Original Article

**Microtubule array observed in the posterior-vegetal cortex during cytoplasmic and cortical reorganization of the ascidian egg**

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Body axis formation during embryogenesis results from asymmetric localization of maternal factors in the egg. Shortly before the first cleavage in ascidian eggs, cell polarity along the anteroposterior (A–P) axis is established and the cytoplasmic domain (myoplasm) relocates from the vegetal to the posterior region in a microtubule-dependent manner. Through immunostaining, tubulin accumulation during this reorganization is observable on the myoplasm cortex. However, more detailed morphological features of microtubules remain relatively unknown. In this study, we invented a new reagent that improves the immunostaining of cortical microtubules and successfully visualized a parallel array of thick microtubules. During reorganization, they covered nearly the entire myoplasm cortical region, beneath the posterior-vegetal cortex. We designated this microtubule array as CAMP (cortical array of microtubules in posterior vegetal region). During the late phase of reorganization, CAMP shrunk and the myoplasm formed a crescent-like cytoplasmic domain. When the CAMP formation was inhibited by sodium azide, myoplasmic reorganization and A–P axis formation were both abolished, suggesting that CAMP is important for these two processes.

**Key words:** ascidian, axis formation, cell polarity, cortical microtubule array, maternal factors.

Introduction

Asymmetric localization of maternal factors in the egg results in clear cell polarity, essential for body axis formation during embryogenesis. In some animal eggs, drastic cytoplasmic reorganization accompanies maternal-factor localization, establishing cell polarity during the first cell cycle. In frog and zebrafish eggs, for example, highly aligned microtubule arrays appear transiently at the vegetal cortex during maternal-factor reorganization; these microtubules are necessary for fixing egg polarity along the dorsoventral (D–V) axis (Elison & Rowning 1988; Jesuthasan & Strähle 1997). However, the exact mechanisms underlying egg polarity establishment and cytoplasmic rearrangement remain unclear.

Ascidian eggs are a typical mosaic type with clearly distinguishable cytoplasmic regions, including the myoplasm, a mitochondria-rich cytoplasmic domain (Conklin 1905). The endoplasmic reticulum and maternal mRNA (i.e. Type I postplasmic/PEM RNA) (Paix et al. 2009; Makabe & Nishida 2012) are localized in the cortical region of the myoplasm (Sardet et al. 2003; Nishida 2005; Prodon et al. 2007). The myoplasm exhibits obvious cell polarity along the animal-vegetal (A–V) axis, being localized to the periphery (except for a small patch on the animal pole) in unfertilized eggs and shifting immediately to the vegetal hemisphere post-fertilization. This first phase of cytoplasmic and cortical reorganization is microfilament-dependent (Sawada & Schatten 1989; Chiba et al. 1999; Roegiers et al. 1999). The microtubule-dependent (Sawada & Schatten 1989; Chiba et al. 1999; Roegiers et al. 1999) second phase begins approximately 30 min post-fertilization, when the sperm aster moves from the vegetal cortex, reaching the equatorial region.
before turning toward the egg center. Sperm-aster movement is accompanied by myoplasm formation into a crescent-like cytoplasmic region, establishing the posterior side and therefore the anteroposterior (A–P) axis (Sardet et al. 1989; Roegiers et al. 1999). The strong correlation between cytoplasm and sperm-aster movements suggested that the latter is responsible for the second segregation (Jeffery & Meier 1983; Jeffery & Swalla 1990), although previous immunostaining experiments have detected only obscure signs of tubulins in the posterior-vegetal cortex (Chiba et al. 1999; Roegiers et al. 1999; Ishii et al. 2014). These relatively weak imaging results prevent us from clearly following myoplasmic movement and thus hamper attempts to clarify regulatory mechanisms of cytoplasmic rearrangement and body-axis determination.

Here, we aimed to improve microtubule visualization through the development of a new immunostaining reagent. Our objective was to provide clear microtubule images during the second phase of reorganization on the posterior-vegetal cortex. Additionally, we tested the involvement of microtubules in myoplasmic reorganization and A–P axis formation.

Materials and methods

Embryos

Ascidian (Ciona intestinalis) adults were provided by the National Bio-Resource Project (NBRP), Japan. Egg and sperm handling, dechorionation, and fertilization followed previous publications (Ishii et al. 2012, 2014). Embryos were reared in filtered seawater at 18°C. At this temperature, the first and second reorganization phases occur immediately post-fertilization and about 30 min post-fertilization (mpf), respectively, while the first cleavage occurs around 60 mpf. The second phase was inhibited by treatment with 5 mmol/L sodium azide (NaN3) from 10 mpf. NaN3 prevents reorganization without depolymerizing microtubules (Ishii et al. 2014).

