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ORIGINAL ARTICLE

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Loss of test cells leads to the formation of new tunic surface cells and abnormal metamorphosis in larvae of *Ciona intestinalis* (Chordata, Ascidiacea)

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Abstract The larvae of the ascidian *Ciona intestinalis* from which the chorion with the test cells and follicle cells were removed developed normally without the test cells until the early tailbud stage. A number of round-shaped cells morphologically similar to the test cells but with different lectin affinities and autofluorescence, then appeared on the neck region of the demembranated embryos. The new cells had three different types: round, particulate, and granular, and these cells increased in number after the late tailbud stage. The morphology of the adhesive papillae, tunic layers and epidermis of the demembranated larvae was similar to that of control larvae; however, the affinity to lectins was different in the swimming period. Control larvae attached to the substratum after the swimming period, resorbed the tail completely and underwent rotation of the visceral organs. Conversely, rotation occurred before completion of tail resorption in the demembranated larvae. Furthermore, the metamorphic events progressed more slowly in the demembranated larvae. These results suggest that the test cells play important roles in normal development and morphogenesis of ascidian larvae.

Key words Test cell · Demembranated larva · Metamorphosis · Lectin · Ascidian

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Introduction

Test cells, which are located on the periphery of the ovarian oocyte in most ascidian species (Conklin 1905; Kowalevsky 1871), are extruded into the perivitelline space upon ovulation and remain there until hatching. A number of roles have been proposed for the test cells in the events that occur between oogenesis and hatching, including provision of nutrients for the oocyte during vitellogenesis (Gianguzza and Dolcemascolo 1978; Harvey 1927; Kessel and Kemps 1962; Mancuso 1965), production of hatching enzymes (Knaben 1936), synthesis of pigment (Kessel and Beams 1965; Kessel and Kemps 1962), and incorporation of vanadium into the oocyte (Kalk 1963).

Kupffer (1870) first suggested that test cells contribute to the formation of the tunic at the tailbud stage, and this has been supported by subsequent observation of inhibition of tunic formation in demembranated embryos of *Ascidia nigra* (Smith 1967), *Corella inflata*, *Ascidia paratropa*, *Styela gibbsii*, *Cnemidocarpa finmarkiensis*, and *Boltenia villosa* (Faulkner 1970). Satoh et al. (1982) described the characteristic locomotion of the test cells at the time of tunic formation and suggested that they participate in tunic differentiation in *Halocynthia roretzi*. Using demembranated *Ascidia* larva and *Corella* larva, Cloney (1990) indicated that the test cells do not participate in fin morphogenesis, but that the contents of the test cell are deposited on the tunic as the "ornaments" in the tailbud stage, making the tunic hydrophilic. This role as ornaments was later shown in many ascidian species (Cloney and Hansson 1995). Experiments using monoclonal antibodies raised against the test cells have recently shown that the cells secrete some materials that participate in the formation of the tunic ornaments before hatching in *C. intestinalis* (Okada et al. 1996).

The test cells peel off with the chorion from the embryo during hatching of the ascidian larvae, but in some species the cells remain on the outer cuticular layer of the larval tunic throughout the swimming peri-

od to the start of metamorphosis. We have previously shown that the test cells, which are distributed equally on the surface of embryo before hatching, become active and migrate across the surface of tunic and finally gather at the tip of the neck of the trunk region, and align along the lateral sides of the tail region at the late stage of the swimming period in *C. intestinalis* (Sato et al. 1997). Furthermore, electron microscopic studies have shown that the test cells protrude pseudopodia and invade through the tunic layers to extend to the surface of the epidermis or the sensory cilium at the late stage of the swimming period in *C. intestinalis* (Sato et al. 1997). Since the sensory cilium is considered to be a mechanoreceptor (Torrence and Cloney 1982), we proposed that the test cells play a role in signaling for the induction of metamorphosis including tunic modification, adhesive papillae retraction, attachment of the larva to substratum, tail resorption, and rotation of the visceral organs.

In the present study we removed the test cells together with the follicle cells, chorion, and fluid of the perivitelline space from fertilized eggs of *C. intestinalis* and cultivated the eggs to the juvenile stage. Morphological and histochemical evidence suggest the role of the test cells for development and metamorphosis in *C. intestinalis*.

Materials and methods

Collection of adults and culture of embryos and larvae

Methods for collecting adult of *C. intestinalis* and gametes and for insemination of eggs were the same as those described previously (Sato et al. 1997). Several fertilized eggs were transferred slowly by mouth pipette into a plastic dish with 20 ml ASW containing 460 mM NaCl, 10.1 mM KCl, 9.18 mM $CaCl_2$, 35.9 mM $MgCl_2$, 17.5 mM $MgSO_4$, buffered with 10 mM HEPES at pH 8.2. The chorions with the follicle cells and test cells were removed from the fertilized eggs (demembranation) with sharpened tungsten needles in the period between the first and second ooplasmic segregations or between the second segregation and the first cleavage. Demembranation by pronase was also carried out by the method described previously (Mita-Miyazawa and Satoh 1986). The eggs were placed in a plastic dish coated with 0.9% agar and filled with ASW and rinsed several times by adding ASW containing 100 U/ml penicillin and 200 µg/ml streptomycin. Residual test cells were completely removed during the washing processes. Each demembranated egg was then transferred to an agar-coated plastic dish 3 cm in diameter, filled with 1.5 ml ASW containing 100 U/ml penicillin and 200 µg/ml streptomycin and the demembranated embryos or larvae were cultivated at 18±1°C until the completion of metamorphosis. Embryos and larvae were observed and photographed with a dissection microscope (SMZ-2T, Nikon, Tokyo, Japan) or Nomarski differential interference contrast optic microscope (Optiphot, Nikon, Tokyo, Japan).

Autofluorescence observation

Living swimming larvae and demembranated larvae at stage 1–3 (see Sato et al. 1997) were placed on a glass slide, covered with a cover slip, and observed under fluorescent microscope with UV excitation (Optiphot, Nikon, Tokyo, Japan).

