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Expession patterns of mesenchyme specification genes in two distantly related echinoids, *Glyptocidaris crenularis* and *Echinocardium cordatum*

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ABSTRACT

The molecular mechanism of the larval mesenchyme cell specification in echinoids has been well analyzed. However, most of the data have been provided by studies of a single group of echinoids, the order Camarodonta. Little is known about this mechanism in other echinoid orders. We examined the expression patterns of mesenchyme specification genes, *micro1*, *hesC*, *alx1*, *tbr*, *ets1*, *cyp1*, and *gcm*, in the two non-Camarodonta echinoids, *Glyptocidaris crenularis* and *Echinocardium cordatum*. We found that the expression patterns of some genes contained characteristics that were unique to one of the species; others were shared by the two species. Some of the shared characteristics of *G. crenularis* and *E. cordatum* are not found in the species belonging to Camarodonta, suggesting the derived status of this order. The expression of *ets1* in *E. cordatum* aboral ectoderm is one of the molecular level modifications possibly related to an evolutionarily novel larval structure, the posterior process. Our results suggest that a considerable number of modifications in the mesenchyme specification mechanisms have been introduced during the echinoid evolution.

The mechanism of mesenchyme specification of echinoids has been actively studied from both embryological and evolutionary point of view (Ettensohn, 2013; McClay, 2011). However, most of these studies are exclusively performed on the species belonging to Camarodonta, one of the three groups in Echinacea (Fig. 1). The Camarodonta-biased information is not sufficient for understanding the evolution of echinoid mesenchyme specification. It is clear that comparative studies of echinoids other than Camarodonta are necessary.

While the data on the ontogeny of Camarodonta larval skeletogenic mesenchyme cells have been recently accumulating, the comparative studies covering all echinoids are still scarce. In the derived echinoid Acroechinoidea, larval skeletons are formed by the primary mesenchyme cells (PMCs) that are descendants of the micromeres at 16-cell stage (Okazaki, 1975). The double-negative gate (DNG), which has been originally discovered in Camarodonta *Strongylocentrotus purpuratus*, is a subnetwork for the micromere-PMC lineage specification found in both Camarodonta and Clypeasteroida (Oliveri et al., 2002, 2008; Revilla-i-Domingo et al., 2007; Yamazaki et al., 2010). However, in the primitive echinoid Cidaroida, typical micromere formation, PMC differentiation, and DNG have not been found (Schroeder, 1981; Wray and McClay, 1988; Yamazaki et al., 2012, 2014). Therefore, it has been suggested that the DNG had been introduced into the Euechinoidea lineage after the divergence from the Cidaroida. The detailed timing of DNG introduction and diversification is still obscure.

The comparative studies of the non-skeletogenic mesenchyme specification are also limited. There are some variations in the pigment cell differentiation in Camarodonta and Irregularia, suggesting that the specification mechanism of non-skeletogenic mesenchyme cells has also undergone evolutionary modifications (Kominami et al., 2001). The expression patterns of *ets1*, a gene related to blastocoelar-cell specification, are different in Camarodonta and Irregularia (Yamazaki et al., 2010). An analysis of the expression patterns of *gcm*, a regulatory gene responsible for pigment cell specification, is indispensable to determine the mechanical differences between the pigment cell specification in Camarodonta and other echinoids. The expression pattern of *gcm* has been described for Camarodonta and Cidaroida (Ransick and Davidson, 2006; Ransick et al., 2002; Yamazaki et al., 2014), but there are no reports for other echinoid orders.

Comparative studies of various diverged lineages of echinoids are necessary to understand the evolution of mesenchyme specification mechanisms. We selected *Glyptocidaris crenularis* and *Echinocardium cordatum* as key species for such evolutionary studies. *G. crenularis* belongs to Stomopneustoida, one of the three groups (Arbacioida, Camarodonta, and Stomopneustoida) of Echinacea.

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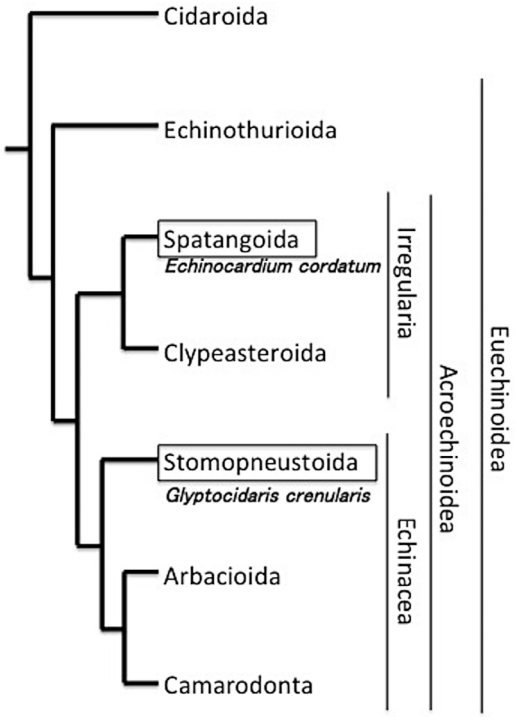

Fig. 1. Schematic drawing of echinoid phylogenic tree consisting of seven orders, based on Kroh and Smith (2010). Boxes represent the orders studied in the present study. Several orders, including Diadematacea, are omitted from the tree. The names of the species examined (*Glyptocidaris crenularis* and *Echinocardium cordatum*), the cohorts (Irregularia and Echinacea), the infraclass (Acroechinoidea), and the subclass (Euechinoidea) are provided.

