 μ m. (I)Stomach and esophagus of the 1st generation, stage 9, phase B. Bar, 50 μ m. bc, branchial chamber; bh, bud hemisphere; bp, bud primordium; bv, bud vesicle; ccm, compact cell mass; en, endostyle, es, esophagus; ilv, inner

longitudinal vessel; in, intestine; ipe, inner pharyngeal epithelium; lcm, loose cell mass; o, oocyte; ope, outer pharyngeal epithelium; pc, peribranchial chamber; sto, stomach; t, testis.

Fig. 10 Summary of cell proliferation activities in *B. primigenus*. (A)Epithelial multipotent cells. (B)Mesenchymal multipotent cells. Orange-colored cells proliferate slowly at first, and then they multiply rapidly (red). In the eldest generation, cells cease to proliferate (black), but exceptionally in some tissues (green) the proliferation activity is kept, which suggests that these cells may possibly be adult stem cells.

Table 1 Chase of labeled cells in various tissues of the 2nd generation in *B. primigenus*.*

	6 hr	12 hr	18 hr	24 hr	96 hr
Epidermis	12.5±0.8	16.6±0.5	18.4±0.8	17.5±4.2	7.1±3.5
Pharynx	16.5±1.4	18.8±2.1	18.5±2.8	21.2±0.3	14.8±4.2
Endostyle	25.4±2.4	22.5±3.4	32.0±4.1	30.5±4.8	8.1±0.9
Stomach	ND	ND	7.5±5.4	8.7±8.9	13.2±6.4
Developing testis (**)	21.8±2.5 (15.5±1.9)	32.5±3.0 (9.9±2.2)	34.5±3.4 (28.2±3.1)	38.8±4.8 (2.2±0.3)	37.0±14.8 (ND)
Blood cells in TV	3.4±0.2	2.9±0.9	3.2±0.4	3.4±1.4	55.4±8.1

*, Data were obtained from experiments in which 1 µl BrdU solution was injected into a single colony.

**, In parentheses the ratios of labeled mitosis to labeled nuclei are shown.

ND, Not determined.

Table 2 Cell proliferation dynamics of epithelial tissues during bud and zoid development in *B. primigenus*

Generation	3 rd (Buds)				2 nd (Prefunctional zooids)				1 st (Functional zooids)			
Phase	A	B	C	D	A	B	C	D	A	B	C	D
Developmental stages	1	2-3	4-5	6	7	8	8	8	9	9	9	11
Epidermis	0	10.0±0.8	18.4±7.5	9.0±0.6	17.6±5.0	19.3±0.7	7.1±2.5	8.3±2.2	0	0	0	0
Bud primordium	14.4±5.9	15.0±3.8										
Pharyngeal rudiment			29.2±2.1	35.3±4.3								
Pharynx					15.7±0.1	19.1±4.8	11.2±4.3	8.9±0.8	5.0±1.2*	4.3±2.5*	ND	0
L. vessel.					32.1±4.8	30.9±3.3	26.3±10.3	16.6±0.4	16.0±1.1	15.0±0.9	4.2±0.4	0
Endostyle					18.2±5.1	34.3±0.5	10.2±0.9	8.2±0.5	7.5±0.6	7.3±0.8	ND	0
A.E.					14.0±3.9	3.5±3.2	1.1±0.9	0	0	0	0	0
Gut rudiment			18.2±5.3	62.4±10.2								
Esophagus					48.8±5.1	38.7±6.1	18.5±7.7	19.1±1.9	5.8±0.8	4.9±2.4	4.1±4.0	0
Stomach					49.5±6.2	20.4±5.6	8.7±8.9	7.5±7.0	3.3±0.3	0	0	0
Intestine					30.1±2.2	9.9±0.9	7.8±2.3	6.5±5.0	0	0	0	0
Neural Complex			20.0±0.8	30.2±0.8	26.2±1.9	28.6±3.5	10.2±4.2	5.4±5.0	0	0	0	0
Ganglion			10.0±1.7	12.4±0.5	10.5±4.2	2.7±2.5	0	0	0	0	0	0

*, Labeled nuclei were exclusively seen on the prepharyngeal epithelium.
 ND, Not determined.

Table 3 Cell proliferation dynamics of coelomic cells and their derivatives during bud and zooid development in *B. primigenus*.

Generation	3 rd				2 nd				1 st			
Developmental stages	1	2-3	4-5	6	7	8	8	8	9	10	10	11
Blood cells	NC	NC	13.3±2.2	14.8±5.1	19.2±5.8	18.5±4.2	13.1±5.7	12.9±6.2	11.5±1.7	10.8±2.5	8.1±2.4	0
Gonad L.C.M.	NC	NC	19.3±2.5	49.6±10.2	50.0±9.9	51.5±12.3	NC	NC	NC	NC	NC	NC
C.C.M.	NC	NC	NC	38.8±6.0	45.5±5.4	NC	NC	NC	NC	NC	NC	NC
Testis	NC	NC	NC	NC	53.5±7.4	30.2±5.8	15.3±4.6	17.5±7.5	0	0	0	0

NC, No cells exist.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Fig. 8

Fig. 9

Fig. 10