Transdifferentiation of pigmented multipotent epithelium during morphallactic development of budding tunicates

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ABSTRACT In the budding tunicate, *Polyandrocarpa misakiensis*, the atrial epithelium is the major formative tissue giving rise to the pharynx, digestive tract, brain and endostyle of a bud. We show here that this multipotent epithelium carries several differentiation markers that are lost in the process of bud development. In both adult animals and growing buds, the atrial epithelium contained orange-pigmented granules in the cytoplasm. In developing buds, on the other hand, the cells committed to organ primordia have lost the granules, taken a cuboidal shape and have a large nucleus with a prominent nucleolus, like undifferentiated cells. The atrial epithelium was also characterized by ALP expressed on the apical surface of the cell. During budding the enzyme activity disappeared from the atrial epithelium and reappeared in the primordial digestive tract. Immunohistochemical studies suggested strongly that during gut formation, ALP antigens had been switched from the epithelial isoform to the intestinal isoform. These results have shown that in *P. misakiensis* budding involves transdifferentiation of multipotent, but differentiated epithelium, confirming our previous results (Fujiwara and Kawamura, *Dev. Growth Differ. 34:* 463-472, 1992).

KEY WORDS: *alkaline phosphatase, budding tunicate, multipotency, transdifferentiation, ultrastructure*

Introduction

In multicellular organisms, the developmental potential of somatic cells is generally restricted to narrow differentiation pathways. Even in epimorphic regenerative fields, the repertoire of each blastema cell is rather limited (Okada, 1991). Not a few marine and freshwater invertebrates take another mode of regeneration, called morphallaxis. Morphallaxis defines a process in which structures to be regenerated arise as the remodeling of remaining tissues over a wide area (Morgan, 1901; Cooke, 1984), which teaches us that some sorts of somatic cells may still possess a wide spectrum of flexibility equivalent to embryonic cells. It provides us with many excellent subjects for studying how and to what extent their flexibility is regulated.

As predicted by the source-sink model of positional information theory (Wolpert, 1969), each cell in the morphallactic field abolishes the previous fate and takes a new fate appropriate to the position in the field. This conversion of cell fate should be equivalent to transdifferentiation, which has been defined clearly in cell culture systems (Okada, 1976; Eguchi, 1979). Alternatively, morphallaxis may involve position-dependent differentiation of pluripotent "reserve cell" such as interstitial cell in hydra (David and Gierer, 1974; Bode *et al.*, 1976) and neoblast in planarian (Baguñà *et al.*, 1989). Thus, in order to understand cellular and molecular natures of morphallaxis, we need to describe accurately the differentiation state of the formative tissues.

Polyandrocarpa misakiensis is a budding tunicate. A bud arises as the outpocketing of the parental mantle wall that consists of the epidermis and atrial epithelium (Kawamura and Watanabe, 1982). It enters the developmental phase after it is isolated from the parent (Kawamura and Nakauchi, 1984). The atrial epithelium is the major formative tissue that gives rise to the pharynx, digestive tract, brain and endostyle of a new asexual individual (Kawamura and Nakauchi, 1986, 1991). It has long been considered as undifferentiated, but we have suggested recently that this epithelium is, although multipotent, not undifferentiated and that the remodeling of the tissue involves a transdifferentiation-like process (Fujiwara and Kawamura, 1992). Unfortunately, we could not identify the molecular nature of the antigen expressed on the atrial epithelium, making our result inconclusive.

The present study was designed to characterize further this multipotent epithelium of *P. misakiensis*. Our strategy was to identify reliable molecular markers of the epithelium and to pursue them in the process of bud development. We show here that the atrial epithelium consists of specialized pigment cells. Special