Immunostaining

Whole Ciona embryos were fixed with 100% methanol, followed by 100% ethanol treatment. Fixed specimens were washed with phosphate-buffered saline containing 0.05% Tween 20 (PBST), then treated with modified ScaleA2 (G1T0; 4 mol/L urea [MP Biomedicals, Solon, OH, USA] and 1% glycerol in distilled water) for 90 min at 4°C, followed by a PBST wash. This treatment did not optically clear the specimens, but led to a sufficient improvement of microtubule immunostaining in the ascidian embryo cortex.

The G1T0-treated specimens were stained with the following antibodies: anti-α-tubulin mouse monoclonal antibody (1:100 dilution, anti-microtubule; CLT9002; Cedarlane Laboratories, Hornby, ON, Canada), anti-MnSOD rabbit antisera (1:40 dilution, anti-myoplasm; Ishii et al. 2012; SPC-117C/D; StressMarq Biosciences, Victoria, Canada), Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (1:1000 dilution, A11001; Molecular Probes, Eugene, OR, USA), and Alexa Fluor 532-conjugated goat anti-rabbit IgG antibody (1:1000 dilution, A11009; Molecular Probes). Stained specimens were cleared with methyl salicylate (Nacalai Tesque, Kyoto, Japan) and observed under a LSM700 confocal microscope (Carl Zeiss, Jena, Germany). Three-dimensional images were rendered from confocal images, and mid-plane images were generated in ZEN (Carl Zeiss).

Image analysis

All analyses were performed in ImageJ (http://imagej.nih.gov/ij/). The egg contour was delineated and its central coordinates calculated from a side-view of the 3D model. The animal pole was defined based on polar body or myoplasm configuration (depending on which was clearer); the image was then rotated to place the animal pole on top for generating the mid-plane view. Sperm-aster position was described based on centrosome coordinates. Myoplasm thickness was measured using the radial line profile per 10°, and its center of mass was calculated. The position of thick microtubule bundles on the posterior vegetal cortex was set as the midpoint of the circular arc formed by the bundles in the side view, equidistant from the upper- and lower-most points of the structures. These positions were determined from three images per time point (30, 45, and 50 mpf), and standard deviations (SDs) were calculated. Microtubule movement was represented in two ways. First, each coordinate was directly plotted on a graph representing the egg’s mid-plane; second, the time course of angles between the animal-vegetal axis and the line from the egg’s center to each coordinate was plotted.

Results

Immunostaining optimization for cortical microtubules

We optimized immunostaining via modifying ScaleA2 reagents to clarify cortical microtubule structures in C. intestinalis eggs. The ScaleA2 reagent (4 mol/L urea, 10% glycerol, and 0.1% Triton X-100 in water) is an optical clearing reagent for whole mount specimens (Hama et al. 2011). While, we found that the
immunostaining of cortical microtubules in the fixed Ciona embryo was improved, when the modified ScaleA2 was used as a pretreatment reagent.

The optimal ScaleA2 recipe was determined through testing a series of glycerol and Triton X-100 concentrations (Table 1) using embryos fixed just before the first cleavage. Optimal glycerol concentrations were measured based on microtubule fluorescent intensity. While 10% glycerol diminished overall microtubule staining, 1% glycerol was sufficient for visualizing astral microtubules in the cortical region (Fig. 1). Next, optimal Triton X-100 concentrations were measured based on the number of microtubule bundles observed on three lines at different depths (Fig. 2; lines 1 to 3). In the control embryo, which was not treated with Scale reagents (non-treated), microtubule staining showed three faintly different layers according to the cytoplasmic depth (Fig. 2A). These lines are drawn in the middle of these three layers. While variation in Triton X-100 concentration did not affect microtubule number on lines 1 and 2, higher Triton X-100 concentrations diminished cortical tubulin staining on line 3. Thus, the modified reagent (G1T0) containing 4 mol/L of urea and 1% glycerol was the most efficient at visualizing microtubule structures in the cortical region.

### Table 1. Composition of modified ScaleA2 reagents

| Modified ScaleA2 | Triton X-100 (%) | Glycerol (%) |
|------------------|------------------|--------------|
| Original         | 0.1              | 10.0         |
| G1T0             | 2.0              | 10.0         |
| G1T0.7           | 0.7              | 1.0          |
| G1T2             | 2.0              | 1.0          |
| G1T5             | 5.0              | 1.0          |
| G1T15            | 15.0             | 1.0          |

†Urea, Triton X-100 (w/v), and glycerol (w/v) were dissolved in distilled water.

