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Mesodermal Cell Differentiation in Echinoid Embryos Derived from the Animal Cap Recombined with a Quartet of Micromeres

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ABSTRACT—Mesodermal cell differentiation in echinoid embryos derived from the animal cap recombined with micromeres was examined. An animal cap consisting of mesomere-descendants was isolated from a 32-cell stage embryo, and recombined with a quartet of micromeres isolated from a 16-cell stage embryo. The recombined embryos were completely depleted of the progenitors of an archenteron, pigment cells, blastocoelar cells and muscle cells. Secondary mesenchyme-like cells (induced SMC) were released from the archenteron derived from the animal cap cells in the recombined embryos. Some induced SMC differentiated into pigment cells, confirming previous data for another echinoid species. Moreover, three different kinds of mesodermal cells –blastocoelar, muscle and coelomic pouch cells– were formed in the recombined larvae. Experiments using a fluorescent probe confirmed that the pigment, blastocoelar, muscle cells and cells in part of the coelomic pouches in the recombined larvae were derived from the animal cap mesomeres. These results indicated that the animal cap mesomere had the potential to differentiate through cell fate regulation into four mesodermal cell types –pigment, blastocoelar, muscle and coelomic pouch cells–.

INTRODUCTION

The 16-cell stage embryo of echinoids consists of 8 mesomeres (animal cap), 4 macromeres, and 4 micromeres. In normal embryos, the developmental fate of animal cap mesomeres is restricted to ectoderm (Cameron and Davidson, 1991). The micromeres give rise to two different cell types: skeletogenic mesenchyme cells and coelomic pouch constituents (Okazaki, 1975; Katow and Solursh, 1980; Pehrson and Cohen, 1986; Tanaka and Dan, 1990; Ettensohn and Ruffins, 1993). The macromeres differentiate into ectoderm, endodermal gut, and mesodermal secondary mesenchyme cells (SMC), from which four different cell types –pigment, blastocoelar, muscle and coelomic pouch cells– are formed (Cameron *et al*., 1991; Ettensohn and Ruffins, 1993).

Embryos derived from the animal cap recombined with micromeres have the potential to differentiate the pigment cells which normally originate from the SMC derived from macromeres (Amemiya, 1996). Thus, recombined embryos devoid of macromeres have the potential to differentiate at least one mesodermal cell type which is derived from the macromeres during normal development. However, it remains to be clarified whether recombined embryos devoid of macromeres have the potential to differentiate the three other mesodermal cell types which are all derived from the macromeres in undisturbed embryos.

In the present study, the developmental potential of animal cap mesomeres recombined with micromeres to differentiate into various mesodermal cell types was examined. The results indicated that such recombined embryos had the potential to differentiate into all mesodermal cell types which were differentiated from SMC in normal embryos, confirming that cell fate in the recombined embryos was completely regulated.

MATERIALS AND METHODS

Animals and embryos

Adults of the sand dollar *Scaphechinus mirabilis* were provided by Asamushi Marine Biological Station and Ushimado Marine Laboratory. The animals were induced to shed gametes by intracoelomic injection of 0.1 M acetylcholine chloride. The eggs were washed several times with artificial seawater (ASW, Jamarin-U, Jamarin Laboratory, Osaka), transferred to ASW containing 1 mM aminotriazole (ATA) to prevent hardening of the fertilization envelopes (Showman and Foerder, 1979), then fertilized with a diluted suspension of sperm. The fertilization envelopes were removed by pipetting the egg suspension in a test tube with a fine-bore pipet. The denuded eggs were cultured at about 18°C.

Manipulation to produce recombined embryos

The recombined embryos were produced according to the method of Amemiya (1996) with some modifications. The fertilized eggs were separated into two groups immediately after removal of the fertilization envelopes. One group was cultured in normal ASW, and the other

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in ASW containing 50 μg/ml rhodamine B isothiocyanate (RITC: R-1755, Sigma) from the 4- to 8-cell stage. At the early 16-cell stage, an embryo labeled with RITC was transferred to another dish filled with calcium-free seawater (CFSW). The embryo was dissected by hand using a fine glass needle to isolate a quartet of micromeres. On the other hand, an unlabeled embryo was transferred at the 32-cell stage from ASW to CFSW, and then dissected by hand through the equatorial plane to isolate an animal cap consisting of 16 sister blastomeres of mesomeres. At this stage, each micromere in the isolated quartets had undergone a division to produce a large micromere and a small micromere. Each unlabeled animal cap and each descendants of the quartet of rhodaminated micromeres were transferred to another petri dish coated with 1.2% agar and filled with ASW supplemented with 100 units/ml penicillin and 50 μg/ml streptomycin sulfate. In the petri dish, an animal cap was recombined with a quartet of rhodaminated micromere-descendants by moving the animal cap with a glass needle onto the micromere-descendants lying on the agar. These recombined embryos were cultured in the same dish under dark conditions.

Staining with rhodamine-phalloidin

The larvae were fixed with 70% ethanol for about 30-60 min at –20°C, washed with phosphate-buffered saline (PBS) and stained with PBS containing 0.5 mg rhodamine-phalloidin (R-415, Molecular Probes) per ml for 30 min under dark conditions. The stained larvae were washed with PBS several times and examined with a fluorescence microscope (Nikon Optiphot).

