In Vitro Culture of Mesenchymal Lineage Cells Established from the Colonial Tunicate *Botryllus primigenus*

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Body trunks were isolated from juvenile zooids of the Japanese colonial tunicate *Botryllus primigenus* and cultured *in vitro* to establish tissue-specific cell lines. Epidermal cells from some explants spread and formed a flat sheet consisting of vacuolated cells. They then dissociated into single cells, and their growth stopped within two weeks. Continuously proliferating cells were established from four explants. After the 20th implantation, nuclear and mitochondrial DNAs were extracted from these cells. The nucleotide sequences of proliferating cell nuclear antigen (PCNA) and mitochondrial large ribosomal RNA (mtlRNA) completely matched the PCNA and mtlRNA taken from living colonies of *B. primigenus*; this shows that the four independently proliferating cells were indeed of the *Botryllus* origin. One cell line (Bp0306E10) comprised round-shaped cells with a diameter of 8–10 µm. These cells have been cultured *in vitro* with a doubling time of approximately 24 hours since June, 2003. The BrdU labeling index was approximately 2%. Monoclonal antibodies raised against the cultured cells recognized a 28 kDa polypeptide and stained free mesenchymal cells *in vivo*. G418-resistant subclonal cells could be established by introducing a tunicate retrotransposon loaded with the neomycin resistance gene into the cells by electroporation. This study is the first to succeed in producing a sustainable cell culture of *Botryllus*.

Key words: *Botryllus*, cell culture, G418, mesenchyme, PCNA, tunicate

INTRODUCTION

Botryllus and *Botrylloides* are well known budding tunicates that form flat colonies with many zooids scattered on a mesh-like common vascular system (Fig. 1A). Their specialized mode of life has attracted the attention of many scientists, for several reasons. A colony always contains three different generations of zooids, and each generation develops in a completely synchronized manner (Cima *et al.*, 2003; Lauzon *et al.*, 1992, 2002; Rinkevich *et al.*, 1992). Furthermore, the lifespan of zooids is very short, *i.e.*, a maximum of two weeks. Adult zooids undergo abrupt degeneration simultaneously accompanied by apoptotic cell death (Lauzon *et al.*, 2000). They are phagocytized and absorbed by the colony. Finally, auto- and allorecognition occurs between neighboring colonies. When two colonies come in contact at their growing edges, the two independent vascular networks either fuse with, or reject, one another, a process similar to the graft-versus-host reaction in vertebrates (Oka and Watanabe, 1957a; Sabbadin, 1962; Mukai and Watanabe, 1974; Scofield *et al.*, 1982; Scofield and Nagashima, 1983; Saito *et al.*, 1994). This phenomenon is referred to as colony specificity (Oka and Watanabe, 1957a) and is interesting from the viewpoint of comparative immunology.

Botryllus primigenus is a Japanese endemic species. It

is commonly found on rocky seashores along the southern coast of the Honshu and Shikoku Islands. This species is well known owing to the discovery of the genetic basis for its colony specificity. Oka and Watanabe (1957a) were the first to suggest that multiple alleles at a single locus govern auto- and allorecognition between different colonies in *B. primigenus*. Further, this species is well known because of presence of stem cells that play a significant role in both sexual and asexual reproductions. Oka and Watanabe (1957b) discovered a new type of budding called vascular budding in *B. primigenus*, wherein hemoblasts (a type of blood cell) aggregate to form new individuals (Fig. 1B). This indicates that hemoblasts act as multipotent somatic cells in the blood (Oka and Watanabe, 1957b, 1959). Germline cells are also derived from blood cells that are very similar to somatic hemoblasts (Mukai and Watanabe, 1976; Sunanaga *et al.*, submitted). Until maturation, they pass through blastogenic generations. Similar observations were made in other species of *Botryllus* (Sabbadin and Zaniolo, 1979; Stoner and Weissman, 1996).

