Establishment of Cell Lines from Multipotent Epithelial Sheet in the Budding Tunicate, Polyandrocarpa misakiensis

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ABSTRACT. We report here the in vitro culture of the atrial epithelium, which is the major formative tissue of the budding tunicate, Polyandrocarpa misakiensis. Preliminary studies suggested that both pH and osmotic pressure of the basic seawater medium should be lowered significantly (pH 6.8, 800–830 mOsm). In the growth medium consisting of modified millipore-filtered seawater, Dulbecco’s modified Eagle medium and 3% fetal bovine serum, cells spread out from the epithelial explant and proliferated with a doubling time of about 13 hours. They could be cloned and cultured successively. They contained the Polyandrocarpa lectin gene, showing that they were indeed of tunicate origin. At a low cell density (< 3 x 10^2 cells/mm²), clonal cells took a spherical form and contained several granules in the cytoplasm. At a high cell density (>3 x 10^4 cells/mm²), on the other hand, they gave rise to smaller cells without any specialized features and, finally, to dark flattened cells. Consistent with this observation, confluent cells lost the atrial epithelium-specific antigen, which reappeared on the cell surface when they were re-plated at a low density. In conclusion, we have established for the first time tunicate cell lines. They appeared to differentiate and dedifferentiate repeatedly in our culture system.

In general, cell and tissue homeostasis governs the post-embryonic life of every multicellular organism, in which cell renewal is moderately balanced with cell loss (11, 29). But, we may find an exceptional case of the homeostasis in asexual reproduction (budding) of marine and freshwater invertebrates. Budding takes place successively throughout their life span (for tunicates, 26, 36). It involves remodeling of the whole body from a piece of individuals (19, 20, 21), referred to as morphalaxis (5, 25). In budding animals, therefore, continuous cell growth that accompanies cell differentiation should be out of harmony with cell decay.

Polyandrocarpa misakiensis is a budding tunicate. The oldest asexual strain that we have was collected in 1970 (40). Many buds arise as the outpocketing of the parental mantle wall that consists of the epidermis, atrial epithelium and mesenchymal cells intervening between them (18, 19, 21). The atrial epithelium is the major formative tissue giving rise to the pharynx, digestive tract, brain and endostyle of a new asexual individual (21). It is interesting to ask what is the motive force of Polyandrocarpa budding that accompanies continuous cell growth and differentiation for more than two decades. Another interesting question is how somatic cells such as the atrial epithelium keep a high degree of developmental plasticity equivalent to embryonic cells. Cell culture may be helpful to answer these questions.

Invertebrate cell cultures are still limited exclusively to molluscs (7, 12, 13). Among several attempts to culture tunicate cells in vitro (32, 33), a striking finding is that interleukin 1-like molecule has been isolated from the solitary tunicate, Styela clava (31). It could stimulate DNA synthesis of tunicate cells in vitro, but cell lines have not yet been established from this animal.

We have also tried to culture Polyandrocarpa cells in vitro for these seven years. A breakthrough was brought about by the discovery of a galactose-binding lectin from P. misakiensis (38). It was a component of extracellular matrix that played a role in bud morphogenesis (16). Interestingly, factors isolated by lectin affinity chromatography showed a conspicuous activity to maintain Polyandrocarpa mesenchymal cells in vitro (the present study). Encouraged by this finding, we improved cell culture conditions by modifying physicochemical properties of the culture medium, and finally we have succeeded in clonal cell cultures from Polyandrocarpa multipotent epithelium.

In this paper, we describe first a preliminary work to determine cell culture conditions, using the lectin-bind-
ing substances. Secondly, we describe a generalized method to culture tunicate cells in the absence of the intrinsic substances. Thirdly, we show several cellular and molecular natures of *Polyandrocarpa* clonal cell lines. The present work is compared with previous cell culture studies and is discussed in the context of cellular flexibility of differentiation that would enable budding of *P. misakiensis*.