RESULTS

In most recombined embryos, ingression of primary mesenchyme cell (PMC) was completed at 11-12 hr after fertilization, and spicule formation started at 18 hr. The period of PMC ingression in the recombined embryos was almost the same as that in normal embryos, but gastrulation of the embryos was delayed in comparison with that of normal embryos. At the gastrula stage, some secondary mesenchyme-like cells (induced SMC) were released from the vicinity of the archenteron tip. Most of the recombined embryos developed into pluteus larvae whose morphology was apparently similar to that of undisturbed embryos, confirming the previous reports (Amemiya, 1996; Minokawa *et al*., 1997). The skeletogenic mesenchyme cells and a proportion of the coelomic pouch constituents in most recombined larvae were labeled with RITC (Fig. 1), indicating that they were derived from the micromeres, whereas the other cells were derived from the mesomeres. In some recombined larvae, the coelomic pouch cells were unlabeled with RITC, whereas the skeletogenic mesenchyme cells were labeled, suggesting that the small micromere-descendants were not incorporated into the larvae.

The pigment cells in echinoid larvae are characterized by the presence of pigment granules (Cameron *et al*., 1991; Ettensohn, 1992; Ettensohn and Ruffins, 1993). The pigment cells in the normal pluteus of *S. mirabilis* were elongated and branched, containing the pigment granules, like those reported for other species (Cameron *et al*., 1991). Some cells morphologically identical to the pigment cells in normal larvae were found in the recombined larvae (Fig. 2A, Table 1). These cells possessed pigment granules and were not labeled with RITC (Fig. 2A, B), indicating that the cells were derived from mesomeres. The number of these cells per a recombined larva

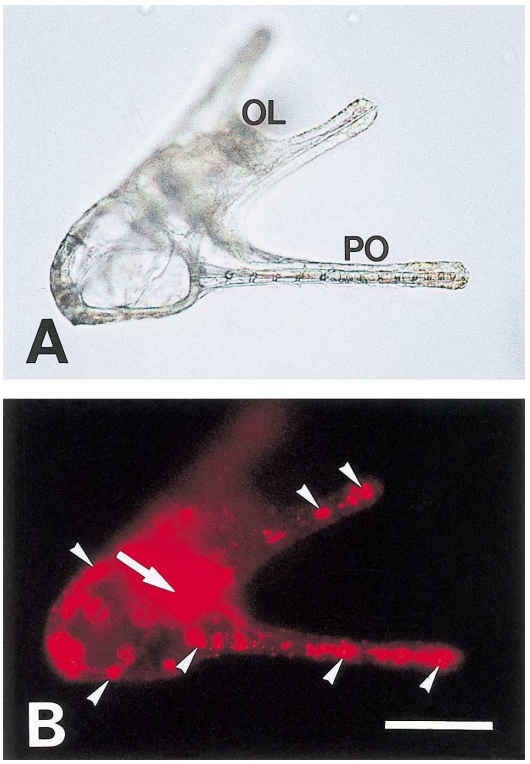

Fig. 1. A four-armed *S. mirabilis* pluteus derived from an animal cap recombined with a quartet of rhodaminated micromeres. The skeletogenic mesenchyme cells (arrowheads) and the coelomic pouch constituents (arrow) were labeled with RITC. (**A**) Light field observation. (**B**) Epifluorescence observation. PO, post-oral arm; OL, oral lobe. Bar represents 50 μm.

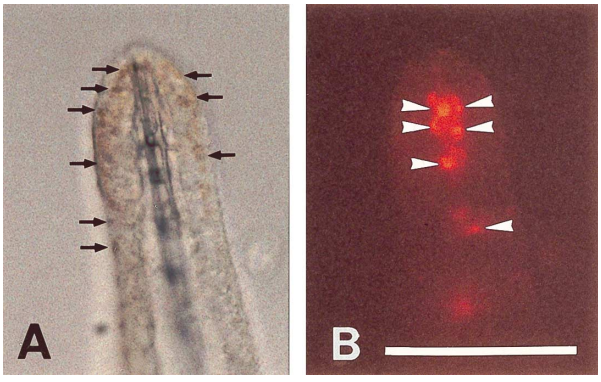

Fig. 2. Pigment cells in a tip of the post-oral arm of the larva derived from an animal cap recombined with a quartet of micromeres. The pigment cells (arrows in **A**) in the ectodermal wall are not labeled with RITC, whereas the skeletogenic mesenchyme cells (arrowheads in **B**) were labeled. (**A**) Light field observation. (**B**) Epifluorescence observation. Bar represents 50 μm.

seemed to be varied. A few recombined larvae did not form pigment cells, although most recombined and all normal ones did so (Table 1). Generally, the recombined larvae formed fewer pigment cells than the normal larvae.