Botryllus primigenus has thus been a pivotal species in fields such as comparative immunology and stem cell biology. However, recent studies on this species have not necessarily utilized the technologies of cell and molecular biology in an efficient manner. This and forthcoming papers are aimed to renew *B. primigenus* as one of the model animals for modern molecular and cellular biology. In this paper, we tested *B. primigenus* cell culture and succeeded in establishing long-term proliferative cell lines. This is the first success in establishing a *Botryllus* cell culture and the second

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Fig. 1. A colony and stages of zooid development of *Botryllus primigenus*. **(A)** A colony, low magnification. Zooids and buds are surrounded by the vascular ampullae (a). e, endostyle; i, intestine; s, stomach. Bar, 2 mm. **(B)** A colony, higher magnification. It contains three asexual generations, each of which is developmentally synchronized. The oldest (o) and middle (m) generations are visible. Vascular buds are located at the colonial periphery (arrowheads). Bar, 1 mm. **(C–E)** The youngest generation. **(C)** A bud of stage 3. Arrowheads show mitotic figures. Bar, 25 μ m. **(D)** A bud of stage 5. Pharyngeal folds (p) are developing. Bar, 50 μ m. **(E)** A bud of stage 6. It forms the neural complex (n) on the dorsal side and the gut rudiment (g) and the heart (h) at the posterior end. Bar, 50 μ m.

one in tunicates, following *Polyandrocarpa misakiensis* (Kawamura and Fujiwara, 1995). We consider the availability of *Botryllus* culture cells from viewpoints of cell biology, biochemistry, and molecular biology.

MATERIALS AND METHODS

Animals

Colonies of *Botryllus primigenus* were collected at Nabeta Bay, Izu Peninsula, Shizuoka Prefecture and at Uranouchi Inlet, Kochi Prefecture. They were attached to glass plates and reared in culture boxes settled near the Usa Marine Biological Institute, Kochi University.

Tissue harvest

Colonies on glass plates were rinsed briefly with 99% alcohol and heated for a few seconds for sterilization (Kawamura and Fujiwara, 1995). They were then incubated in millipore-filtered seawater (MS). Zooids were cut carefully with razor blades, and tissue fragments were picked up with forceps and transferred to cell culture medium in 96-well multiplates. The multiplates were incubated in a CO₂ incubator in 4% CO₂ at 25°C.

Cell culture medium

Modified MS (MMS) consisted of 90 ml MS and 30 ml distilled water (cell culture grade) containing 10 mM HEPES (N-(2-hydroxy-ethyl) piperazine-N'-2-ethanesulfonic acid). After autoclaving, antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin) were added to the MMS. The growth medium for *Botryllus* contained five volumes of MMS, one volume of RPMI 1640 (Invitrogen), and 3% fetal bovine serum (FBS, Wheaton). Medium was prepared immediately before use and was added to each well (dish) every three days.

MTT assay

After harvesting from the subconfluent culture, cells were resuspended in culture medium at a density of 5×10^5 cells/ml. The

cell suspension (0.5 ml each) was dispensed in 24-well multiplates. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay for cell-number quantification was modified as described elsewhere (Kawamura *et al.*, 1999). In brief, MTT (5 mg/ml) was dissolved in PBS. One tenth volume of MTT was added to the cell suspension and incubated for four hours at 25°C. The reaction product (formazan) was dissolved overnight with the same volume of 10% SDS in 0.01N HCl. It was monitored for absorbance at 600 nm and the reference wavelength of 690 nm.

BrdU incorporation

Cells were plated on a chamber slide and cultured in growth medium for 24 hours. They were then incubated with 10 μ M 5-bromo-2'-deoxyuridine (BrdU) for 15 minutes. The medium was replaced with new medium, and 30 minutes later cells were fixed

Fig. 2. Mitotic indices of differentiating tissues and organs in developing zooids of *B. primigenus*. For developmental stages of the oldest, middle and youngest (1st, 2nd and 3rd) generations, see Kawamura and Sugino (1999).

Fig. 3. *In vitro* proliferating cells in *B. primigenus* (1). (**A–C**) Epithelial sheets of vacuolated cells, perhaps epidermal in origin. (**A**) One week after tissue culture. Bar, 50 μ m. (**B**) 1.5 weeks later. Bar, 50 μ m. (**C**) Two weeks later. Bar, 50 μ m. (**D–F**) Cell growth and morphology of cell line Bp0407B1. (**D**) Cells spreading from the explant (e), two days after tissue culture. Bar, 100 μ m. (**E**) Proliferating cells and aggregates after five platings. Bar, 100 μ m. (**F**) Higher magnification of (**E**). Note that the aggregates contain larger cells. Bar, 50 μ m. (**G–I**) Cell growth and morphology of another cell line, Bp0310A6 after more than 10 platings. (**G, H**) Cell aggregates radiating fibrous matrices. Bars, 100 μ m and 50 μ m, respectively. (**I**) Small cell aggregates interconnected by fibrous matrices. Bar, 25 μ m.