Abbreviations used in this paper: ALP, alkaline phosphatase; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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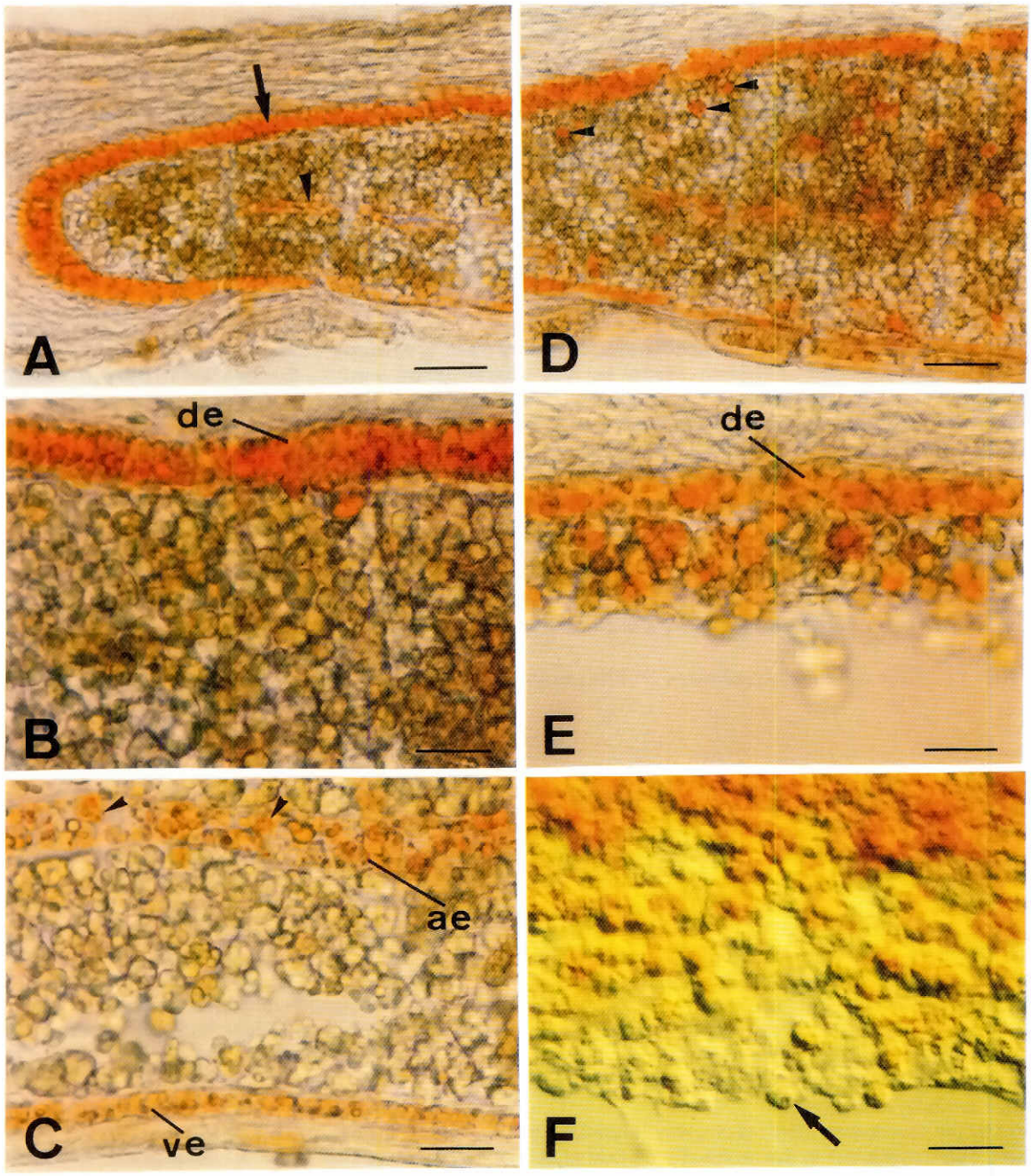

Fig. 1. Cryosections of buds in *P. misakiensis*, cut longitudinally. (A-C) *Buds just after being isolated from the parent.* **(D-F)** *Buds, 2 days after the isolation.* **(A)** *Distal area. Arrow and arrowhead show, respectively, the epidermis and atrial epithelium that are two epithelial components of the bud.* **(B)** *Dorsal area.* **(C)** *Ventral area. Arrowheads show pigment granules.* **(D)** *Middle area. Arrowheads show pigmented bodies in the mesenchymal space.* **(E)** *Proximal area. Note that pigmented bodies are abundant.* **(F)** *Nomarski's optics, the proximal area. The atrial epithelium (arrow) does not contain pigment granules. ae, atrial epithelium; de, dorsal epidermis; ve, ventral epidermis. Bars, A,D, 100 μm; B,C,E, 50 μm; F, 40 μm.*

attention has been paid to ALP, which is shared with many differentiated cells and tissues. We would like to stress that *Polyandrocarpa* budding offers an invaluable developmental system in which somatic cells other than reserve cells still carry pluripotency.

Results

The multipotent epithelium is granulated

A growing bud consists of a double-walled vesicle elongated proximo-distally and collapsed dorso-ventrally (Fig. 1A). The outer