Lectin and Alcian blue staining

Fluorescein-labeled lectins, dolichos biflours agglutinin (DBA), jack bean agglutinin (ConA), peanut agglutinin (PNA), wheat germ agglutinin (WGA), soy bean agglutinin (SBA), and *Ulex euroaeus* agglutinin (UEA) (Vector Laboratories, Calif., USA) were diluted with ASW at a ratio of 1:2000. Live swimming larvae at stage 1, stage 2, or stage 3 were incubated in ASW containing a fluorescent lectin for 1 h in a moist chamber at 18±1°C in the dark. The larvae at each stage were also fixed with 4% formalin in ASW for 10 min, and then lectin treatment was performed following the same procedure. Fixed larvae were washed extensively before staining with fluorescent lectins. About 30 stained larvae were used in each experiment. After extensive washing of the larvae with ASW they were placed on a glass slide and covered with a cover slip. Staining of the test cells and new cells, tunic layers and epidermis was observed with fluorescent microscopy (Optiphot, Nikon, Tokyo, Japan). For analysis of Alcian blue staining about 30 larvae from each stage were fixed with 4% formalin in ASW for 10 min, washed with ASW several times and stained with Alcian blue at pH 1.0 (Pavão et al. 1994). The cells and the layers of the specimens were observed under a microscope (Optiphot, Nikon, Tokyo, Japan).

Statistics

The two-tailed *t*-test or χ^2 contingency test was carried out with Instat 2.1 programs. The χ^2 contingency test was used except when indicated.

Results

Generation of the new cells in the demembranated larvae

No solitary cells similar to the test cells were found on the surface of the demembranated embryos until the late tailbud stage (about 17 h after fertilization, AF; Fig. 1A). Solitary cells began to emerge from the right side of the embryo's trunk near the neck region about 17.5 h AF (Fig. 1B). The newly generated cells had the same round shape (round cell; Fig. 2A, rc) and size as those of test cells. Two types of smaller cells of different shapes (Fig. 2B, C) then appeared at the proximal end of the tail at about 17.5–19 h AF, although only at a low frequency (data not shown). These smaller cells contained either round distinct particles and were named granular cells (Fig. 2A, gc), or they included small granules and were named particulate cells (Fig. 2C, pc). The numbers of all three types of new cells increased until 20 h AF and then slightly decreased (Figs. 1C, 3). The numbers of new cells began to increase again at 22.5 h AF (stage 3; Fig. 3) until the cells covered the whole surface of the body (Fig. 2). The number of new cells on the demembranated larvae was 1/30 of that of test cells on control developing larvae. When fertilized eggs were demembranated by pronase, the new cells also appeared in the larvae although at lower numbers than in the larvae demembranated with a needle. The nuclei of all three types of cells stained with Hoechst 33258 (data not shown). These phenomenon were consistently seen in 400–500 larvae that were examined.

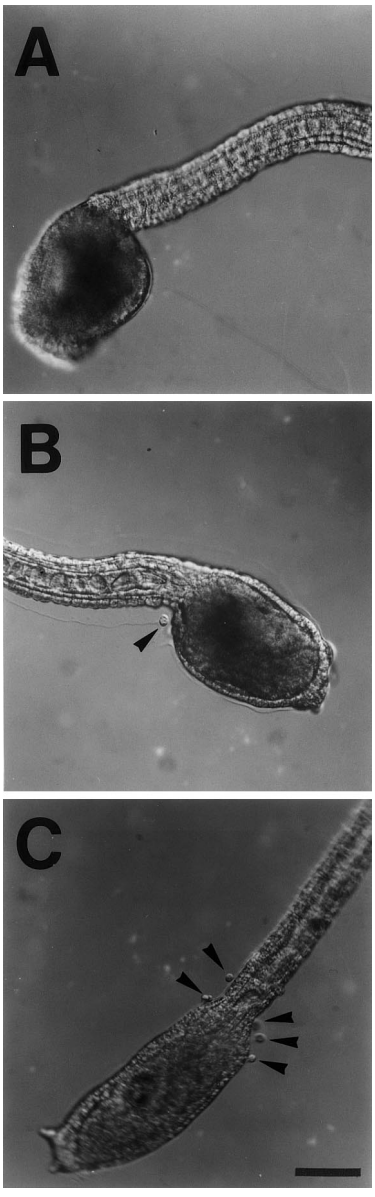

Fig. 1A–C The first appearance of the new cells on the surface of a demembranated larva during the tailbud and swimming stages. **A** Embryo at the tailbud stage 17 h AF. No cells can be seen on the surface of the demembranated larva. **B** Embryo 17.5 h AF. A new cell is apparent (*arrowhead*). **C** Larva during the swimming stage at 21.5 h AF. Many new cells (*arrowheads*) are apparent. *Bar* 50 μm

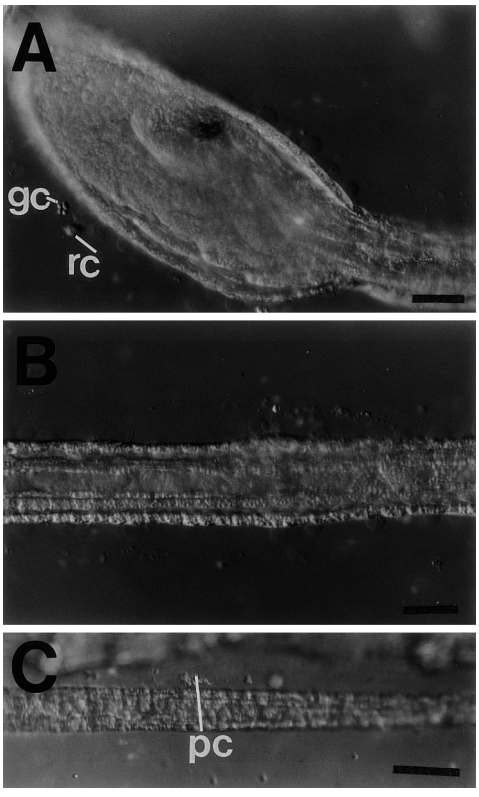

Fig. 2A–C Appearance of three types of new cells on a demembranated embryo. A number of new cells are apparent on the whole body. Round cells (*rc*), granular cells (*gc*), and particulate cells (*pc*). **A** Trunk region of embryo. *Bar* 20 μm **B** Middle part of the tail region. *Bar* 20 μm **C** Posterior end of the tail region. *Bar* 50 μm