The effects of original and modified Scale reagents were compared using embryos at 45 mpf (Fig. 4). In non-treated specimens, the microtubule array in the posterior-vegetal cortex was hardly observed, while the sperm aster was evidently observed (Fig. 4A). In contrast, the sperm aster was observed at 45 mpf in non-treated specimens, while the background is lower than that of non-treated specimens (Fig. 4B). In contrast, G1T0 treated embryo showed clear microtubule array on the posterior-vegetal cortex, however, the sperm aster was appeared to be smaller than that in the non-treated control and the original ScaleA2 treated specimens (Fig. 4C).

### Changes in cortical microtubule structures during cytoplasmic and cortical reorganization

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In unfertilized G1T0-treated eggs, short and faint microtubule fragments were observed beneath the cortex (Fig. 5A, G, M, M'). At 15 mpf, the dome-like sperm aster appeared on the vegetal cortex (Figs. 5B, H, N); microtubule fragments accumulated there as well, forming a coarse meshwork to which some astral microtubules connected (Fig. 5N'). At 30 mpf, the now larger and spherical sperm aster had already migrated to the posterior equatorial region, after which the microtubule meshwork also shifted posteriorly (Fig. 5C, I, O, O'). At 45 mpf, obviously visible thick microtubules formed a parallel array beneath the posterior-vegetal cortex (Fig. 5D, J, P, P') that covered nearly the entire region. By this point, the sperm aster had shifted toward the egg center. Although previous reports have suggested that astral microtubules and cortical tubulin accumulation form a continuous structure (Chiba et al. 1999; Roegiers et al. 1999; Ishii et al. 2014). In contrast, when the specimens are treated with G1T0, we observed thick microtubule bundles positioned in parallel arrays to the mid-plane arc (Fig. 3B, D, F).

The effects of original and modified Scale reagents were compared using embryos at 45 mpf (Fig. 4). In non-treated specimens, the microtubule array in the posterior-vegetal cortex was hardly observed, while the sperm aster was evidently observed (Fig. 4A). In the specimens treated with original ScaleA2, overall microtubule staining was similar to the non-treated specimen, while the background is lower than that of non-treated specimen (Fig. 4B). In contrast, G1T0 treated embryo showed clear microtubule array on the posterior-vegetal cortex, however, the sperm aster was appeared to be smaller than that in the non-treated control and the original ScaleA2 treated specimens (Fig. 4C).

### Correlation between myoplasm reorganization and microtubule structures

To understand whether the sperm aster or the CAMP microtubule system is involved in myoplasm
Fig. 1. Evaluation of glycerol concentration in modified ScaleA2 reagents. Ciona embryos fixed at 60 min post-fertilization (mpf) were treated with modified ScaleA2 reagents (Table 1) and immunostained for microtubule visualization. (A, B) Horizontal optical sections at the equatorial region of each specimen were compared. Photographs were taken at the same exposure. Scale bar, 50 µm. (A’, B’) Enlargement of the indicated region (white rectangle) in A and B, respectively. Scale bar, 20 µm. (C) Tubulin staining intensity was evaluated. Average fluorescent intensities of 100-pixel widths along the indicated arrows were calculated. These graphs represent the fluorescent intensity relative to the centrosome region (basal point of arrows). G10T2 showed faint microtubule staining, whereas G1T2 staining was stronger. (—, G1T2; —, G10T2).