Fig. 4. *In vitro* proliferating cells in *B. primigenus* (2). Cell line Bp0306E10 and its subclones are shown. (**A–C**) Primary cell culture. (**A**) Cells spreading from the explant (e), two days after tissue culture. Bar, 100 μ m. (**B, C**) Proliferating cells 10 and 15 days after implantation, respectively. Bars, 50 μ m. (**D–F**) Subcultures of proliferating cells. (**D**) Adherent cells, round or somewhat amorphous in shape. Bar, 25 μ m. (**E**) Cell aggregates detached from the substratum. Bar, 50 μ m. (**F**) Heterogeneous cell population appearing after long-term culture without plating. Bar, 50 μ m. (**G**) A large cell with several vacuoles (arrowheads) in the cytoplasm after long-term culture. Bar, 20 μ m. (**H**) Cell morphology of subclone Bp0306E10D1. Bar, 25 μ m. (**I**) BrdU-incorporating cells in culture (arrowheads). Bar, 50 μ m.

with 70% ethanol in 50 mM glycine buffer (pH 2.0). Labeled cells were stained with anti-BrdU antibody followed by anti-mouse IgG-AP, using a BrdU detection kit (Boehringer).

Genomic DNA

Cultured cells ($\sim 10^8$) were harvested in a conical tube and washed twice with cold PBS. They were solubilized with 0.5% SDS and 0.01% proteinase K in 10 mM Tris/HCl (pH 8.0) and 100 mM EDTA for 20 hours at 50°C. Colonies of *B. primigenus* were frozen and crushed in liquid N₂. The resultant powder was solubilized in the same manner as cultured cells. The extracts were further denatured in 80% formamide. Genomic DNA was stored in the refrigerator until use. For the construction of a genomic library, DNA was cut by the restriction enzyme *Sau*3A. Both ends of DNA fragments were filled with A and G. These partial fill-in products were ligated to λ Fix II vector (Stratagene), then packaged using a λ Packaging Kit (Amersham Pharmacia Biotech.).

PCR amplification and DNA sequencing

The polymerase chain reaction (PCR, Saiki *et al.*, 1988) was performed with genomic DNA as the template and primers we designed from cDNA nucleotide sequences: BpPCNA1 (5'-GGATATGCTAGAACTTCAGTTTGA), BpPCNA2 (5'-GTTAATATGCGCTGTCATTATGTG), BpYC1 (5'-TGAGTGATACCTATAGTTGGTG), and BpYC2 (5'-ATAAGTCGACATCGAGGTCGTAAA). Ex Taq DNA polymerase (Takara Bio Inc.) was used under the following reaction conditions: 94°C for 0.5 min (one cycle); 94°C for 0.5 min, 53°C for 1 min, 72°C for 1.5 min (30 cycles); 72°C for 4 min (one cycle).

For cycle sequencing, a BigDye Terminator (v3.1) Cycle Sequencing Kit (Applied Biosystems) was used. Reaction products were analyzed with an ABI Prism 3100 DNA sequencer (Applied Biosystems).

Preparation of monoclonal antibody

Confluent cells of *Botryllus* cell line Bp0306E10A6 were harvested from two 24-well multiplates, washed once with PBS, resuspended in 1 ml PBS, and frozen until use. BALB/c 3T3 mice were immunized five times with 0.2 ml of cell suspension conjugated with the same volume of Freund's adjuvant. Spleen cells were fused with NS1/AG4 myeloma cells and screened by HAT medium. Positive hybridomas were cloned, and the culture media were stored in a refrigerator in the presence of 0.05% NaN₃.