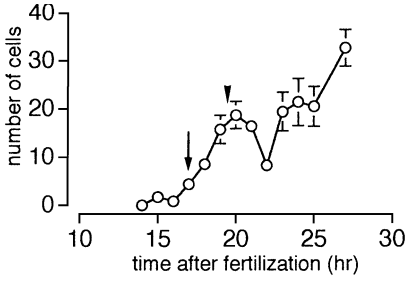

Fig. 3 Change in the number of new cells on a demembranated larva. The new cells begin to appear in the late of tail bud stage (*arrow*). The number of cells gradually increases with a brief transient decrease. *Arrowhead* the beginning of swimming. *Bar* mean±SD (*n*=78)

Lectins binding to the test cells, new cells, and adhesive papillae

All the new cells had blue autofluorescence when excited by UV. Autofluorescence of a round cell was shown in Fig. 4A, and other types of new cells were the same color as the round cell, whereas the test cells showed yellow auto fluorescence (Fig. 4B; see Deno 1987). Lectin binding to the new cells was different from that of the test cells (Table 1). To investigate lectin binding in greater detail, the swimming period of the demembranated larvae was divided into three stages (Tables 1, 2, 3, 4), which are comparable to the staging of control larvae (see Sato et al. 1997). In the control larvae there are drastic changes in the tunic layers and in the test cells in the swimming period so the division into three stages is based on morphological changes in tunic layers and distribution of the test cells around the surface of larva (see Sato et al. 1997). However, since the demembranated larvae has no test cells, the stage depends only on the

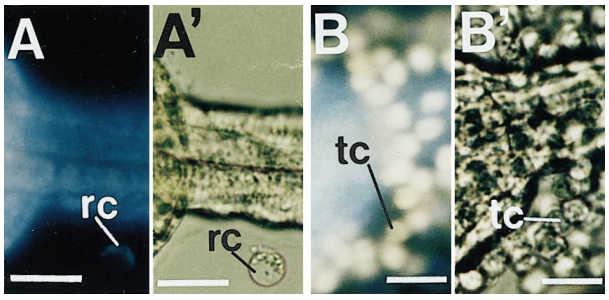

Fig. 4A–B' Autofluorescence of the new cell and the test cells. **A** A fluorescence micrograph of dorsal view of the demembranated larvae at neck region. Round cell (*rc*) shows blue autofluorescence. *Bar* 20 μm. **A'** A light micrograph of **A**. *rc* Round cell. *Bar* 20 μm **B** A fluorescence micrograph of ventral view of control larva at neck region. Test cells (*tc*) show yellow autofluorescence as described by Deno (1987). *Bar* 20 μm. **B'** A light micrograph of **B**. *tc* Test cell. *Bar* 20 μm

morphological feature of the tunic. ConA bound to the new cells but did not bind to the test cells at stages 1 and 3. In contrast, SBA did not bind to the new cells but bound to the test cells at the both stages. Binding of DBA, UEA, PNA, and WGA to the test cells and the new cells differed depending on the stage. Alcian blue staining of both test cells and new cells changed from positive to negative as the larva progressed from stages 1 to 3. At the beginning of stage 3, when the test cells had migrated and accumulated on a specific region of the control larvae (see Sato et al. 1997), the cells retained their affinity for WGA (Fig. 5A) at stage 1, but this was lost by stage 3 (Fig. 5B). When the test cells spread out across the whole of the tunic again just before tail resorption (Fig. 5C'), they regained the ability to bind WGA (Fig. 5C). Intense staining was observed on the test cells at the end region of the tail.

The demembranated larvae showed well-developed adhesive papillae, and binding of lectins to the adhesive papillae in these larvae was almost equivalent to control larvae (Table 2).