**MATERIALS AND METHODS**

**Strains.** Four asexual strains of *Polyandrocarpa misakien- sis* were used in this study. The oldest one was collected in Sagami Bay in 1970 (40). The second one was collected at Izu, near the Shimoda Marine Research Center, the University of Tsukuba. They were named, respectively, spotless and white spot after phenotypes expressed on the dorsal surface of adult animals (14). The third one named *kagoshima* was collected in Kinko Bay, Kagoshima Prefecture, in 1985. The last one, *ushi- mado*, was collected near the Ushimado Marine Laboratory, Okayama University, in 1987. They were attached to glass slides and have been reared in Uranouchi Inlet near the Usa Marine Biological Institute, Kochi University, as described previously (17).

**Preparation of lectin affinity column.** *Polyandrocarpa* colonies (about 100 g) were homogenized. After gel filtration chromatography (Ultrigel AcA 44, IBF, France), the lectin (TC-14) was purified, as described previously (16, 38). Other fractions were stored at −25°C before affinity chromatography. The amount of protein was determined by the method of Lowry et al. (24). TC-14 (1.8 mg) was equilibrated against the coupling buffer (0.1 M NaHCO₃ containing 0.5 M NaCl, pH 8.0). One gram of activated CH Sepharose 4B (Pharmacia, Sweden) was suspended in 1 mM HCl. It was packed in the column (16 × 40 mm) and washed with 200 ml of 1 mM HCl. TC-14 was passed slowly through the column for 2 hours at room temperature. The eluate was collected and unbound protein was estimated at 78.4 µg (about 4.4% of the original amount), showing that more than 90% of TC-14 had been coupled with the column. The column was washed thoroughly with 0.1 M Tris-HCl (pH 8.0) for 1 hour in order to block remaining active groups. It was further washed with three cycles of altering pH (0.1 M acetate buffer (pH 4.0) containing 0.5 M NaCl and 0.1 M Tris-HCl (pH 8.0) containing 0.5 M NaCl).

**Affinity chromatography.** Fractions after gel filtration were thawed and dialyzed against 20 mM Tris-HCl (pH 8.0) containing 10 mM CaCl₂. The sample was applied to the TC-14 affinity column equilibrated with the same buffer. The column was eluted with the same buffer for 2 hours at a flow rate of 0.5 ml/min. It was then washed in a stepwise manner with the buffer containing 10 mM EDTA instead of CaCl₂ for 2 hours (fraction 1) and eluted finally with 10 mM EDTA + 0.5 M NaCl (fraction 2). The eluate was monitored for absorbance at 280 nm. Total amounts of proteins in fraction 1 and fraction 2 were estimated as 900 µg and 180 µg, respectively.

**SDS-PAGE.** SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 12% acrylamide gel containing 0.1% SDS in 0.375 M Tris-HCl (pH 8.8) (23).

**Culture media.** In preliminary works, millipore-filtered seawater (MS) was used as a basic medium for cell culture in the presence or absence of antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). Lectin-binding fractions after affinity chromatography were concentrated by lyophilization and dialyzed against ion-exchanged water (cell culture grade). Aliquots of them were added to MS. Modified millipore-filtered seawater (MMS, pH 6.8, 800–830 mOsm) consisted of MS (100 ml), H₂O (20 ml), and 10 mM HEPES (N-(2-hydroxyethyl) piperazine-N’-2-ethanesulfonic acid). Antibiotics were added to MMS, unless denoted elsewhere. The growth medium contained five volumes of MMS, one volume of Dulbecco’s modified Eagle medium (DMEM, Nissui, Japan) and 3% fetal bovine serum (FBS, Gibco, USA). It was prepared immediately before use.

**Preparation of mesenchymal cells.** Animals were rinsed with 99% alcohol for 5–10 seconds, as described previously (10). They were burned for a few seconds and then incubated in MS. They were cut carefully with a razor blade and squeezed with a forceps to collect free mesenchymal cells.

Mesenchymal cell suspension was first centrifuged manually in order to remove the debris. Then, the cells were pelleted twice by the centrifugation at 450 × g for five minutes. They were resuspended in MS at the concentration of 3 × 10⁶ cells/ml. The cell suspension, 100 µl each, was plated in a single well (about 1 × 10⁴ cells/mm²) of a 96-well plate (Corning 25860, USA). Culture plates were incubated in a moist chamber at 20°C. Cell number was counted every day with the aid of ocular quadrate micrometer (0.06 mm²).