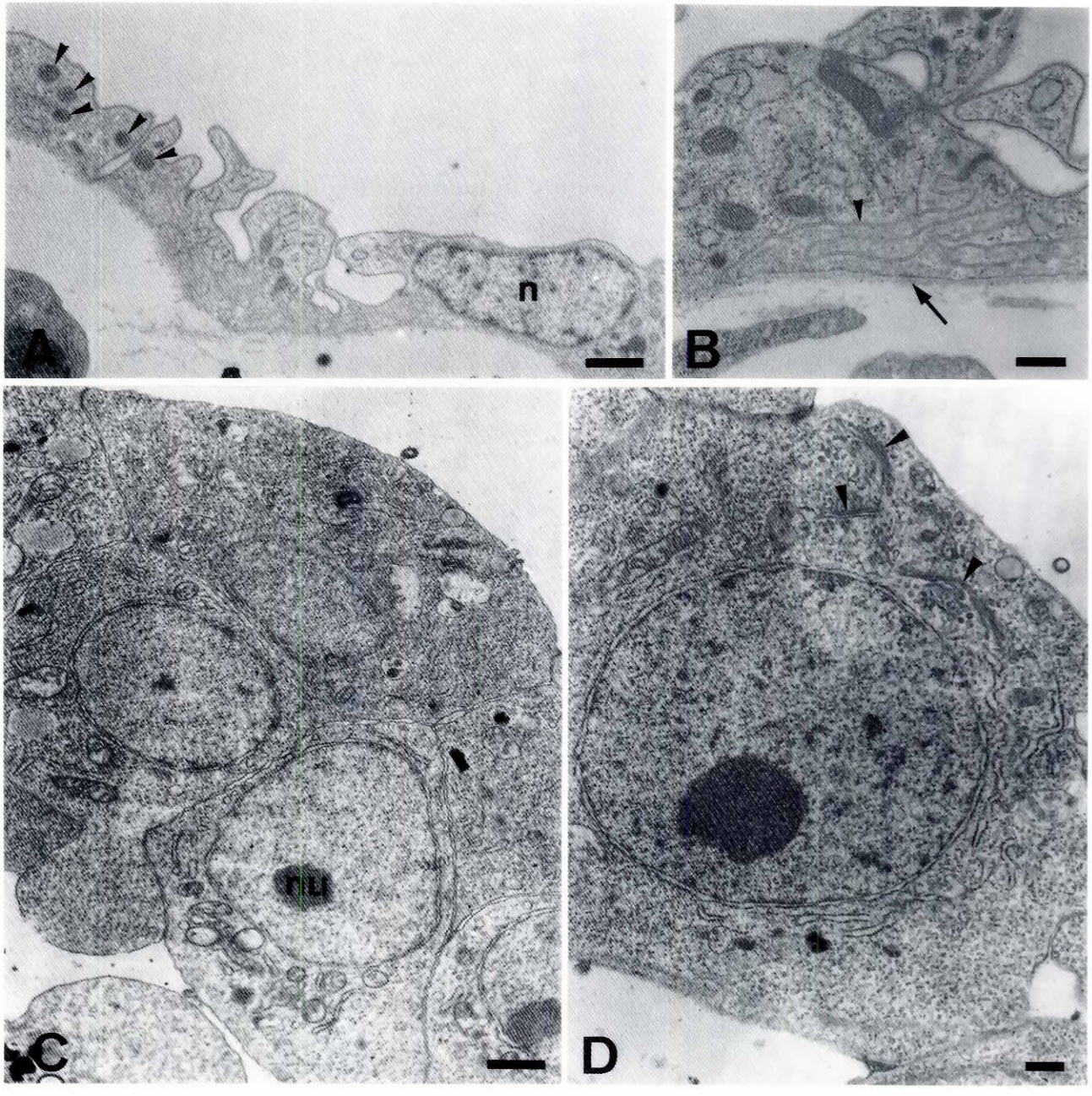

Fig. 2. Ultrastructure of the atrial epithelium in *P. misakiensis*. **(A)** *Growing bud. Squamous epithelial cell contains moderately electron-dense granules (arrowheads).* **(B)** *Cell boundary, growing bud. Plasma membrane is highly indented (arrowhead). Arrow shows the basal lamina underlying the atrial epithelium.* **(C)** *Multi-layered epithelium, the proximal area of a 1.5-day-old bud. Note that the basal lamina is not observable (bottom).* **(D)** *Single-layered epithelium, the proximal area of a 2-day-old bud. Golgi complexes (arrowheads) are well-developed. n, nucleus; nu, nucleolus. Bars, A,C, 1 μm; B,D, 0.5 μm.*

vesicle, epidermis, was colored orange (Fig. 1 arrow), as described previously (Kawamura and Watanabe, 1982). The dorsal epidermis was pigmented more strongly than the ventral epidermis (Fig. 1B,C). This was also the case in adult animals (not shown). The atrial epithelium underlying the epidermis was pigmented faintly (Fig. 1A arrowhead, 1C). It carried minute orange granules in the cytoplasm anywhere along the proximal-distal and dorso-ventral bud axes (Fig. 1C arrowheads).

The growing bud enters the developmental phase by extirpating it from the parent. Large pigmented bodies appeared in the mesenchymal space of the bud (Fig. 1D arrowheads). They were more abundant in the proximal area than in the distal area (Fig. 1E).