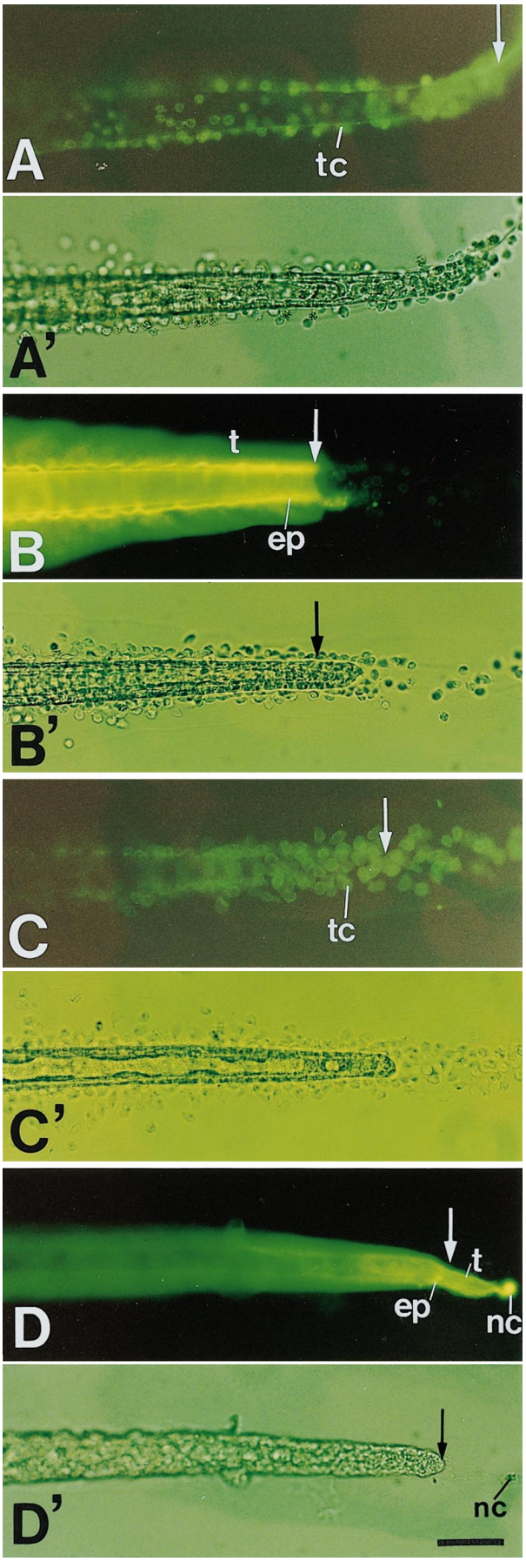

Fig. 5A–D' Change in WGA staining of the control and demembranated larvae in the swimming stage. **A** A fluorescence micrograph of the dorsal view of control larva at stage 1. All test cells (*tc*) are stained with fluorescent WGA. *Arrow* Posterior end of the tail. **A'** A light micrograph of **A**. **B** A fluorescence micrograph of the lateral view of the control larva at stage 3. The tunic (*t*) and epidermis (*ep*) are strongly stained, but the staining missing posterior part of *white arrow*. **B'** A light micrograph of **B**. *Black arrow* the same position of the white arrow in **B**. **C** A fluorescence micrograph of the lateral view of a control larva just before tail resorption. Only the test cells (*tc*) are stained. *Arrow* The posterior end of the tail. **C'** Light micrograph of **C**. **D** A fluorescence micrograph of the lateral view of a demembranated larva at stage 3. Tunic (*t*), epidermis (*ep*), and a new cell (*nc*) are strongly stained with fluorescent WGA. *Arrow* posterior end of the tail. **D'** Light micrograph of **D**. *Black arrow* shows the same position of the *white arrow* in **D**. *Bar* 50 μm

Table 1 Changes in lectin binding affinities and Alcian blue staining in the test cells and new cells during the swimming period of *C. intestinalis* larvae (– no staining, ± a part of body stained, *x* staining time is transient, ++ almost all body stained, *AB* Alcian blue)

Lectin	Test cell	New cell	Stage
ConA	–	±	1
ConA	–	++	3
DBA	–	±	1
DBA	–	–	3
PNA	–	±	1
PNA	±	–	3
SBA	++	–	1
SBA	±	–	3
UEA	–	–	1
UEA	–	++	3
WGA	++	++	1
WGA	x	++	3
AB	++	++	1
AB	–	–	3

Table 2 Changes in lectin binding affinities and Alcian blue staining of the adhesive papillae during the swimming period of *C. intestinalis* larvae (– no staining, ++ almost all body stained, *AB* Alcian blue)

Lectin	Control larva	Demembranated larva	Stage
ConA	++	++	1
ConA	++	++	3
DBA	–	–	1
DBA	–	–	3
PNA	++	++	1
PNA	++	++	3
SBA	–	–	1
SBA	++	–	3
UEA	–	–	1
UEA	–	–	3
WGA	–	–	1
WGA	–	–	3
AB	–	–	1
AB	–	–	3

Table 3 Changes in lectin binding affinities and Alcian blue staining of the tunic during the swimming period of *C. intestinalis* larvae (– no staining, ± a part of body stained, +/- part of body stained transiently; ++ almost all body stained, *AB* Alcian blue)

Lectin	Control larva	Demembranated larva	Stage
ConA	++	++	1
ConA	++	++	3
DBA	++	++	1
DBA	++	++	3
PNA	–	–	1
PNA	–	–	3
SBA	–	++	1
SBA	++	±	3
UEA	++	–	1
UEA	++	–	3
WGA	–	++	1
WGA	+/–	++	3
AB	–	–	1
AB	++	++	3

Table 4 Changes in lectin binding affinities and Alcian blue staining of the epidermis during the swimming period of *C. intestinalis* larvae (– no staining, +/– part of body stained, transiently, ++ almost all body stained, *AB* Alcian blue)

Lectin	Control larva	Demembranated larva	Stage
ConA	++	++	1
ConA	++	++	3
DBA	++	++	1
DBA	++	++	3
PNA	–	–	1
PNA	–	–	3
SBA	–	++	1
SBA	++	++	3
UEA	++	–	1
UEA	++	++	3
WGA	–	++	1
WGA	+/–	++	3
AB	–	–	1
AB	–	–	3

Lectin binding to the tunic layers and epidermis in the demembranated larvae

The demembranated larva formed the tunic layers, outer cuticular layer (C1) and inner cuticular layer (C2), similar to control swimming larva, and could swim vigorously. Formation of the double-humped shape of the C2, which is observed in control swimming larvae (see Sato et al. 1997) was also observed in the demembranated larvae. The dorsal and ventral fins of the demembranated larva were smaller than those of control larva when the demembranated egg was cultured alone in plastic dishes, as described by Cloney (1990).

The affinities of the C1 to ConA, DBA, PNA, and Alcian blue at stages 1 and 3, and of C2 to these lectins at stage 3 of the demembranated larvae were similar to those of control larvae (Table 3), whereas the affinities of these layers to SBA, UEA or WGA were varied between the demembranated and control larvae during the swimming stage.

As shown in Fig. 5, WGA binding changed drastically in the C1 in almost all of the tail region except for the tip of the tail, and in the C1 and C2 on the surface of the trunk of control larvae at stage 3; WGA staining in the layers of the tail disappeared when the test cells spread out just before tail resorption (Fig. 5C). At the posterior region of the tail, the C1 remained unstained throughout the swimming stage. These changes were not found in the demembranated larvae (Fig. 5D).