**Preparation of epithelial explants.** *Polyandrocarpa* colonies attached to a glass plate were sterilized, as described above. Only growing buds were used as the source of explants. They were cut with a razor blade into squares of less than 1 mm² in order to remove the dorsal tunic and epidermis. The atrial epithelium appeared on the attached surface. It was isolated carefully from the ventral epidermis, using forceps. Single pieces of the epithelial sheet, although a small number of mesenchymal cells were attached to them, were transferred to each well of a 96-well plate that contained 100 µl of MMS.

From the fourth day of the primary culture onward, the growth medium (20 µl) was added to the explant every two days for a week. Thereafter, 50 µl of the growth medium was exchanged every week. If cells began to proliferate, the medium was added every two days.

**PCR and DNA blot hybridization.** Primer-N [5’-AT-GGA(TC)TA(TC)GA(AG)AT(TC)(TC)T(TCAG)TT-3’] and primer-C [5’-(AG)TC(A)TC(TCAG)A(AG)(TC)(TC)TT (TC)T-3’] were synthesized, the sequences of which correspond to the amino- and carboxy-terminal regions of TC-14 protein, respectively (38). Using these primers, the entire translated region of TC-14 gene(s) was amplified by the polymerase chain reaction (PCR) (35). Genomic DNA samples purified
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from respective culture cells and adult animals were used as
templates for the PCR. Gene amplification was performed in
50 μl of 50 mM KCl, 2 mM MgCl2, 10 mM Tris-HCl (pH 9.0)
and 0.1% Triton X-100, with 0.2 mM each dNTP, 1 μM each
primer, 250 ng of template DNA and 0.02 units/μl Taq DNA
polymerase (Promega, USA). The temperature regimen for 30
cycles was 1 min at 94°C, 2 min at 45°C, and 3 min at 72°C.
Products of the PCR was separated on an agarose gel and
transferred to Hybond-N+ membrane (Amersham, UK),
using 0.4 N NaOH as the transfer medium. Antisense RNA
probe was synthesized, using a cloned fragment of TC-14
cDNA as a template (Shimada et al., submitted). Labeling of
the probe with digoxigenin (DIG), hybridization of the DNA
blot with the DIG-labeled probe, and immunological detec-
tion of hybrids were performed in accordance with the proto-
col supplied by Boehringer Mannheim. Anti-DIG Fab frag-
ment conjugated with alkaline phosphatase and chemilumines-
cent substrate Lumigen PPD (Boehringer Mannheim, FRG)
were used for detection.

Determinaiton of doubling time. Sub-confluent cultures
were treated with 2 mM thymidine for 24 hours. The cells
were then washed thoroughly with the growth medium. They
were harvested every two hours and attached to coverslips
coated with 0.05% poly-D-lysine. The specimens were fixed in
alcohol/acetic acid (3/1) for 10 minutes and then stained with
1% orcein in 45% acetic acid for 15 minutes. They were
washed briefly with 45% acetic acid and mounted with glycer-
ol. Mitotic index was counted in order to determine the doubl-
ing time of synchronized cells.

Immunohistochemistry. Culture cells were mounted on
cover slips and fixed in alcohol/acetic acid, as described
above. Blocking was carried out in 2% dry milk in phosphate-
buffered saline (PBS, pH 7.4) for 30 minutes. AP-E2 antibody
recognizes alkaline phosphatase expressed on the atrial epithe-
lium (15). The specimens were stained with a primary anti-
body for 30 minutes, followed by the wash with 0.1% Tween
20 in PBS. Goat anti-mouse secondary antibody labeled with
gold particles and silver enhancing kit (Biocell, USA) were
used, as described previously (15).

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Fig. 1. Gel filtration and lectin affinity chromatographies of Polyandrocampa extracts. (A) Elution profile of AcA 44 gel filtration column eluted
with 0.1 M ammonium acetate containing 0.5 mM EDTA. The second peak (arrowhead) was used for further chromatography. (B) Elution pro-
file of lectin-bound CH Sepharose 4B column. The column was eluted with 10 mM EDTA and later with 10 mM EDTA + 0.5 M NaCl, as indi-
cated by arrows. For details, see the text. (C) SDS-PAGE of lectin-binding fractions. Lane 1, fraction 1 eluted by EDTA. Lane 2, fraction 2
eluted by EDTA + NaCl.
RESULTS