Stomopneustoida are considered as the basal group of Echinacea and were separated from the common ancestor of Arbacioida and Camarodonta (Kroh and Smith, 2010). Because of its phylogenic position, Stomopneustoida sea urchins are important for understanding the ancestral mechanism of mesenchyme specification in Echinacea.

The order of Spatangoida (heart urchins) is another lineage important in the studies of the evolutionary changes, especially for analyzing the newly acquired structures and their evolutionary origins. Spatangoid urchins commonly form the posterior process, a larval arm-like unpaired structure in the aboral apex, which does not exist in Clypeasteroida and Echinacea (Emlet et al., 2002). Little is known about the molecular mechanism of the posterior process formation. Studies of Spatangoida will help to clarify the evolutionary changes responsible for such newly acquired structures. The results should shed some new light on the ancestral specification mechanism of the Irregularia and the evolutionary modifications after the divergence of Spatangoida and Clypeasteroida in the Lower Jurassic epoch (Smith, 1984).

The purpose of the present study was to examine the expression patterns of mesenchyme specification genes in the two species, *G. crenularis* and *E. cordatum*. Our results suggest a substantial variation in mesenchyme specification mechanism in echinoids. The comparative analysis showed that the DNG functions in *Glyptocidaris*, *Echinocardium*, and other echinoids including Clypeasteroida and Camarodonta. However, we found that the regulatory relationship between *hesC* and the downstream genes was only partially conserved. The expression patterns of *tbr*, *ets1*, *cyp1*, and *gcm* in *Glyptocidaris* and *Echinocardium* were different from those of Camarodonta orthologs, suggesting that the Camarodonta are particularly specialized echinoids. Furthermore, we reported *Echinocardium*-characteristic expression patterns that might be related to the posterior process formation. Our results suggest that the posterior process was formed by means of a co-option of larval-arm formation mechanism. We also discuss a possible scenario for the evolution of mesenchyme specification mechanism in echinoids.

1. Results

1.1. *Expression patterns of mesenchyme specification genes in* G. crenularis

1.1.1. *Ortholog of* micro1

We could not isolate the ortholog of *micro1/pmar1* from this species.

1.1.2. GchesC, *the* hesC *ortholog in* G. crenularis

The QPCR results showed a trace amount of *GchesC* transcript in the embryo until 10 hours post-fertilization (hpf) at around 6th cleavage (60 cells, Fig. 2A7). Whole-mount *in situ* hybridization (WMISH) experiments did not detect the mRNA until 10 hpf. Newly synthesized mRNA of *GchesC* was found at 14 hpf (200 cells, Fig. 2A1, A7). During the blastula stages (18 hpf to 28 hpf), the expression of *GchesC* mRNA was observed in the whole vegetal hemisphere (Fig. 2A2, A3), except for a doughnut-like region, which presumably consists of the large micromere descendants (inset Fig. 2A2). The expression in small micromeres was substantial (an asterisk in Fig. 2A2 and the inset) and a faint signal was detected in the animal hemisphere. At the mesenchyme blastula stage, the spatial expression pattern became complicated (Fig. 2A4); the whole vegetal hemisphere, except for the vegetal pole region, showed a strong signal. The PMCs also expressed *GchesC* mRNA. The animal hemisphere, however, showed a patchy, relatively weak expression. In the gastrula, the ectodermal region expressed *GchesC* weakly (Fig. 2A5). The expression in the animal pole region was stronger than in the other ectodermal regions. Weak expression was also observed in a part of the archenteron and in mesenchyme cells. In the pluteus larva, *GchesC* expression was observed in the oral lobe, a part of the digestive tract, and the coelomic pouches (Fig. 2A6).

1.1.3. Gcalx1, *the* alx1 *ortholog in* G. crenularis

The QPCR results showed a trace amount of *Gcalx1* transcript in the embryo until 14 hpf (200 cells, Fig. 2B7). WMISH experiments did not detect *Gcalx1* mRNA before 14 hpf in the majority of the examined embryos (Fig. 2B1). Small number of 14 hpf embryos (3/21) showed a patch of expression in the vegetal pole region. At 18 hpf (650 cells), all the embryos showed *Gcalx1* expression in the vegetal hemisphere. This spatially restricted expression continued during the blastula stages (18 hpf–28 hpf, Fig. 2B2, B3). The cells in the center of the expression patch, presumably small-micromere descendants, showed weaker expression of *Gcalx1* than the surrounding region (inset Fig. 2B3). The PMCs continued to express *Gcalx1* during the stages between mesenchyme blastula (Fig. 2B4) and gastrula (Fig. 2B5). At the pluteus stage (120 hpf), the expression of *Gcalx1* was not observed in the larval skeletogenic mesenchyme cells, but it was detected in a part of the coelomic pouches (Fig. 2B6).

1.1.4. Gctbr, *the* tbr *ortholog in* G. crenularis

Both QPCR and WMISH results indicated that there was a maternal *Gctbr* transcript that ubiquitously distributed in the embryo until 14 hpf (200 cells, Fig. 2C1, C7). The spatially restricted expression started at around 18 hpf (650 cells). In the early blastula (20–24 hpf), the expression of *Gctbr* was observed in a solid circular region, probably corresponding to the micromere descendants (Fig. 2C2). At the hatching blastula stage (28 hpf), the expression in the vegetal regions was still visible, but the area of the expression became wider (Fig. 2C3). In the mesenchyme blastula, the expression was observed in both the PMCs and the vegetal plate region