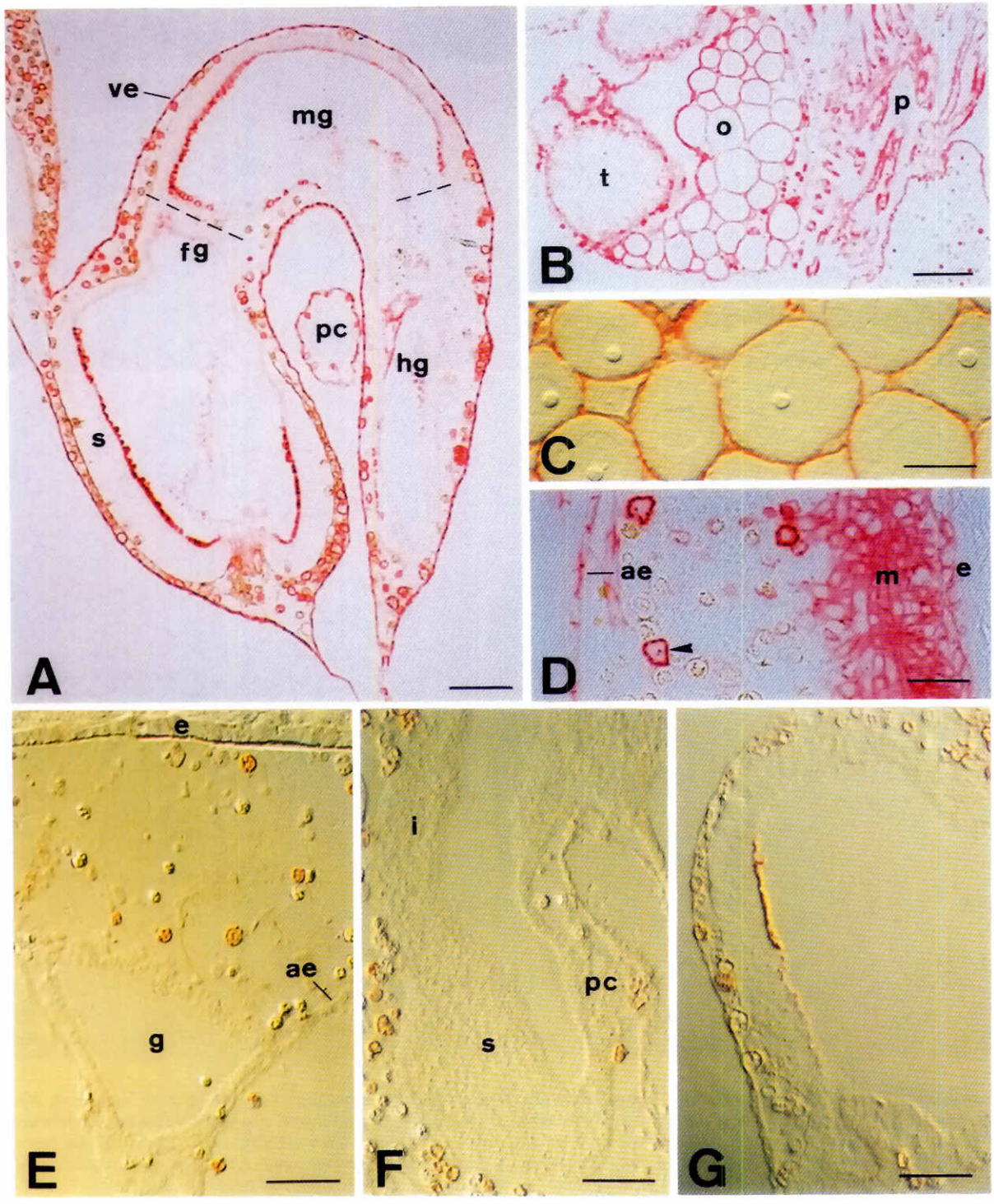

Fig. 3. Histochemistry of ALP in *P. misakiensis*. *The enzyme is colored red. (C,E-G) Nomarski's optics.* **(A)** *Digestive tract of a 7-day-old young adult. The section was also stained with the substrate of peroxidase (brown). ALP is distributed in the stomach, mid-gut and visceral envelope that is a derivative of the atrial epithelium. The mid-gut is discernible from the hind-gut by the absence of the pyloric gland.* **(B)** *Ventral area of an adult animal.* **(C)** *Ovary.* **(D)** *Body wall of an adult animal. Arrowhead shows granular leucocyte. Note that the atrial epithelium shows the enzyme activity.* **(E)** *Proximal morphogenetic area of a 3-day-old bud. Note that neither the atrial epithelium nor the gut rudiment derived from it show the enzyme activity.* **(F)** *Developing gut rudiment of a 4-day-old bud. It is differentiating into the stomach, pyloric caecum and intestine.* **(G)** *A portion of the stomach of a 5-day-old bud. The enzyme activity appears on the luminal surface. ae, atrial epithelium; e, epidermis; fg, fore-gut; g, gut rudiment; hg, hind-gut; i, intestine; m, muscle; mg, mid-gut; o, ovary; p, pharynx; pc, pyloric caecum; s, stomach; t, testis; ve, visceral envelope. Bars, A,C,E,F,G, 50 μm; B, 100 μm; D, 25 μm.*

It is at this proximal area that morphogenetic events take place (e.g. Kawamura *et al.*, 1993). The atrial epithelium in this domain has lost the minute pigment granules (Fig. 1F arrow), although it was still pigmented in the remaining area. The depigmentation began within 12 hours of the onset of bud development. The epithelial sheet became thickened and folded to form organ rudiments about 2 days later (Kawamura and Nakauchi, 1984).

Figure 2 shows ultrastructures of the atrial epithelium. In growing buds, the cell took a squamous shape (Fig. 2A). The nucleus was usually ellipsoidal in shape. The endoplasmic reticulum was poorly developed. Variable numbers of moderately electron-dense granules could be seen in the cytoplasm (Fig. 2A arrowheads). The boundary of neighboring cells was highly indented (Fig. 2B arrowhead). The epithelial sheet was underlaid by the basal lamina (Fig. 2B arrow).

In 1.5-day-old developing buds, the epithelial cells became round or took a somewhat amorphous shape at the morphogenetic area (Fig. 2C). Multi-layered epithelial sheet was often observable transiently. It lacked the basal lamina. The large nucleus had a prominent nucleolus. The moderately electron-dense granules were scarcely visible in the cytoplasm. By two days of bud development, the cell became cuboidal in shape and single-layered (Fig. 2D). Golgi complex was prominent at the apical area of the cell (Fig. 2D arrowheads). The basal lamina reappeared on the basal surface of the epithelial sheet.

Developmental expression of ALP

In *P. misakiensis*, ALP was expressed by many differentiated adult tissues, such as the digestive tract (Fig. 3A), pharynx (Fig. 3B), ovary (Fig. 3C), body muscle and granular leucocyte, a kind of mesenchymal blood cell (Fig. 3D) (Kawamura *et al.*, 1992). The atrial epithelium also expressed the enzyme on the cell surface (Fig. 3D). This enzyme activity became weakened or often undetectable during bud formation. In the developing bud, it was lost completely from organ rudiments such as the gut rudiment (Fig. 3E). The gut rudiment differentiated morphologically into the stomach, pyloric caecum and intestine in 4 days of bud development (Fig. 3F). In 5-day-old buds, ALP reappeared on the luminal surface of the stomach (Fig. 3G). By 7 days it had spread to the whole area of the stomach and to the mid-gut (Fig. 3A). This distribution pattern was the same as that of the fully-differentiated gastric epithelium (not shown).