Isolation of Polyandrocarpa lectin-binding substances. Figure 1A shows a gel filtration profile of Polyandrocarpa crude extracts. Only the second peak was dealt with in this study. It was applied to the affinity column of Polyandrocarpa lectin (TC-14) in the presence of CaCl₂, as TC-14 is a calcium-dependent, galactose-binding lectin (38). After thorough washing, the column was eluted by EDTA (Fig. 1B). The eluate (fraction 1) gave a band of 20 kDa and an upper broad band of about 24 kDa under a reduced condition (Fig. 1C lane 1). Cross-linking experiments showed that both 20 k and 24 k are subunits of a glycoprotein of about 100 kDa (GP-100) (not shown).

The lectin affinity column was further eluted by EDTA and NaCl (Fig. 1B). The eluate (fraction 2) gave a weak, but evident band of 44 kDa (P-44) and other minor bands (Fig. 1C lane 2). These proteins might bind to TC-14 via a bond other than a calcium-dependent lectin-carbohydrate interaction, as it was hardly eluted by EDTA.

Preliminary mesenchymal cell culture using lectin-binding fractions. Polyandrocarpa mesenchymal cells were used to improve cell culture media. From one gram of adult animals 1–3 × 10⁷ cells were usually collected. They were plated at the density of 3 × 10⁵ cells per well (96-well plate) (Fig. 2A). If the culture medium, millipore-filtered seawater (MS), was devoid of either antibiotics or Polyandrocarpa fractions, every well was filled with microbial organisms within two days of culture, and mesenchymal cells decreased conspicuously in number (Fig. 3). When penicillin (100 U/ml) and streptomycin (100 μg/ml) were added to the culture, the decrease in cell number was retarded to some extent (Figs. 2D, 3).

Each fraction after affinity chromatography was applied to the culture instead of the antibiotics. It showed a remarkable anti-bacterial activity at the concentration of 0.2–0.4 μg proteins/100 μl MS/well (Fig. 2B, E). The fraction 2 appeared more effective than either the fraction 1 or antibiotics for the viability of tunicate cells (Fig. 3), although the difference was not statistically significant. It blocked even the growth of protozoa, which could never be achieved by antibiotics. More interestingly, it showed cell growth activity in the modified millipore-filtered seawater (MMS) that contained 10 mM HEPES, pH 6.8 and one fifth volume of ion-exchanged H₂O (Figs. 2C, 3). The cells approximately duplicated within 2 days and kept a high density for more than a month (Figs. 2F, 3). They became small in size and were attached firmly to the substratum.

The result mentioned above suggested that both pH and osmotic pressure of the basic medium should be

Fig. 2. Effects of lectin-binding fractions and culture media on mesenchymal cell survival. Cells were plated in a 96-well plate at the density of 3 × 10⁵ cells per well. Bar, 50 μm. (A, D) Cells were cultured in millipore-filtered seawater (MS) containing antibiotics. (B, E) Cells were cultured in MS containing fraction 2 after lectin affinity chromatography. (C, F) Cells were cultured in modified millipore-filtered seawater (MMS, pH 6.8, 830 mOsm) containing fraction 2. (A) Immediately after plating. (B, C) 3 days after plating. (D, E, F) 14 days after plating. Fraction 2 showed the anti-microbial activity, like antibiotics. Note that in MMS + fraction 2 cells become smaller, increase in number and survive for a long time.
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lowered to keep Polyandrocarpa cells in vitro. On the basis of these preliminary findings, we have undertaken the main subject to culture multipotent epithelial sheets, using defined media.

Cell growth in the primary culture of epithelial sheets. Figure 4A shows an explant of epithelial sheet immediately after plating. A small number of mesenchymal cells attached to the explant were shed off on the bottom of the culture plate, but they did not increase in number, as far as we observed. In one or two months, cells began to spread from 8–9% of the explants in all four spotless, white spot, kagoshima and ushimado strains (Fig. 4B, Table I). They were accompanied with fibrous extracellular matrix at their margin (Fig. 4B arrowhead).