Similar staining patterns of the epidermis and tunic layers (Tables 3, 4) to all of the fluorescent lectins examined except for SBA, UEA, and alcian blue staining were seen in both demembranated and control larvae. No binding of alcian blue to the epidermis of the demembranated larvae was seen (Table 4), but there was some binding to the tunic layers at the stage 3 (Table 3). Fur-

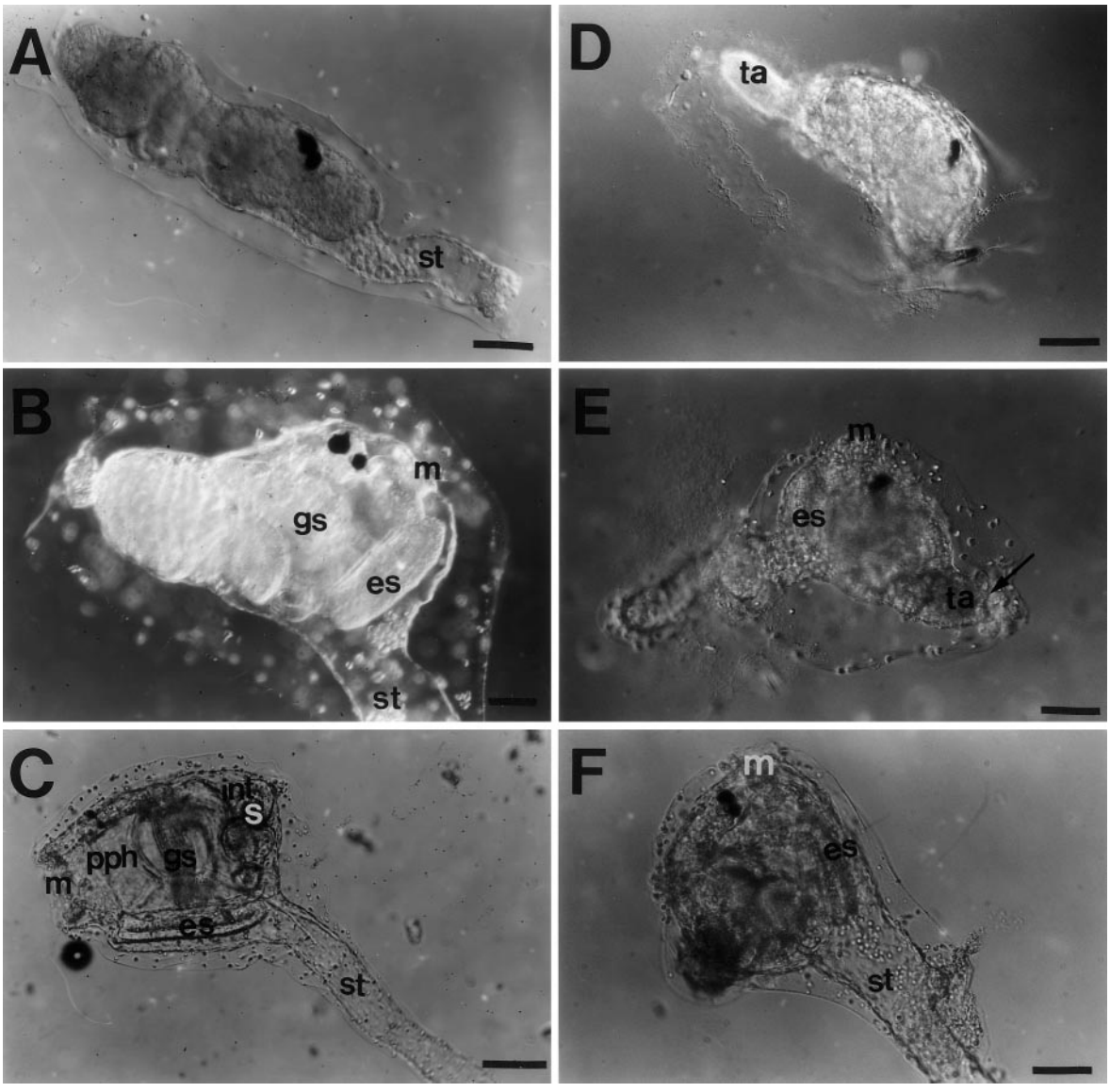

Fig. 6A–F Metamorphosis in the control and demembranated larvae. **A** A control juvenile has already attached to the substratum just after tail resorption but the axis of the body remained unchanged (juvenile 1). *Bar* 50 µm. **B** Rotation of the visceral organs begins after completion of tail resorption in a control juvenile (juvenile 2). *Bar* 20 µm. **C** The rotation and opening of the siphon is complete in a control developed juvenile (juvenile 3). *Bar* 50 µm. **D** The rotation of the visceral organs begins without complete tail resorption in a demembranated juvenile (juvenile 1). *Bar* 50 µm. **E** The rotation of the axis is completed without complete tail resorption in a demembranated juvenile (juvenile 2). *Arrow* The remaining tail. *Bar* 100 µm. **F** Tail resorption is completed after the rotation of the axis in a demembranated juvenile (juvenile 3). Common feature of both juveniles are no differentiation of organs and opaque body (juvenile 1), start of differentiation of organs and opaque body (juvenile 2) and transparent body (juvenile 3). *Bar* 50 µm. *es* Endostyle; *gs* gill slit; *int* intestine, *m* (both black and white) siphon; *pph* peripheral phalynx; *s* (white) stomach; *st* stron; *t* tunic; *ta* tail

thermore, WGA binding to the epidermis in the posterior end of the tail in control larvae changed drastically in a similar manner to that seen in the tunic layers (Fig. 5).