We have raised several kinds of monoclonal antibodies against *Polyandrocarpa* ALPs (Fig. 4A). Respective AP-G and AP-E were named after the tissues that they recognized (shown later). AP-G1 (lane a) and G4 (lane c) could bind ALPs on the nitrocellulose membrane, as evident from the positive spots in the color development solution for ALP. AP-E2 also gave a clear, although weak, spot (lane b). As a control of dot blot analysis, Pae 1 antibody, which recognizes the atrial epithelium, was used (cf., Fujiwara and Kawamura, 1992). It did not bind ALPs at all (lane d). On SDS-PAGE (Fig. 4B), AP-G1 recognized several bands (lane a), whereas AP-G4 reacted with bands of about 27 kDa (lane c). The latter was used for the immunohistochemistry. AP-E2 showed a smeary staining pattern (lane b).

AP-E2 recognized strongly with the apical surface of the atrial epithelium and with the granular leucocyte in the mesenchymal space (Fig. 5A), whereas it did not react with the viscera such as digestive tract (Fig. 5B). AP-G4 stained heavily the luminal surface of the stomach (Fig. 5G) and mid-intestine (not shown), the pattern

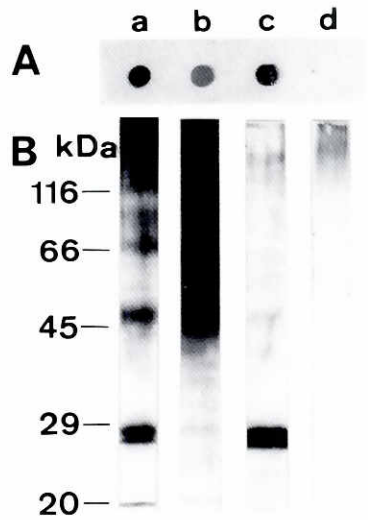

Fig. 4. Immunoblotting of *Polyandrocarpa* ALPs. (A) *Dot blot analysis of anti-ALP activity of monoclonal antibodies. As for the method, see Materials and Methods.* **(B)** *SDS-PAGE and immunostaining of Polyandrocarpa crude extracts. (Lane a) AP-G1; (lane b) AP-E2; (lane c) AP-G4; (lane d) Pae 1 (cf., Fujiwara and Kawamura, 1992). Note that although both AP-E2 and Pae 1 recognize the atrial epithelium similarly, the latter does not bind ALP* **(A)** *and shows the staining pattern of the antigen different from the former* **(B)**.

being quite similar to that of the enzyme activity (cf., Fig. 3A). These results showed that ALP of the atrial epithelium had an antigenic epitope different from that of the gastric epithelium.

As soon as the bud entered the developmental phase, the granules that showed the AP-E antigenicity appeared, at first, just beneath the plasma membrane of the atrial epithelium (Fig. 5C arrowheads). On the other hand, the antigenicity became weakened on the apical surface of the cell. This phenomenon was observable over the whole area along the proximo-distal axis of the bud, although it was most prominent in the proximal area. In 3-day-old buds, numerous AP-E-positive granules could be seen in the gut rudiment (Fig. 5D). In 5-day-old buds, the stomach not only contained the vestigial AP-E antigen (Fig. 5E arrowhead), but also expressed, for the first time, the AP-G4 antigen on the luminal surface (Fig. 5F arrowhead). By 7 days, the antigenicity became strong and restricted to the apical surface, probably the brush border, of the stomach (Fig. 5G).

Discussion

Considering the source of regenerative potential of multicellular organisms, we often encounter a serious problem of whether it should be attributed to the multipotency of undifferentiated reserve cells or the transdifferentiation capacity of specialized cells (Okada, 1991). The answer is important, as it inevitably influences our strategy to study how the developmental flexibility of somatic cells is regulated. The present study has dealt with budding of *Polyandrocarpa misakiensis*, which is equivalent to morphallactic regeneration (Kawamura and Nakauchi, 1986). First of all, we discuss several cellular and molecular features of the multipotent, atrial epithelium.

Polyandrocarpa multipotent epithelium is differentiated both structurally and functionally

In budding of *P. misakiensis*, the atrial epithelium is the primary formative tissue to form many organ rudiments of the bud (e.g. Fujiwara and Kawamura, 1992), although it does work in collaboration with mesenchymal reserve cells (Kawamura *et al.*, 1991). Previous studies have shown that the atrial epithelium consists of