Proliferating cells were harvested and re-plated. They scattered soon on the bottom of a 96-well plate (Fig. 4C). They became confluent within a week (Fig. 4D). The cell was about 5 μm in diameter and contained several granules in the cytoplasm, when cell density was low (<3 x 10^2 cells/mm^2) (Fig. 4E). At a high cell density (>3 x 10^4 cells/mm^2) they gathered to form spherical aggregates of variable size (Fig. 4F). When the aggregate was re-plated, it became loose and smaller cells, about 3 μm in diameter, crept out from it (Fig. 4G). They looked blue in color and appeared undifferentiated (Fig. 4H).

Re-examination of cell culture condition. MMS in the growth medium was replaced by MS. Cells, although low in density, aggregated precociously and they did not increase in number (Fig. 4I). Low pH as such did not improve the results, while cell growth was restored partially by lowering the osmotic pressure of seawater to about 750 mOsm (Fig. 4J). A desirable culture condition was obtained again by lowering both osmotic pressure (720–830 mOsm) and pH (6.0–7.0) (Fig. 4K).

Cell growth was retarded significantly in the absence of FBS. It was also blocked by the excess DMEM of more than one-third volume of MMS (not shown).

Establishment of Polyandrocarpa cell lines. A single cell was isolated and cultured clonally. At first, every clonal cell from four respective strains took a yellowish, large spherical form with several granules, like the primary culture (Fig. 4L, see also Fig. 4E). Successive cultures gave rise to smaller pale cells and a few dark cells (cf., Fig. 4H arrowhead). In a confluent culture, the dark flattened cells increased in number. Unless re-plating, they occupied exclusively the culture dish in about a month (Fig. 4M).

The cells from four strains showed somewhat different behaviors with one another. The derivatives of spotless and kagoshima aggregated easily (cf., Fig. 4F), while those of white spot and ushimado were apt to keep a monolayer even in a confluent culture.

Genomic DNA was extracted from in vitro clonal cells and in vivo tissues, both of which were of white spot strain. Using these DNA samples as templates, the entire translated region of the gene(s) for tunicate lectin TC-14 was amplified by the PCR. The PCR products were separated on an agarose gel, blotted on a nylon membrane, and hybridized with a probe prepared from a cloned fragment of TC-14 cDNA. They had two hybrid signals of about 0.4 kb and 0.6 kb, which were quite similar in the pattern of mobility between the in
Cell proliferation from explants of the multipotent atrial epithelium. (A) Explant of the atrial epithelium. Bar, 100 μm. (B) Spreading cells around the explant (e). Arrow shows fibrous matrix at the periphery. Bar, 100 μm. (C, D) Secondary culture of spreading cells. One day (C) and four days (D) after inoculation. Asterisks show the same reference points. Bars, 100 μm. (E) Cells proliferating at low density. Note that every cell contains yellowish granules in the cytoplasm. Bar, 25 μm. (F) Cell aggregate at high cell density. Arrow shows fibrous matrix arranged radially. Bar, 100 μm. (G) Cells re-spreading from an aggregate. Bar, 100 μm. (H) Smaller cells in successive culture. They looked undifferentiated. Arrows show dividing cells. Arrowhead shows a dark cell. Bar, 25 μm. (I, J, K) Effect of osmotic pressure and pH of the growth medium on cell growth, 7 days after plating. Bar, 100 μm. (I) MMS was replaced by MS (about 1,100 mOsm, pH 8.2). Note that cells do not proliferate and that they aggregate precociously. (J) Only osmotic pressure was lowered (750 mOsm, pH 8.2). Cell growth was restored partially. (K) Both osmotic pressure and pH were lowered (830 mOsm, pH 6.8). Cell growth was restored completely. (L) Cells after cloning. Bar, 25 μm. (M) Long-term culture of clonal cells. Note that the well is occupied exclusively by dark flattened cells. Bar, 25 μm.

vitro cell line and in vivo tissues (Fig. 5).

Growth and differentiation of clonal cells. Smaller pale cells in a sub-confluent culture divided actively (cf., Fig. 4H arrows). Mitotic index was 8.5% in a certain spotless cell line. Excess thymidine (2 mM) prevented the cells from entering cell division cycle (see 0 hr of
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Fig. 5. Genomic Southern hybridization of the TC-14 lectin gene. DNA was extracted from in vitro clonal cells and adult animals of white spot strain, respectively. The lectin gene was amplified by the PCR and hybridized with a probe prepared from TC-14 cDNA. Lane 1, adult animals. Lane 2, a clonal cell line. Note that both lanes contain two quite similar PCR products of about 0.4 kb and 0.6 kb.