Effects of demembranation on metamorphosis

Control larvae hatched and started swimming at 19–19.5 h AF at 18±1°C and then attached to the substratum with their adhesive papillae. The demembranated larvae started swimming at the same time that the control larvae hatched and started swimming, and their body length became the same as that of the control larvae (t=1.71, P=0.09) at the end of the period (23–24 h AF). The percentage of attached larvae was not significantly different between the demembranated and control larvae at 23–24 h AF (P=0.99). Retraction of the adhesive papillae and tail resorption began just after attachment in control and demembranated larvae (Fig. 7A). The percentage of the events between them was not different (P=0.99). These

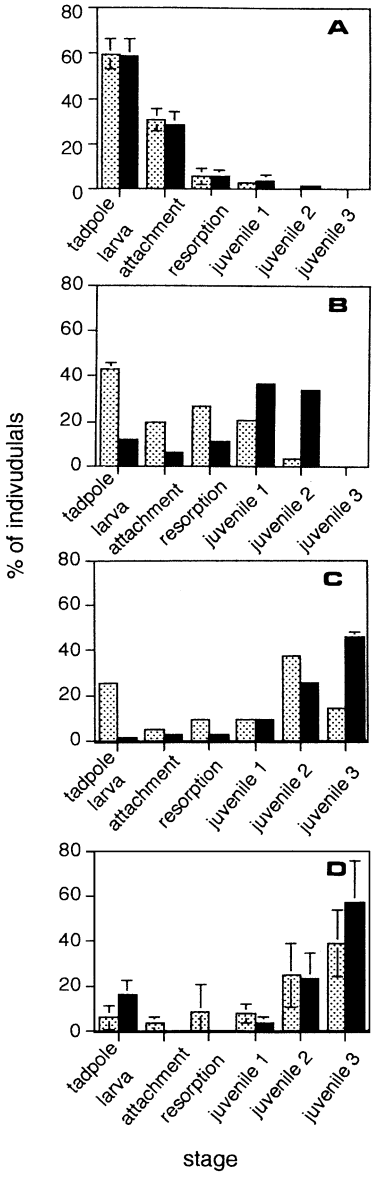

Fig. 7A–D Transition of metamorphosis in the control and demembranated larvae. **A** 4–5 h AH. **B** 2 days AH. **C** 3 days AH. **D** 4 days AH. Percentage of tadpole larvae, larvae attached to substratum (attachment), larvae began resorption of tail (resorption), juvenile 1, juvenile 2, or juvenile 3, respectively in the control (*black bars*) and demembranated (*shaded bars*). *Bar* mean±SD (*n*=85)

results suggest that the chorion, follicle cells, test cells, and fluid of the perivitelline space are not required for development before tail resorption.

Some perturbation of the development process, however, occurred during and after tail resorption. All control larvae examined (*n*=51) completed tail resorption within 1–3 days after hatching (AH) (Figs. 6A, 7A–C) and then entered into the juvenile stage in which rotation of the visceral organs started. The rotation completed between 3–4 days AH (Figs. 6C, 7C, D). In the demembranated larvae, however, a longer period was required for tail resorption and the rotation of the visceral organs. In all demembranated larvae (*n*=65), rotation of the visceral organs started 1–3 days AH (Figs. 6D, 7A–C), but the tail was still maintained beyond 3 days AH. Therefore the demembranated larvae entered the juvenile stage, with rotating visceral organs, although they still kept the tail. The percentage of control larvae with tail and demembranated larvae or juveniles with tail was significantly different at 3 days AH (P=0.02), however, there was no significant differences between them at 4 days AH (P=0.82). After finishing the rotations all the demembranated juveniles (*n*=37) with tails completed tail resorption by 3–6 days AH and became juveniles with the same features (Figs. 6F, 7D) as control juveniles. The percentage of demembranated and control juveniles showing rotations at 2 days AH (P=0.01) was significantly different, but no difference was present between them at 3 days AH (P=0.12). The timing of the rotation of the visceral organs in the demembranated juvenile with tail was delayed compared to that of control juveniles, and tail resorption finished after completion of the rotation in demembranated juveniles. The completely different order of tail resorption and rotations is the most characteristic feature of the effect of demembranation on *Ciona* development. These juveniles had an open siphon and started feeding (Fig. 6C, F).

The juvenile stage of control development was divided into juvenile 1 (J1), 2 (J2), and 3 (J3), depending on their morphology (see Fig. 6). The primordial organs began to differentiate between 1–3 days AH, and J1 became J2 in the control (Figs. 6B, 7A–C), while demembranated J1 became J2 with organ differentiation between 3–4 days AH (Figs. 6E, 7C, D). The percentage of demembranated and control juveniles (J2 and J3) with organs at 2 days AH (P<0.0001) and at 3 days AH (P=0.02) was significantly different, but no significant difference was present at 4 days AH (P=0.27). When the J1 became J2 during 1–3 days AH, part of the bodies became transparent (Fig. 6B, E) and the whole body became transparent between 3–4 days AH in the control or 3–6 days AH in the demembranated J3 (Fig. 6C, F). The percentage of transparent juveniles (J3) was significantly different between demembranated and control at 3 days AH (P<0.0001) but was not significant after 4 days AH (P=0.10). These abnormalities occurred in larvae demembranated with pronase in the same manner as those in larvae demembranated by manual dissection.

Discussion

Conflicting views have been expressed on the role of the test cells in ascidian development, based on experiments using demembranated larva in which the egg coat was removed at the neurula stage. Several studies, in which the neurula of *Ascidia callosa* was demembranated and embryos were cultured in small dishes, suggest that the test cells are not required for tunic formation. Submicroscopic materials called ornaments are present in the test cells of this species (Cloney 1990) and in other species

such as *Distaplia occidentalis* and *C. inflata.* The ornaments are deposited on the surface of the tunic layer (Cavey and Cloney 1983; Cloney and Cavey 1982), making the tunic hydrophilic (Cloney 1990; Cloney and Hannson 1995). This conclusion was supported by Okada et al. (1996) in *C. intestinalis*, using a test cell monoclonal antibody.