SDS-PAGE and western blotting

Colonies of *B. primigenus* (1.5 g, wet weight) were powdered in liquid N₂. The powder was mixed with 2 ml of 10 mM TrisCl (pH 8.0) and homogenized in a glass homogenizer on ice. After centrifugation at 15K rpm for 5 minutes, the supernatant was separated from the pellet, and aliquots were mixed with an equal volume of SDS sample buffer. The pellet was dissolved directly in SDS sample buffer. Electrophoresis was done on a 15% polyacrylamide gel containing 0.1% SDS in 0.375 M Tris-HCl (pH 8.8) (Laemmli, 1970). Polypeptide bands were electrically blotted onto nitrocellulose (NC) membrane (Biorad) at 300 mA for 1.5 hours in Tris-glycine buffer (25 mM Tris, 195 mM glycine, 20% methanol). The NC membrane was blocked with 2% skim milk and stained with the primary antibody for 30 min at room temperature. It was washed twice with PBST (PBS+0.1% Tween 20), stained with the anti-mouse secondary antibody labeled with HRP (Vector), and colored by diaminobenzidine.

Immunohistochemistry

The cell suspension was left for 30 minutes on cover slips coated with poly-L-lysine. Cells were fixed in 2% paraformaldehyde at 4°C for 30 minutes and washed with PBS. Subsequent procedures were the same as those for the NC membrane. Colonies of

B. primigenus were fixed in 2% paraformaldehyde at 4°C for 60 minutes. After washing with PBS, they were dehydrated with ethanol, embedded in Technovit 8100 (Kulzer, Wehrheim), serially sectioned, and mounted on cover slips. The subsequent procedure of immunostaining was the same as that for the NC membrane, except that the secondary antibody was labeled with Texas Red (Vector) instead of HRP.

Electroporation

Cells (about 5×10^6) mixed with 20 μ g of plasmid were preincubated for 10 minutes in 400 μ l saline G, then transferred to a sterilized cuvette with a 0.4 cm gap. Electroporation was carried out with a Gene Pulser Xcell (Bio-Rad) at 200 V and 100 μ F.

RESULTS

Mitotic activity of cells and tissues

Similarly to other botryllid tunicates, a colony of *B. primigenus* consists of three generations of asexual individuals (Fig. 1B; Kawamura and Sugino, 1999). The youngest generation begins with a disc-like bud primordium, forms a bud vesicle (stage 3), and commences organogenesis (stage 5; Fig. 1C, D, E). The middle generation undergoes morphogenesis most actively, and the oldest generation attains functional maturity. Each generation lasts for 4–5 days; hence, the maximum lifespan of a single zooid is two weeks. Figure 2 shows the mitotic indices (MI) of developing zooids. A double-walled bud vesicle (stage 3) showed an MI of 1.5–2.5 in the inner multipotent epithelium (arrowheads, Fig. 1C). At stage 5, the MI reached a maximum (approximately 5.5) in the gut rudiment and neural complex (precursor of the neural gland and ganglion; Fig. 1D, E). Thereafter, the MI of the ganglion cells decreased rapidly to nearly zero; however, the MI of the neural gland, pharynx, and gut remained stable until the mid-phase of the middle generation. Finally, the MI of all tissues and organs reached zero by the end of this generation. Based on these observations, we chose the middle generation for the *Botryllus* cell culture.

Establishment of cell lines

Body trunks were isolated from the middle generation of colonies and consecutively implanted in each well of a 96-

Fig. 5. Cell growth curve of subclonal cell line Bp0306E10D1. The cell number was estimated by MTT assay. Means $\pm$ SD.

single cells and finally disappeared (Fig. 3B, C). In several wells, continuously proliferating cells of four types appeared. One type (Bp0407B1) spread away from the explant at first (Fig. 3D) and later grew as loose aggregates in which larger

cells appeared (Fig. 3E, F). The second and third types (Bp0310A6 and Bp0310C10, respectively) formed aggregates of various cell sizes. The aggregates extended matrix substances in a radial manner when they were distant from each other (Fig. 3G, H). If small aggregates were located near one another, they were interconnected (Fig. 3I). The fourth type of cell (Bp0306E10) appeared as cell aggregates (Fig. 4A, B, C). These cells were round or somewhat amor-

phous in shape, with a diameter of 8–10 μm , and had no apparent vacuoles in their cytoplasm (Fig. 4D). They adhered firmly to the substratum, and when the cells became subconfluent, they formed large cell masses that were often detached from the substratum (Fig. 4E). Cumulative cultures gave rise to a smaller and a larger type of cell (5–6 μm and 12–15 μm in diameter, respectively; Fig. 4F). Unlike the smaller cells, the larger cells contained several