Fig. 6). Six hours after the release from the thymidine treatment, the mitotic index attained the first peak. There was about 13 hours between the first and second peaks (Fig. 6), showing roughly the cell doubling time.

The monoclonal antibody, AP-E2 recognizes specifically alkaline phosphatase expressed on the atrial epithelium (15). This antibody did not react with the confluent culture that was occupied mainly by smaller pale cells (Fig. 7A). On the other hand, it reacted with a lot of cells re-plated at a low density from the confluent culture (Fig. 7B). They were stained heavily, weakly or partially to various extent (Fig. 7C).

DISCUSSION

Culture condition of tunicate cells. In several attempts to keep tunicate cells in vitro, artificial seawater or tunicate physiological saline has been used as a basic medium (31, 32, 33, 39). From the data of ionic concentration of the blood, the osmotic pressure was adjusted to 1,100–1,200 mOsm (33, 39), roughly isotonic to that of seawater. The pH was adjusted to 7.3 (30, 33, 39). In our early studies of Polyandrocarpa cell culture starting from 1988, the basic medium with similar osmolarity and pH has been used. Cell viability was variable, ranging from a few days to a few weeks (the present study and unpublished data).

Preliminary studies in this paper suggested that both the osmotic pressure and pH of the basic medium should be lowered significantly, inconsistent with the consensus of the above mentioned previous works. It should be noted that even the proliferating Polyandrocarpa cells have ceased to grow under the high osmotic pressure (about 1,000 mOsm). At present, we can not give a satisfactory explanation as to why cell growth is enhanced by lowering the osmolarity and pH of the culture medium.

The lectin-binding fractions isolated by affinity chro-
matography showed both anti-microbial activity and colony maintenance activity when applied to mesenchymal cells in vitro. Recently, interleukin 1 (IL-1)-like cytokine has been isolated from the blood plasma of the solitary tunicate, Styela clava (31). It stimulated the uptake of \(^{3}H\)-thymidine into pharyngeal explants, although continuous cell growth has not been achieved. Anti-microbial tetrapeptides (halocymesin) have been isolated from the blood cells of the solitary tunicate, Halocynthia roretzi (1, 2). In P. misakiensis, the lectin-binding fractions showed both cell growth activity and anti-microbial activity. They contained GP-100, P-44 and other minor components. Physiological activities of each component will be published elsewhere.

Fortunately, we could prepare a satisfactory cell growth medium without lectin-binding fractions (5 volumes of MMS + 1 volume of DMEM + 3% FBS). We found that, if DMEM was added excessively (more than one third of MMS), the cell never proliferated in vitro (the present study and our unpublished data). In previous works, the culture media for tunicate cells contained a high concentration of DMEM (33, 39) or RPMI 1640 (32), equivalent to that used in mammalian cell cultures. In such a medium, blood cells of Pyura stolonifera remained viable for at least 9 days (39). In Botryllus schlosseri, blood cells could be cultured up to several months in the presence of 5% FBS (33). In S. clava, pharyngeal explants remained viable in vitro for up to 72 days in the presence of tunicate plasma (30). In contrast, the oldest Polyandrocarpa cell lines that we established from epithelial sheets have continued to proliferate from June, 1993, the longest record for tunicate cell culture.

**Cellular and molecular characterization of Polyandrocarpa cell lines.** A calcium-dependent (C-type), galactose-binding lectin (TC-14) has been isolated from P. misakiensis (38). It is composed of 125 amino acid residues. It forms the extracellular matrix and plays a role in morphogenesis of proliferating stem cells during Polyandrocarpa budding (16). A recent study has shown that P. misakiensis has two closely related lectin genes (Shimada et al., submitted). PCR products of genomic DNA from an in vitro cell line had two DNA fragments that could hybridize with the TC-14 probe, like those from Polyandrocarpa adult animals. The size of the smaller band (0.4 kb) was consistent with the deduced length of the TC-14 translated region without long introns. It is unlikely that the probe has cross-hybridized with genes of other contaminating organisms, as the similarity of the amino acid sequences between TC-14 and any known member of C-type lectin family does not exceed 30% (38). It is, therefore, concluded that in vitro proliferating cells have the TC-14 lectin gene of P. misakiensis.