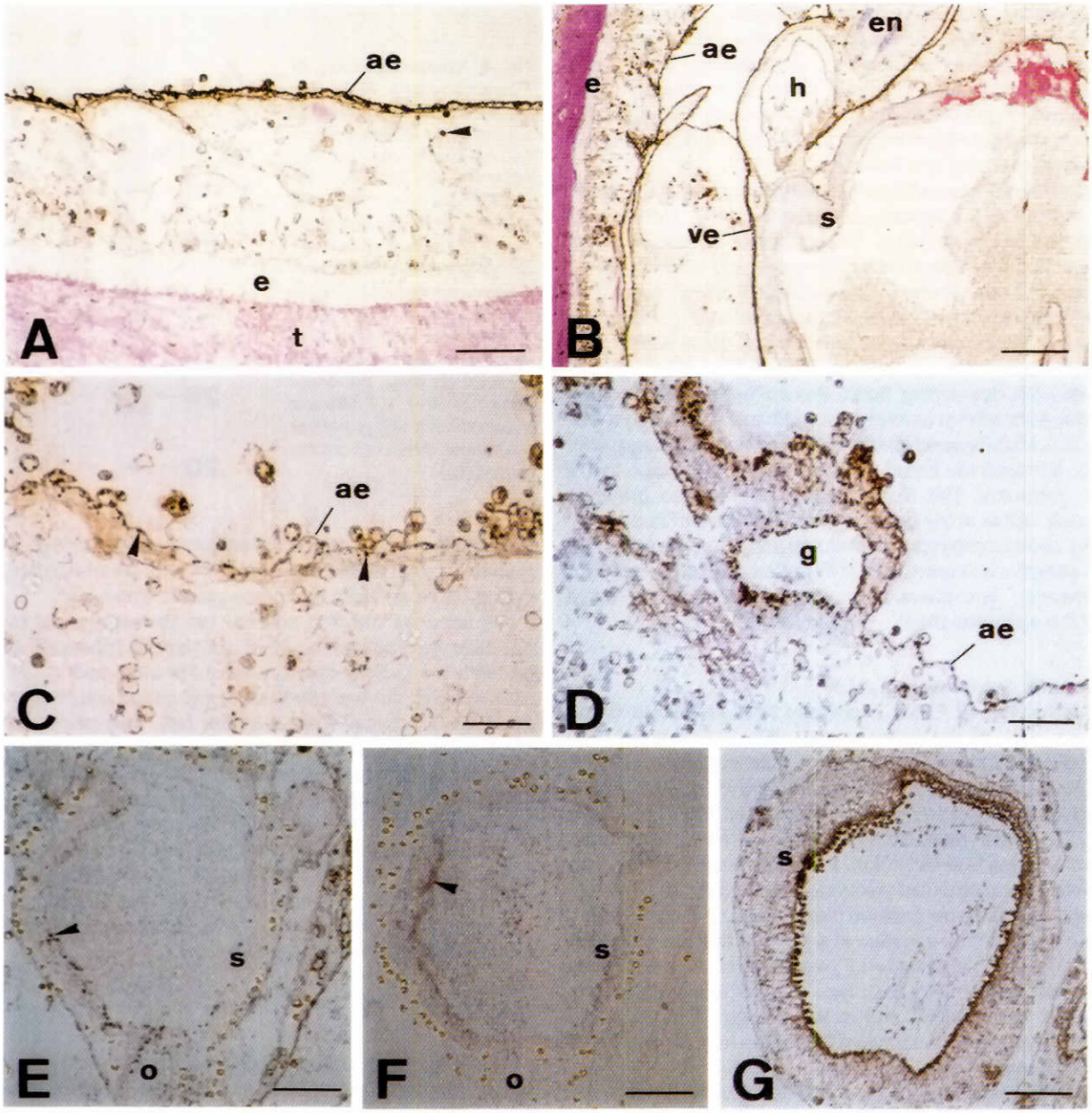

Fig. 5. Developmental expression of ALP antigens in *P. misakiensis*, sagittal sections. (A-E) *Immunostaining of AP-E2.* **(F,G)** *Immunostaining of AP-G4. Sections of A,B and D were counterstained with Giemsa's solution.* **(A)** *Growing bud. Arrowhead shows the granular leukocyte in the mesenchymal space.* **(B)** *Adult animal. Note that the stomach is non-reactive.* **(C)** *Proximal end of a bud, 12 h after being isolated from the parent. Arrowheads show immunoreactive granules in the atrial epithelium, of which the apical surface is stained weakly.* **(D)** *Gut rudiment of a 3-day-old bud.* **(E)** *Stomach of a 5-day-old bud. Note that the AP-E antigen still remains (arrowhead).* **(F)** *Stomach, the same specimen as E. Note that the AP-G4 antigen appears (arrowhead).* **(G)** *Stomach of a 7-day-old young adult. Note that the luminal surface, probably the blush border, is stained heavily, consistent with the result of histochemistry. ae, atrial epithelium; e, epidermis; en, endostyle; g, gut rudiment; h, heart; o, esophagus; s, stomach; t, tunic; ve, visceral envelope. Bars, A,E,F,G, 50 μm; B, 100 μm; C, 25 μm; D, 40 μm.*

squamous cells (Kawamura and Nakauchi, 1984), it has a cell cycle time of about 170 h (Kawamura and Nakauchi, 1991), and it expresses a tissue-restricted antigen, named Pae 1 (Fujiwara and Kawamura, 1992). The present study yielded a new finding that the atrial epithelium is pigmented. Ultrastructural studies showed that it indeed contained moderately electron-dense granules in the cytoplasm. The granules became electron-dense by imidazole, suggesting that they contained liposoluble substances (Y.M. Sugino, personal communication).

Recently, several kinds of retinoids have been extracted from *Polyandrocarpa* colonies (Kawamura *et al.*, 1993). ß-Carotene and other unidentified carotenoids were also detected by high performance liquid chromatography (our unpublished data). It is well known that ß-carotene is a major source of vitamin A and related

retinoids. Anti-retinoid monoclonal antibody reacted with the epidermis and atrial epithelium of developing *Polyandrocarpa* buds (Hara *et al.*, 1993). These results suggest that the atrial epithelium may reserve carotenoids in the pigment granules.

The present study also showed that in *P. misakiensis*, many differentiated cells and tissues expressed ALPs. The enzyme of the atrial epithelium (E-type) was discernible serologically from the isoform of the digestive tract (G-type). Our suggestion that several isoforms may exist in *Polyandrocarpa* ALPs is consistent with the findings in vertebrates, in which ALPs have been classified into four groups, that is, intestinal, placental, placental-like or germ cell and the tissue-nonspecific liver/bone/kidney types (Watanabe *et al.*, 1989). As discussed below, several physiological functions have been attributed to ALPs of differentiated endodermal and mesodermal tissues. In tunicate embryos, the enzyme is an endoderm-specific marker (cf., Whittaker, 1973; Nishida, 1993).