Fig. 2. Evaluation of Triton X-100 concentration in modified ScaleA2 reagents. Ciona embryos fixed at 60 mpf were treated with modified ScaleA2 reagents (Table 1) and immunostained for microtubule visualization. (A–F) Horizontal optical sections at the equatorial region of each specimen were compared. Control embryos were not treated with any Scale reagents. All photographs were taken with the same exposure. Scale bar, 50 µm. (A’–F’) Enlargement of the indicated region (white rectangle) in A–F. Scale bar, 20 µm. (G) Comparison of microtubule bundles detected along the indicated red lines. The G1T0 reagent caused a significant increase in the number of microtubule bundles in the outermost region (*P < 0.001). Error bars represent SDs (n = 6). (—) Control; G1T0; G1T0.7; G1T2; G1T5; G1T15.
movement, we double-stained microtubules and myoplasm in G1T0-treated specimens. The myoplasm of the unfertilized egg was localized only in the outermost layer of the cortical cytoplasm (except at the animal pole), whereas the microtubule meshwork spread deeper in the peripheral cytoplasm (Fig. 6A, G, M). At 15 mpf, the microtubule meshwork was restricted to the vegetal pole, while the myoplasm was more widely distributed on the vegetal cortex (Fig. 6B, H, N), with the sperm aster nearby. At 30 mpf, the myoplasm remained at the vegetal hemisphere, but the sperm aster had separated from the myoplasm and moved to the equatorial region (Fig. 6C, I, O). At 45 mpf, CAMP formed from densely aligned cortical microtubules, covering nearly the entire cortex of the myoplasm (except the equatorial region), which had moved to the posterior-vegetal region (Fig. 6D, J, P) while the sperm aster had begun moving toward the egg center. At 50 mpf, the sperm aster was localized to the egg center and CAMP had shrunk into the posterior pole within the myoplasm, which moved posteriorly to form a crescent-like cytoplasmic domain (Fig. 6E, K, Q). Mitosis began at 60 mpf; myoplasm rearrangement in the posterior region was complete, and CAMP was no longer detectable (Fig. 6F, L, R). The appearance and shrinkage of CAMP around 45 mpf at the posterior-vegetal cortex coincided with the second phase of reorganization.

When we analyzed the relationship between myoplasm and microtubule structures through mapping their relative positions (see Materials and Methods), we found that the sperm aster was positioned far ahead of the myoplasm during 30–50 mpf (Fig. 6S). Furthermore, myoplasm movement was accompanied by CAMP shrinkage from 45 mpf to 50 mpf. This correlation was confirmed with similarities in the angle trajectories between the A–V axis and the lines from the egg center to the myoplasm center or the CAMP midpoint (Fig. 6T). Overall, the data suggest that CAMP shrinkage during 45–50 mpf contributes to myoplasm movement. However, the CAMP shrinkage solely might not drive the movement of the subcortical myoplasm to form a crescent like cytoplasmic domain. Although we did not observe direct contact between sperm astral microtubules and CAMP, we feel confident in proposing that the direction of sperm-aster movement and subsequent CAMP formation defines the A–P axis.

Myoplasm reorganization failed in CAMP-disturbed eggs

Previously, we reported that NaN₃ treatment during the second phase of reorganization disturbed cortical tubulin accumulation but not astral microtubule structure (Ishii et al. 2014), suggesting a specific inhibition of CAMP formation. So we visualized the effects of NaN₃ on the CAMP formation by immunostaining using G1T0 (Fig. 7). The results of NaN₃ treatment on embryos indicated that CAMP was abolished, but the sperm aster remained in the egg center (Fig. 7B). Additionally, the myoplasm did not move to the posterior region and the A–P axis was not observed (Fig. 7B; Ishii et al. 2014). On the other hand, the sperm aster was appeared to be smaller than that in our previous study (Ishii et al. 2014), because the G1T0 treated embryo has small sperm aster compared to the untreated embryo as shown in Figure 4.

Discussion

In this study, we optimized microtubule immunostaining using a modified ScaleA2 reagent and described
the presence of CAMP during the second phase of reorganization in the ascidian egg. While ScaleA2 was typically used as an optical clearing reagent (Hama et al. 2011), our modified scale reagent was used for the pretreatment of immunostaining and successfully improved the immunostaining of thick microtubule
structures on the cell cortex. The G1T0 reagent was very simple to formulate and effective in *Ciona*; thus, it might be useful for clarifying cortical microtubule structures in eggs and embryos of other species.

Previous researchers had considered that sperm-aster microtubules were responsible for myoplasm movement during the second phase of ascidian-egg reorganization, because it was the only microtubule structure thought to be present during that period (Sawada & Schatten 1988; Sardet et al. 1989, 2007). However, in this study, we successfully visualized CAMP, demonstrating that its movements were coordinated with those of the myoplasm. In addition, we showed that CAMP is responsible for myoplasm movement, as abolishing the former arrested the latter. Moreover, similar to the sperm aster movement, CAMP is likely also essential for determining the posterior pole because the second phase establishes the A–P axis.