The atrial epithelium is composed of granular cells that express Pae 1 antigen (9) and alkaline phosphatase (AP-E antigen) (15). Not a few culture cells expressed the AP-E antigen when the cell density was low. This has afforded another line of evidence that the in vitro cell is of Polyandrocarpa origin.

During the budding of P. misakiensis, a definite number of the epithelial cells enter rapid division cycle to establish organ primordia (19, 21). Autoradiographic studies showed that the cell cycle time was 12.5 hours long (\(T_{G1}=2.3\) h, \(T_S=5.0\) h, \(T_{G2}=4.9\) h, \(T_M=0.3\) h) at the morphogenetic area (21, 22). In the present study, the doubling time of a spotless cell population was estimated as about 13 hours, consistent with the cell cycle time determined by autoradiography.

At least three types of cell were discernible light-microscopically from one another in our culture system. One was the granular cell that expressed the AP-E antigen to variable extent. It always appeared after cell clon- ing or plating at a low density. The smaller pale cell was the second type of cell, which appeared after cell aggregation in a confluent culture. They did not contain any apparent granules in the cytoplasm (the present study and unpublished EM data). They looked hemoblasts that have been considered as undifferentiated cells in the blood (16, 37; as for the nomenclature, 41). These first and second types of cell should be interchangeable with each other, depending on cell density. The dark flattened cell was the third type of cell. It seemed not to proliferate any more. There is a good possibility that they might be derived from the smaller pale cells (cf., Fig. 4H).

**Homeostatic cell growth and differentiation in budding tunicates: perspective from cell culture.** In the present study, 8-9% of epithelial explants began to proliferate when they were dissociated into single cells. In living animals, the mitotic index of the atrial epithelium was unexpectedly low (0.15%, see ref. 21), the cell cycle time being 170-200 hours long (22). As already mentioned, a definite number of the epithelial cells is released from this homeostatic cell growth and committed to primordial organs. The rapid cell cycling is accompanied by the transient disappearance of the basal lamina that underlies the atrial epithelium (15). Interestingly, the cell in culture was blocked to proliferate on the collagen-coated dish (unpublished data). These results suggest that in P. misakiensis the basal lamina regulates the homeostatic growth of both in vivo and in vitro multipotent cells.

In P. misakiensis, many buds arise in sequence from the parental body wall (14, 26). How does this occur in spite of homeostatic cell growth? A possible resolution is that there might exist progenitor cells of the atrial epithelium in the mesenchymal pool. This is undoubtedly the case in vascular budding of Botryllus and Botryloides (27, 28), in which an aggregate of free mesen-
chymal cells gives rise to the atrial epithelium of a bud (see Figs. 4E and 4F of ref. 21). As already mentioned, in P. misakiensis the in vitro smaller pale cell resembles the hemoblast in living animals. It is changeable into the granular cell that expresses the AP-E antigen. These results may afford, for the first time, in vitro substantial evidence for the progenitor cell of the atrial epithelium in P. misakiensis. This notion is consistent with our recent observation that in P. misakiensis mesenchymal hemoblasts undergo epithelial transformation during bud development (16).

It appears that both budding and regeneration of aquatic animals depend on undifferentiated stem cells such as interstitial cell of hydra (4, 6) and neoblast of planarian (3). In tunicates, hemoblast is assigned to such a stem cell (41). The stem cell is characterized by a large nuclear-cytoplasmic ratio, a prominent nucleolus, and a large number of free ribosomes (34). Ermak (8) found a nest of stem cells in various parts of body in S. clava and Ciona intestinalis. The nest is called a hematopoietic nodule, in which the stem cells are surrounded by differentiating blood cells. In the present study, hemoblast-like smaller cells appeared in vitro after the aggregation of granular cells, suggesting strongly that dedifferentiation takes place. As already mentioned, cell density may regulate in vitro differentiation and dedifferentiation of Polyandrocarpa cells. If this is also the case in living animals, it is possible to assume that the hematopoietic nodule (8), if any in P. misakiensis, may not be the nest of stem cells but the aggregate of dedifferentiating cells. In any case, our result has raised an important question of whether multipotent stem cells are indeed reserved throughout the post-embryonic life of budding tunicates.

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