In the present study, when the test cells, follicle cells, and chorion were removed from the fertilized eggs of *C. intestinalis* and each demembranated larva was allowed to develop under similar conditions to those used by Cloney (1990), fins developed at the stage comparable to late tailbud stage of control larvae. Although the fins were reduced in size when the demembranated larva was cultured alone, the larva could swim normally. These results suggest that the test cells, follicle cells, and chorion are not necessary for the formation of the tunic and fin.

There were no solitary cells present on the surface of the demembranated embryo until the tailbud stage, at which point new cells resembling test cells in shape (round cell) were produced at the region near the junction of the trunk and tail (Fig. 1). Two other types of cells, granular and particulate cells, subsequently appeared (Fig. 2), but it is unclear whether these two types of cells had differentiated from the round cells. All three new cell types had the same lectin affinities, but the outer matrix of the new cells had different lectin affinities from that the test cells. Furthermore, all three new cell types showed different autofluorescence from the test cells (Fig. 4), indicating that the contents of the new cells was different from those of the test cells. These new cells were not produced during normal development, since no cells with the same autofluorescence and lectin binding as the new cells were observed on the surface of control larvae. Thus these new cells are considered to be different from test cells.

It is well known that several types of solitary cells appear during metamorphosis in ascidians. Button cells, which are derived from B 4.1 cells and stain with three kinds of vital dyes (Materazzi 1967; Patricolo et al. 1981; Reverberi et al. 1969; Ries 1939) appear at the space between the epidermis and primordium of the pharynx in *Phallusia mammillata* (Sotgia et al. 1993). B-cells, which originate from the ectoderm in the body cavity of *C. intestinalis*, migrate toward the outermost layer of the larva, crossing through the epidermal mantle and the cuticle (Mancuso 1986). Trunk lateral cells of the larvae of *H. roretzi*, derived from A6.3 and A.7.6 blastomeres, are located underneath the epidermis at the neck region and migrate toward the surface of the tunic layer crossing the epidermis (Nishida 1987; Nishikata and Satoh 1991). Although it is still unknown whether these cells are identical to the new cells which appear on the surface of larvae of *C. intestinalis* (Fig. 3), the position and the timing of their appearance was different from those of the new cells that we observed.

The C1 larval tunic layer differentiates on the surface of the epidermis at the tailbud stage in *C. intestinalis* (Cavey and Cloney 1983; Gianguzza and Dolcemascolo 1980; Mancuso 1973). The layer is gradually separated from the epidermis and a matrix-filled space forms between the tunic and the surface of the epidermis called the outer compartment (Cloney 1990). C1 finally forms the fins in the tail region. We observed here that the adult tunic (C2) then appears in the trunk region of the demembranated larvae at a comparable stage to the control larvae. In the present study, the epidermis and tunic layer of both the control and demembranated larvae showed no Alcian blue staining at stage 1, suggesting that no sulfate glycosaminoglycans are present on the surface of larvae. The epidermis was Alcian blue negative at stage 3, but Alcian blue staining appeared on the tunic layer in both the control and demembranated larvae at stage 3. Both test cells and new cells, which were stained with the dye from stages 1 and 2, lost Alcian blue reactivity at stage 3. We have recently shown that the test cells migrate and gather on the tunic at the tail region, and that some cells ruptured at stage 3 (Sato et al. 1997). Some intracellular contents of the test cells and new cells stain with Alcian blue, and it is possible that these components are released from the test cell of control larvae or from the new cells of the demembranated larvae and are deposited on the surface of the tunics. However, the autofluorescence results from the test cells and the new cells were quite different, suggesting that the constituents of the tunic in control and demembranated larvae are different.

The ability of the test cells, tunic layer, and epidermis of control larvae to bind WGA changed during the swimming period (Tables 1, 3, 4; Fig. 5), concomitantly with the occurrence of drastic migration of the cells (see Sato et al. 1997). The test cells exhibited WGA binding affinity in stage 1 before migration, and this affinity was lost in stage 3 when the cells migrated and aligned in the lateral side of the tail regions. In contrast, the tunic layer and epidermis acquired WGA binding capacity between stages 1 and 3, suggesting the appearance of glycosaminoglycans on the surface. WGA binding affinities of the tunic layer and epidermis remained unchanged in the demembranated larvae during the swimming period. Cell surface glycosaminoglycan are considered to be a key factor in the control of the migration of the mesenchyme cells on the surface of the coelomic wall in the sea urchin embryo (Katow and Amemiya 1986) and may also contribute to the migration of the neural crest cells in the mouse (Derby 1978). Therefore the changes in the appearance of the glycosaminoglycan on the surface of the epidermis in the swimming larvae of the ascidian may relate to the migration of the test cells.

Metamorphosis of ascidians is characterized by settlement of swimming larva, tail resorption, and rapid changes in body shape; reorientation of organs, emigration of blood cells, disintegration of larval nervous, sensory, muscular and skeletal tissues, and replacement of these larval tissues with adult tissues. We showed that the removal of the test cells, follicle cells, and chorion

from the fertilized eggs delays (Fig. 7) and perturbs (Fig. 6) the process of metamorphosis. In the normal metamorphosis in *C. intestinalis*, rotation of the visceral organ began after completion of tail resorption. However, in the demembranated larvae, partial tail resorption occurred first, then the visceral organs rotated, and the tail finished resorption (Fig. 6). This result and the characteristic transient migration of the test cells on the larva suggest that the test cells play some role in the control of the order of complete resorption of the tail and rotation of the visceral organs.

The test cells migrate and gather around the adhesive papillae at the trunk region, suggesting a role of these cells in the formation or behavior of this organ (Sato et al. 1997). The demembranated larvae also had well-developed adhesive papillae, and retraction occurred in the same manner as in the control larvae. However, no migration of the new cells forward to the adhesive papillae was observed although the cells showed some locomotive activity, suggesting that the test cells as well as the new cells make no contribution to the formation and behavior of the adhesive papillae.