Fig. 6. Correlations between microtubule structures and myoplasm reorganization. *Ciona* eggs during the first cell cycle were double-stained for microtubules (green) and myoplasm (red) using G1T0. In all photographs, the animal pole faces upward. In mid-plane optical sections and 3D-model side views, the posterior pole is on the right. In the unfertilized egg (radial symmetry) and the 15-mpf embryo (posterior side unclear), corresponding views are simply perpendicular to the tentatively assigned posterior view. (A, G, M) Unfertilized egg. (B, H, N) 15 mpf. (C, I, O) 30 mpf. (D, J, P) 45 mpf. (E, K, Q) 50 mpf. (F, L, R) 60 mpf. (A–F) Mid-plane optical sections. (G–L) Side views. (M–R) Posterior views. Arrowheads indicate sperm asters. Scale bar, 50 µm. (S, T) Analysis of sperm aster, myoplasm, and microtubule movements from 30 to 50 mpf. Plots include the coordinates of the sperm-aster centrosome (♦), myoplasm center of mass (●), and the middle of the cortical microtubule meshwork (*; 30 mpf) and CAMP (▲; 45 mpf, 50 mpf). Error bars represent SDs (n = 3). (S) Vertical and horizontal axes represent animal-vegetal and anteroposterior axes, respectively. The origin of the coordinate axes is the egg center, and the egg radius is represented as 1.0. (♦, Sperm aster; ●, Myoplasm; *, CAMP). (T) Movement time courses are represented as the angle (+90° to −90°) from the animal-vegetal axis from the animal-vegetal axis (♦, Sperm aster; ●, Myoplasm; ▲, CAMP).
During the second reorganization phase, two types of microtubule-dependent translocations occur in the ascidian Phallusia mammillata (Sardet et al. 1989; Roegers et al. 1999). The first is a slow translocation (25–40 mpf) associated with posterior cortex oscillation accompanying female pronucleus migration. The second is a fast translocation (40–45 mpf) characterized by movement of the duplicated male centrosome, asters, and myoplasm toward the egg center. Given the timing, our results suggest that myoplasm-associated CAMP shrinkage to the posterior pole (at 45 mpf and 50 mpf) may be responsible for fast translocation.

Due to the close association between CAMP and myoplasm movement, the former probably also influences microtubule-dependent relocation of type I postplasmic/PEM RNA to the posterior cortex (Sasakura et al. 2000; Tanaka et al. 2004). These RNAs play pivotal roles in body axis formation and tissue differentiation during embryogenesis (Sardet et al. 2003; Prodon et al. 2007; Makabe & Nishida 2012). Additionally, CAMP may also affect the posterior transport of cortical endoplasmic reticulum (cER)-mRNA domains. These structures form from the anchoring of some type I postplasmic/PEM RNA (e.g. macho-1) to the cER (Sardet et al. 2003). Thus, studies of the interaction between the CAMP and type I postplasmic/PEM RNA or the cER should increase current understanding of mRNA transport mechanisms during the second phase of reorganization.

Although novel to ascidian eggs, similar microtubule structures to CAMP have been observed in other animals. For example, parallel arrays of microtubule bundles appear in the vegetal cortex of fertilized zebrafish eggs (Jesuthasan & Strähle 1997; Tran et al. 2012). This cortical microtubule array is responsible for maternal mRNA (e.g. maternal dorsalizing factor, wnt8a) transport (Tran et al. 2012; Ge et al. 2014). Microtubule array formation is mediated by Ca2+ signaling, but independent of sperm entry and fertilization (Tran et al. 2012). In fertilized frog eggs, a parallel microtubule array forms beneath the vegetal cortex during cortical rotation (a form of cytoplasmic reorganization) (Elinson & Rowning 1988; Houliston & Elinson 1992; Elinson & Ninomiya 2003), without requiring the presence of a sperm aster (Elinson & Rowning 1988). Microtubule plus ends indicate the future dorsal side, and a dorsal determinant candidate (disheveled protein) was reported to be translocated dorsally along the array (Miller et al. 1999; Weaver & Kimelman 2004). Including the newly reported CAMP in ascidian eggs, these arrays are similar to each other in the following aspects: (i) localized to the vegetal cortex, (ii) comprise parallel bundles extending along the future body axis, and (iii) contribute to maternal factor transport. These similarities strongly support the notion that the cortical microtubule array is a conserved structure in early development, functioning to relocate maternal factors for future body axis determination. Therefore, cross-chordate comparisons of the microtubule array formations will contribute to our knowledge of conserved mechanisms in cytoplasm reorganization and body axis formation during embryogenesis.

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Author contribution

TN conceived and designed the study, and assisted in manuscript preparation. HI and TG performed the experiments and wrote the manuscript draft. All authors contributed to data analysis and interpretation, and critically reviewed the manuscript. The final version of the manuscript was approved by all authors.

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