Fig. 8. Immunohistochemistry using anti-P28 monoclonal antibody. (A, C, E, G, I) Bright field microscopy. Bar, 50 μm . (B, D, F, H, J) Fluorescence microscopy. Bar, 50 μm . (A, B) Coelomic cells in the vascular ampullae. (C, D) Body trunk. e, endostyle; pe, peribranchial epithelium. (E, F) Pharynx (p). (G, H) Oesophagus (o). (I, J) A developing zooid with gonads (g).

vacuoles in the cytoplasm (Fig. 4G). After cloning, medium-sized cells were obtained without any apparent vacuoles in the cytoplasm (Fig. 4H).

Clonal cells (Bp0306E10D1) proliferated with a doubling time of approximately 24 hours (Fig. 5). Immediately after plating, the cells grew slowly. However, after 2 days of culturing, the growth curve became steep; this suggested that the conditioned culture medium might facilitate cell growth. DNA synthesis was assessed by immunocytochemistry of BrdU-incorporating cells. Positive cells had a variable size ranging from 5 to 10 μm in diameter (Fig. 4I). The labeling index was $2.1\% \pm 0.8\%$, which suggested that the S phase had lasted for approximately 30 min.

Comparison of genomic sequences

Genomic DNA was extracted from colonies of *B. primigenus*, and the gene for proliferating cell nuclear antigen (BpPCNA) was cloned and sequenced (Accession Number, AB239703). This sequence was 75% identical with homologous gene sequences from *Styela clava* and *P. misakiensis* (Accession number, AB239704). Four types of cell lines (Bp0407B1, Bp0310A6, Bp0310C10, and Bp0306E10) and a subclone of Bp0306E10 (Bp0306E10D1) were harvested at the 20th subculture (6 months later). Nuclear DNA was extracted from these cells, and the gene for PCNA was cloned and sequenced. As the sequences were identical with one another, only that of Bp0306E10D1 was used for alignment (Fig. 6). The result showed that the sequence from cultured cells was completely consistent with that from living colonies. An identical sequence was obtained from the cells of the 50th subcultures (one year later). A gene encoding the mitochondrial large ribosomal RNA was cloned from all four cell lines and compared with that from colonies of *B. primigenus*. Except for base substitutions at several sites, the sequences were identical with one another (data not shown).

Preparation of monoclonal antibody recognizing *Botryllus* cell lines

A monoclonal antibody (3E9A5) was raised against the cell line Bp0306E10. The antibody stained dissociated cells in the culture (Fig. 7A), but not the cytoplasm of aggregated cells, except at the periphery of the aggregate (Fig. 7B). On performing SDS-PAGE and western blotting of the *Botryllus* homogenates, we found that the antibody recognized a single band of 28 kDa. This band appeared in the water-insoluble fraction of the homogenate (Fig. 7C).

In the colonies, the anti-P28 antibody heavily stained mesenchymal cells in the vascular ampullae (Fig. 8A, B). It weakly stained the endostyle (Fig. 8C, D), but did not recognize the epidermis, peribranchial epithelium, pharynx (Fig. 8E, F), oesophagus (Fig. 8G, H), stomach, intestines, and gonads (Fig. 8I, J).

Establishment of neomycin-resistant subclonal cell lines

A *Polyandrocarpa* retrotransposon loaded with the neomycin resistance gene was prepared (Kawamura *et al.*, unpublished data). The construct was introduced into clonal Bp0306E10D1 cells by electroporation. Such cells not only survived but also proliferated in the presence of 0.2 mg/mL G418 for at least 10 months (Fig. 9B, C). In the negative

Fig. 9. Cell growth of a G418-resistant cell line. Retrotransposon loaded with the neomycin resistance gene was introduced into Bp0306E10D1, after which the cells (Bp0306E10D1neo) were treated with 200 $\mu\text{g}/\text{mL}$ G418. (A) A control without electroporation, one week later. All cells were dead. Bar, 25 μm . (B) Bp0306E10D1neo, one week later. Bar, 25 μm . (C) Bp0306E10D1neo, three weeks later. Bar, 25 μm .

control without electroporation, cells died within about a week in the presence of G418 (Fig. 9A). These results indicated that neomycin-resistant subclonal cell lines had been established successfully.