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References

Cavey MJ, Cloney RA (1983) Development of the larval tunic in a compound ascidian: morphogenetic events in the embryos of *Distaplia occidentalis.* Can J Zool 62:2392–2400

Cloney RA (1990) Larval tunic and the function of the test cells in ascidians. Acta Zool 71:151–159

Cloney RA, Cavey MJ (1982) Ascidian larval tunic: extraembryonic structures influence morhogenesis. Cell Tissue Res 222: 547–562

Cloney RA, Hansson LJ (1995) Ascidian larvae: the role of test cells in preventing hydrophobicity. Acta Zool 157:1–7

Conklin EG (1905) Organization and cell lineage of the ascidian egg. J Acad Natl Sci Philos 8:1–119

Deno T (1987) Autonomous fluorescence of eggs of the ascidian, *Ciona intestinalis.* J Exp Zool 241:71–79

Derby MA (1978) Analysis of glycosaminoglycans within the extracellular environments encountered by migrating neural crest cells. Dev Biol 66:321–336

Faulkner GT (1970) Formation and fine structure of the larval tunic in simple ascidians. Thesis, University of Wisconsin

Gianguzza M, Dolcemascolo G (1978) On the ultrastructure of the test cells of *Ascidia malaca* during oogenesis. Acta Embryol Exp 2:173–189

Gianguzza M, Dolcemascolo G (1980) Morphological and cytochemical investigations on the formation of the test during the embryonic development of *Ciona intestinalis.* Acta Embryol Morphol Exp 1:225–239

Harvey LA (1927) The histology of cytoplasmic inclusion of the egg of *Ciona intestinalis* during oogenesis and fertilization. Proc R Soc Lond B Biol Sci 101:137–161

Kalk M (1963) Cytoplasmic transmission of vanadium compound in a tunicate oocyte, visible with electron microscopy. Acta Embryol Morphol Exp 6:289–303

Katow H, Amemiya S (1986) Behavior of primary mesenchyme cells in situ associated with ultrastructural alteration of the blastocoelic material in the sea urchin, *Anthocidaris crassisoina*. Dev Growth Differ 28:31–42

Kessel RG, Beams HW (1965) An unusual configuration of Golgi complex in pigment-producing "test" cells of the ovary of the tunicate *Styela*. J Cell Biol 25:55–67

Kessel RG, Kemps NE (1962) An electron microscope study on the oocyte, test cells and follicular envelope of the tunicate, *Morgula manhattensis*. J Ultrastruct Res 6:55–76

Knaben N (1936) Über entwicklung und Function der testazellen bei *Corella parallelogramma*. Müll Beregens Mus Årb Nat Rek 1:1–33

Kowalevsky A (1871) Weitere Studien über die Entwicklung der einfachen Ascidien. Mem Acad St Petersburg 7:101–130

Kupffer C (1870) Weitere Studien über die entwicklung der einfachen Ascidien. Mem Acad St Petersburg 7:115–172

Mancuso V (1965) An electron microscope study of the test cells and follicle cells of *Ciona intestinalis* during oogenesis. Acta Embryol Morphol Exp 8:239–266

Mancuso V (1973) Changes in fine structure associated with the test formation in the ectoderm cells of *Ciona intestinalis* embryo. Acta Embryol Exp 3:247–257

Mancuso V (1986) Type B cells in metamorphosing *Ciona intestinalis* embryos. Acta Embryol Morphol Exp 7:27–33

Materazzi G (1967) Further observations on the embryonic development of ascidians in artificial sea water devoid of sulfate ions. Acta Embryol Morphol 10:101–107

Mita-Miyazawa I, Satoh N (1986) Mass isolation of muscle lineage blastomeres from ascidian embryos. Dev Growth Differ 28:483–488

Nishida H (1987) Cell lineage analysis in ascidian embryos by intracellular injection of tracer enzyme. III. Up to the tissue restricted stage. Dev Biol 121:526–541

Nishikata T, Satoh N (1991) Expression of an antigen specific for trunk lateral cells in quarter embryos of the ascidian, *Halocynthia roretzi*. J Exp Zool 258:344–352

Okada T, Takamura K, Yamaguchi Y, Yamamoto M (1996) Secretory function of the test cell in larval tunic formation in the ascidian *Ciona intestinalis*: an immunoelectron microscopic study. Zool Sci 13:253–262

Patricolo E, Ortolani G, Cascio A (1981) The effect of L-thyroxine on the metamorphosis of *Ascidia malaca*. Cell Tissue Res 214: 289–301

Pavão MSG, Lambert CC, Lambert G, Mourão PAS (1994) Comparison between the sulfated polysaccharides from larval and adult ascidians. J Exp Zool 269:89–94

Reverberi G, Ortolani G, Fenandez M (1969) Su alcune singolari cellule "a peossidasi" delle larve di Ascidie. Rend Acc Naz Lincei 47:375–380

Ries E (1939) Versuche über die Bedeutung des Substanzmosaic für die embryonale Gewebedifferenzierung bei Ascidien. Arch Exp Zellforsch 23:95–121

Sato Y, Terakado K, Morisawa M (1997) Test cell migration and tunic formation during post-hatching development of the larva of the ascidian, *Ciona intestinalis*. Dev Growth Differ 39:117–126

Satoh N, Numakunai T, Hori R (1982) Behavior and cellular morphology of the test cells during embryogenesis of the ascidian *Halocynthia roretzi*. J Morphol 171:219–223

Smith K (1967) Genetic control of macromolecular synthesis during development of an ascidian: *Ascidia nigra*. J Exp Zool 164:393–406

Sotgia C, Fascio U, Ortolani G, Benardi FD (1993) Behavior of endodermal "button cells" during metamorphosis of ascidian larvae. Int J Dev Biol 37:547–553

Torrence SA, Cloney RA (1982) Nervous systems of ascidian larvae: caudal primary sensory neurons. Zoomorphology 99: 103–115