ALPs constitute a family of isozymes capable of hydrolyzing organic monophosphate esters *in vitro*. It is generally believed that they are involved in the transport of low molecular weight substances (Register *et al.*, 1984, 1986). According to Bernard *et al.* (1986), ALP is a calcium-binding glycoprotein. The calcification of bones is blocked in the missense mutation of the human liver/bone/kidney ALP gene (Weiss *et al.*, 1988). Human intestinal ALP may serve as a phosphate binding protein and play a key role in its uptake (Hirano *et al.*, 1985). In *P. misakiensis*, the G-type isoform of ALP expressed in the stomach and mid-gut might work in such a way. In *Botryllus schlosseri*, these organs are able to absorb exogenous peroxidase into the cell bodies (Burighel, 1979). The cells are characterized ultrastructurally by brush borders and large vacuoles (Burighel and Milanesi, 1975, 1977). As already mentioned, in *P. misakiensis* the G-type ALP seems to be localized in the brush border.

Unlike the G-type, the physiological function of the E-type isoform of ALP is uncertain. The E-type seems to be a weak enzyme. It could easily have lost the enzyme activity when animals were transferred to the laboratory condition, although the antigen was observable even after starvation (our unpublished data). It was inactivated enzymatically by acetone, although the G-type was still alive (our unpublished data). It is noteworthy that ALP may act as a protein phosphatase that regulates the phosphorylation state of membrane-bound proteins (Chan and Stinson, 1986; Meijer *et al.*, 1986; Pondaven and Meijer, 1986). Interestingly, the E-type ALP was blocked by calyculin A, a specific inhibitor of protein phosphatase-1 and 2A (our unpublished data). It is possible, therefore, that in *P. misakiensis* the enzyme acts as a negative regulator of phosphorylation of the membrane proteins in the atrial epithelium. Whatever the physiological function of the E-type isoform is, it is evident that ALP can be used as a reliable differentiation marker of the atrial epithelium.

Evidence shows that the atrial epithelium should be considered differentiated from both structural and functional viewpoints. It is unlikely that the atrial epithelium contains a small number of undifferentiated, reserve cells that carry developmental multipotency (Fujiwara and Kawamura, 1992). This is because every cell of the atrial epithelium expressed ALP, as far as we examined.

Transdifferentiation of the pigmented epithelium during morphallaxis

We have recently found that in developing *Polyandrocarpa* buds Pae 1 antigen disappears from the atrial epithelium at the site of presumptive organ rudiments (Fujiwara and Kawamura, 1992). This antigen, of which the molecular nature could not be determined, was shared by a few differentiated tissues such as the pharynx and phagocyte-like cells. This was the first indication of transdifferentiation in tunicate budding. The present study confirmed and extended this previous notion.

First, the atrial epithelium has lost pigment granules from the cytoplasm prior to the organogenesis. The cell had the large nucleus with a prominent nucleolus, like undifferentiated cells. During lens regeneration of newt, the iris pigment epithelium discharges pigment granules that are phagocytized by the macrophage (Eguchi, 1963). In *P. misakiensis* the depigmentation of the atrial epithelium may have taken place in such a way, as supported by large pigmented bodies that appeared in the mesenchymal space of developing buds. Alternatively, the depigmentation may have come from the *in situ* metabolism of the pigment granule containing carotenoids. Our recent study suggested that in the developing bud, endogenous retinoids were metabolized enzymatically at the morphallactic field (Kawamura *et al.*, 1993). A potent product, retinoic acid, can induce the field ectopically (Hara *et al.*, 1992; Kawamura *et al.*, 1993).

Secondly, the atrial epithelium has lost the ALP activity during budding. The AP-E antigen also disappeared from the apical surface of the epithelium, like the Pae 1 antigen (Fujiwara and Kawamura, 1992). Instead, it appeared in a granular form in the cytoplasm, at first, just beneath the plasma membrane, suggesting that the antigen may have undergone endocytosis. The development of Golgi complex suggested the active transport of the unit membrane.

In the developing gut rudiment, the AP-E antigen was displaced by the AP-G4 antigen, which strongly suggested that during gut formation the gene encoding the E-type ALP had been switched off and then the gene for the G-type isoform switched on. In 5-day-old buds, both types of antigen coexisted in the stomach. This situation is analogous to the regeneration of jellyfish, in which the striated muscle cell is able to transdifferentiate into 7-8 new cell types *in vitro*, leaving the muscle phenotype (Schmid and Alder, 1984). In the same context, transdifferentiation occurs in the atrial epithelium during *Polyandrocarpa* budding.

Epimorphic fields of regeneration contain a variety of dedifferentiated cells with different histories of their life (e.g. Kintner and Brockes, 1984). They also contain reserve cells such as the satellite cell, which is undifferentiated but committed to muscle cell (Cameron *et al.*, 1986), making epimorphic regeneration very complicated. In contrast, morphallactic fields seem simple. In *P. misakiensis* the hemoblast, a reserve cell in the mesenchymal space, takes part in organogenesis (Kawamura *et al.*, 1991). Fujiwara and Kawamura (1992), however, suggested that most cells of the gut rudiment have been derived not from the hemoblasts but from the atrial epithelium. The behavior of AP-E antigen in the present study confirmed this notion. The interstitial cell, a reserve cell of hydra, plays a key role in homeostatic cell differentiation (e.g. Bode *et al.*, 1976). However, epithelial hydra that lacks it is still able to regenerate the head and foot (Marcum and Campbell, 1978; Sugiyama and Fujisawa, 1978). This mode of regeneration is carried out through transdifferentiation of epitheliomuscular cell (Bode *et al.*, 1986).