DISCUSSION

Differences in cell culture method from previous studies

In tunicates, blood cells were used for early attempts at cell culture because they can be collected and handled with ease. Blood cells from some adult tunicates continue to exhibit proliferative potential *in vivo* and *in vitro*. In *S. clava*, for example, the hematopoietic nodule incorporates [³H] thymidine *in vivo* (Ermak, 1977), and allogeneic stimuli and

cytokines induce blood cell proliferation *in vivo* and *in vitro* (Raftos and Cooper, 1991; Raftos *et al.*, 1991). In *Botrylloides diegensis*, a large number of free blood cells are labeled with [³H] thymidine (Ermak, 1981). In *Ciona intestinalis*, lymphocyte-like cells show cytotoxic activity (Peddie and Smith, 1993) and are capable of mitogen-induced cell proliferation *in vitro* (Peddie *et al.*, 1995). Despite these findings, attempts to culture blood cells *in vitro* ended in failure for a long time.

Budding tunicates are known to have proliferative epithelial cells other than blood cells. The peribranchial (atrial) epithelium is a multipotent tissue in *Symplesma reptans* (Kawamura and Nakauchi, 1986a), *B. schlosseri* (Berrill, 1941; Izzard, 1973), *B. primigenus* (this study), and *P. misakiensis* (Kawamura and Nakauchi, 1986b). In fact, proliferative cell lines were established successfully for the first time from the peribranchial epithelium of *P. misakiensis* (Kawamura and Fujiwara, 1995). In the present study, we did not try to isolate the peribranchial epithelium from *B. primigenus* because it was too small to manipulate. Instead, to establish *Botryllus* cell lines, we used body trunks of the middle generation that contained a developing pharynx, gut, and neural complex.

In previous studies, the cell culture media contained seawater or a salt solution of the same osmolarity as seawater (Warr *et al.*, 1977; Rinkevich and Rabinowitz, 1993). They were mixed with nutrients such as DMEM (Rinkevich and Rabinowitz, 1993) and RPMI 1640 (Raftos *et al.*, 1990; Peddie *et al.*, 1995). The pioneering success obtained with the *Polyandrocarpa* cell culture was primarily due to the low osmotic pressure (830 mOsm) and low pH (6.8) of the culture medium (Kawamura and Fujiwara, 1995). Although this medium could also be used for the *Botryllus* cell culture, many cells underwent morphological changes and cell growth retardation in a long-term culture (data not shown). In the present study, the osmotic pressure of the basal medium was further lowered to approximately 600 mOsm. Instead of DMEM, RPMI 1640 was used as the nutrient source. This new culture medium required 4% CO₂ as a supplement. These revised methods effectively supported the morphology and proliferative activity of *Botryllus* cells for more than two years. This medium was also effective in the cell culture of *C. intestinalis*; however, it was not very effective in culturing *Polyandrocarpa* cell lines (data not shown).

Proliferative cells in the body trunk of *B. primigenus*

In this study, we chose the middle generation as the source of tissue explants, because in *B. primigenus* the neural complex, pharynx, and gut maintained high mitotic activities (the MI was approximately 2.5) until the mid-stage of the middle generation. In *B. schlosseri*, the cells of the neural complex divide and differentiate into nerve cells in the middle generation (Burighel *et al.*, 1998). Similarly, in *P. misakiensis*, the dorsal strand (posterior extension of the neural complex) consists of undifferentiated cuboidal cells that differentiate into nerve cells (Koyama, 2002). In *B. diegensis*, cell proliferation in the gut occurs only during bud development (Ermak, 1981). In *B. primigenus*, the gut rudiment showed the highest mitotic activity (MI=5.5) at stage 5 of the youngest generation. Thereafter, it maintained a moderate mitotic activity during the middle generation, branching

into three lobes that differentiate into the stomach, pyloric caecum, and intestine (*cf.* Berrill, 1941; Burighel, 1970; Izzard, 1973; Burighel *et al.*, 1977, 1984, 1998; Casagrande *et al.*, 1993; for review see Kawamura and Sugino, 1999). In the pharynx consisting of outer and inner branchial epithelia (*cf.* Casagrande *et al.*, 1993), mitotic figures were observable until the stigmata were perforated.