Regulation of developmental flexibility of the atrial epithelium

In *P. misakiensis*, transdifferentiating cells are characterized by the cuboidal form, the large nucleus with a prominent nucleolus (Kawamura and Nakauchi, 1984, 1986; Hara *et al.*, 1992 and this

issue), the active cell cycling (Tc= 12.5 h) (Kawamura and Nakauchi, 1986; Kawamura *et al.*, 1988) and a large amount of RNA in the cytoplasm (Kawamura and Nakauchi, 1984). As shown in this paper, they are also marked by the transient disappearance of the basal lamina underlying the atrial epithelium. Retinoic acid can induce ectopically the morphallactic development of *Polyandrocarpa* buds (Hara *et al.*, 1992; Kawamura *et al.*, 1993). Many features of transdifferentiation mentioned above were observable in the ectopic field induced by retinoic acid (Hara *et al.*, 1992). This result strongly suggests that retinoic acid influences the differentiation state of the atrial epithelium either directly or indirectly (Kawamura *et al.*, in preparation).

In jellyfish, transdifferentiation needs enzyme digestion of the striated muscle layer with collagenase or hyaluronidase, otherwise the muscle cell isolate is maintained stably (Schmid and Alder, 1986). Mechanochemical interactions between the muscle cells and their substrate seem to be responsible for transdifferentiation (Schmid *et al.*, 1992). It is interesting to ask if in *P. misakiensis* the loss of the basal lamina is a necessary prerequisite for transdifferentiation of the atrial epithelium.

Materials and Methods

Animals

Polyandrocarpa misakiensis was used (for taxonomic description, see Watanabe and Tokioka, 1972). Animals were cultured on glass slides in a culture box settled in Uranouchi Inlet near the Usa Marine Biological Institute, Kochi University. Growing buds were extirpated from the parent and allowed to develop for definite time periods in the laboratory at 20°C.

Cryosection

Histological preparations have already been described elsewhere (Kawamura and Watanabe, 1982). In brief, both growing and developing buds were fixed overnight in 10% formaldehyde in seawater, followed by thorough washing with tap water. They were infiltrated with graded sucrose up to 20% in PBS and embedded in O.T.C. compound (Miles, Inc.). After freezing, the samples were cryocut at 12 μm in thickness.

Electron microscopy

Specimens were prefixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.4) containing 8% sucrose for 2 h in an ice bath (Kawamura *et al.*, 1991). Alternatively, they were prefixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 5 mM $CaCl_2$ and 0.34 M NaCl for first 10 or 20 minutes at room temperature and for over 2 h in an ice bath (Sugino *et al.*, 1993). After washing with the buffer, they were postfixed in 1% OsO_4 for 2 h. They were dehydrated and embedded in a modified Spurr's resin (Kushida, 1980). Sections were stained with uranyl acetate and Reynolds' lead citrate (Reynolds, 1963). They were observed with a JEOL JEM 100U electron microscope.

Histochemistry and immunohistochemistry

Specimens were fixed in Zamboni's fixative (Zamboni and DeMartino, 1967) at 4°C for 30 min, as described previously (Kawamura *et al.*, 1991). After dehydration, they were embedded in JB-4 plastic medium (Polyscience, Inc.) and sectioned with glass knives at 2 μm. Sections were mounted serially on coverslips.

Endogenous ALP of the sectioned materials was stained with the color development solution for ALP (kit I, Vector Lab.) in an incubation buffer (0.1 M Tris-HCl, pH 8.2) for about 20 min at room temperature.

For the immunohistochemistry, sections were incubated in the blocking solution containing 2% skim milk in PBS for 30 min. Goat anti-mouse secondary antibody labeled with gold particles and a silver enhancing kit were purchased from Biocell Research Laboratories. The secondary antibody was diluted 100-fold with PBS.

Monoclonal antibodies

Polyandrocarpa ALPs used as immunogens were extracted and purified partially, as described previously (Kawamura and Fujiwara, 1993). A female BALB/c mouse was immunized five times with about 20 μg of ALPs. Three days after the last booster, 6.8×10^7 spleen cells were isolated and fused with 1.2×10^7 P3X63Ag8U1 myeloma cells. Hybridomas were produced, as described previously (Fujiwara and Kawamura, 1992).

The screening of hybridomas was carried out, as described previously (Kawamura and Fujiwara, 1993). Briefly, 100 μl of culture media was loaded on each hole of the dot-blot apparatus (AE-6190, Atto Co. Ltd.) and blotted to nitrocellulose membrane. The membrane was incubated for 30 minutes in the blocking solution containing 2% skim milk in PBS. It was then reacted with the antigen (*Polyandrocarpa* ALPs) for 30 min, followed by thorough washing with 0.1% Tween 20 in PBS. It was stained with the color development solution mentioned above.

SDS-PAGE and immunoblotting

Total homogenates of *Polyandrocarpa* colonies were extracted with the sample buffer of SDS-PAGE. SDS-PAGE was carried out on 10% acrylamide gel containing 0.1% SDS in 0.375 M Tris-HCl (pH 8.8) (Laemmli, 1970). The gels were transferred electrically to nitrocellulose for 1.5 h at 30 volts. Immunostaining was made as described previously (Kawamura *et al.*, 1991).

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