Developmental synchrony is one of the most fascinating features of *Botryllus* and *Botrylloides* (Cima *et al.*, 2003; Lauzon *et al.*, 1992, 2002; Rinkevich *et al.*, 1992). At the end of the life span of two weeks, adult zooids undergo degeneration accompanied by apoptotic cell death (Lauzon *et al.*, 2000). As the explants were chosen from the middle generation in this study, the expected remaining life span of explants was a maximum of 4–7 days. In fact, some explants dissociated into single cells in a few days; however, others remained intact for approximately a month. These observations suggest that programmed cell death (Lauzon *et al.*, 1992, 2000) may not occur *in vitro*.

Characterization of *in vitro* proliferative cells

In this study, we established four proliferative cell lines from *Botryllus* explants. Partial nucleotide sequences for PCNA and mtDNA obtained from cultured cells were consistent with those obtained from colonies of *B. primigenus*. For reference, we compared *Botryllus* PCNA with that of related species of *Polyandrocarpa* and *Styela*, and found 75% similarity among them. These results indicate that all four cell lines are of *Botryllus* origin. The cell line Bp0306E10D1 comprised adherent cells that could form aggregates of various sizes. Initially, these were believed to be epithelial cells, as we had previously established a similar type of epithelial cell line from *P. misakiensis* (Kawamura and Fujiwara, 1995). However, the monoclonal antibody raised against the cultured cells did not recognize any epithelial tissues, but did stain free mesenchymal cells in the hemocoel *in vivo*. These mesenchymal cells, especially abundant in the vascular ampullae, were 8–10 µm in diameter and had many granules in the cytoplasm, features that are similar to those of granulocytes (Hirose *et al.*, 2003).

Unlike the larger cells in the culture, the aggregated cells did not stain with the anti-28kDa antibody. This may indicate that the aggregated cells are indeed epithelial cells differentiating *in vitro* from round and somewhat amorphous cells. In budding tunicates, undifferentiated blood cells, *i.e.*, hemoblasts, are known to adhere to form the epithelial tissues (Kawamura *et al.*, 1991; Kawamura and Sugino, 1999). Hemoblasts that aggregate under the vascular epidermis form vascular buds (Oka and Watanabe, 1957b, 1959; Sabbadin *et al.*, 1975), while those that aggregate in the gonadal space form a loose cell mass (*i.e.*, germline precursor cells; Mukai and Watanabe, 1976; Sunanaga *et al.*, unpublished data). If we can prepare antibodies that recognize the aggregating cells, we can further our understanding of hemoblast adhesion.

Establishment of G418-resistant subclonal cells

In this study, the cultured cells of *Botryllus* could be rescued from the lethal effect of the antibiotic G418 when a retrotransposon loaded with the neomycin resistance gene was introduced into them by means of electroporation. The

retrotransposon was approximately 6.5 kb long. It has been isolated from the colonies of *P. misakiensis* (Kawamura *et al.*, unpublished data), indicating that it works effectively across species. This is not unexpected, because recently it was discovered that the *Drosophila* transposable element Minos could be integrated efficiently into the genome of germ cells in *C. intestinalis* (Sasakura *et al.*, 2003). Minos is transmitted stably to subsequent generations (Matsuoka *et al.*, 2004), thereby enabling insertional mutagenesis (Awazu *et al.*, 2004). Although we do not have any evidence for the integration of the retrotransposon into the genome of *Botryllus* culture cells, the transgenic cells continued to divide for at least 10 months. In the natural host *P. misakiensis*, the retrotransposon is expressed during both sexual and asexual reproduction (Kawamura *et al.*, unpublished data). Therefore, *Botryllus* cell lines and retrotransposons may be useful as new tools for investigating gene function.

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