The blastocoelar cells of echinoids are characterized by a fibroblast-like phenotype (Cameron *et al*., 1991; Ettensohn, 1992; Tamboline and Burke, 1992; Ettensohn and Ruffins,

Table 1. The potential of recombined and undisturbed larvae for differentiation into mesodermal cell types derived from SMC

cell types examined	types of larvae	No. examined	No. positive (%)
pigment cells	recombined	44	38 (86)
	undisturbed	66	66 (100)
blastocoelar cells	recombined	19	17 (89)
	undisturbed	66	66 (100)
muscle cells	recombined	13	12 (92)
	undisturbed	24	24 (100)
coelomic pouch cells	recombined	19	18 (95)
	undisturbed	66	66 (100)

The larvae were examined between 48 and 72 hr after insemination for the differentiation of pigment, blastocoelar and coelomic pouch cells. The muscle cells were examined at 72 hr after insemination, because muscle cells differentiate somewhat later than the other cell types.

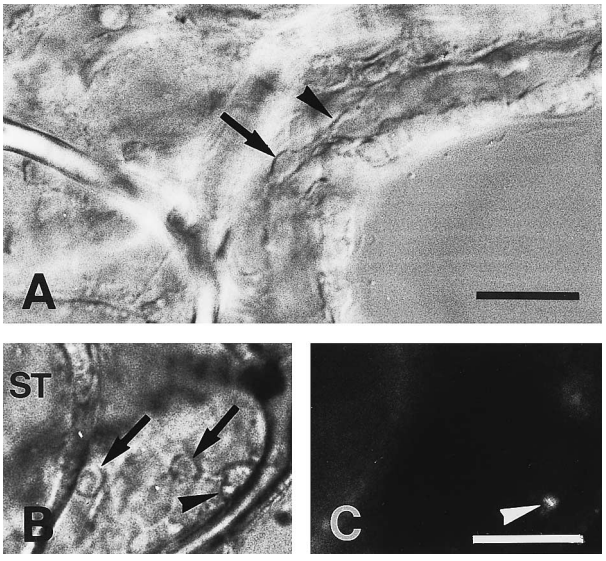

Fig. 3. Blastocoelar cells in a larva derived from an animal cap recombined with a quartet of micromeres. (**A**) Observation using differential interference contrast (DIC) optics. A filopodium (arrowhead) extends from a blastocoelar cell (arrow). (**B**, **C**) Light (**B**) and epifluorescence (**C**) observation of blastocoelar cells. Two blastocoelar cells (arrows in **B**) were not labeled with RITC (**C**), whereas the skeletogenic mesenchyme cell (arrowhead in **B**) was labeled (arrowhead in **C**). ST, stomach. Bars represent 20 μm.

1993). These cells were morphologically distinct from PMC, which have a spherical body and possess thin filopodia. Cells with the fibroblast-like phenotype were found in all normal and most of the recombined larvae examined using differential interference contrast optics (Fig. 3A, Table 1). The blastocoelar cells in the recombined larvae were not rhodaminated (Fig. 3B, C), indicating that the cells were derived from the animal cap mesomeres.

The circumesophageal muscle of echinoid larvae consists of contractile strands containing actin filaments and tropomyosin (Ishimoda-Takagi *et al*., 1984; Ettensohn and Ruffins, 1993). Examination of the muscle was performed at 72 hr after insemination, because this tissue differentiates somewhat later than other cell types derived from SMC (Ettensohn and Ruffins, 1993). The bands of actin filaments composing the muscle in the normal pluteus of *S. mirabilis* were stained with rhodamine-phalloidin (Fig. 4D), as in those of other echinoid species reported previously (Harris, 1986). Muscle stained with rhodamine-phalloidin was found in all but one (12 out of 13 larvae) of the recombined larvae (Fig. 4B, Table 1). There were considerably fewer actin bands in the muscle around the esophagus of the recombined pluteus than in undisturbed larvae (Fig. 4B, D), reflecting the small size of the muscle in the recombined larvae. The observation using epifluorescence microscope demonstrated that muscle cells in the recombined larvae without rhodamine-phalloidin staining were not rhodaminated (Fig. 4E, F), indicating that the cells were derived from animal cap.

The coelomic pouches are structures located at both sides of the esophagus in the echinoid pluteus (Gustafson and Wolpert, 1963; Cameron *et al*., 1991). In the normal pluteus of *S. mirabilis*, the coelomic pouches were formed as clumps of cells (Fig. 5A). Coelomic pouches morphologically identical to those in the normal larvae were found in most of the recombined larvae (Figs. 4E and 5B, Table 1). However, the coelomic pouches along the larval axis in the recombined larvae were shorter than those in the undisturbed plutei. In normal larvae, the coelomic pouches are formed by cells derived from the small micromeres and SMC. The coelomic pouches in larvae derived from the animal cap and rhodaminated micromeres appeared to be composed of rhodaminated and non-rhodaminated cells (Fig. 4E, F). This suggested that the pouches in the recombined larvae were formed by cells descended from the small micromeres and SMC derived from the animal cap mesomeres.

DISCUSSION

Classic studies have indicated that an embryo derived from an animal cap recombined with a quartet of micromeres has the potential to form an archenteron and to develop into an apparently normal pluteus (Hörstadius, 1973). Recently,

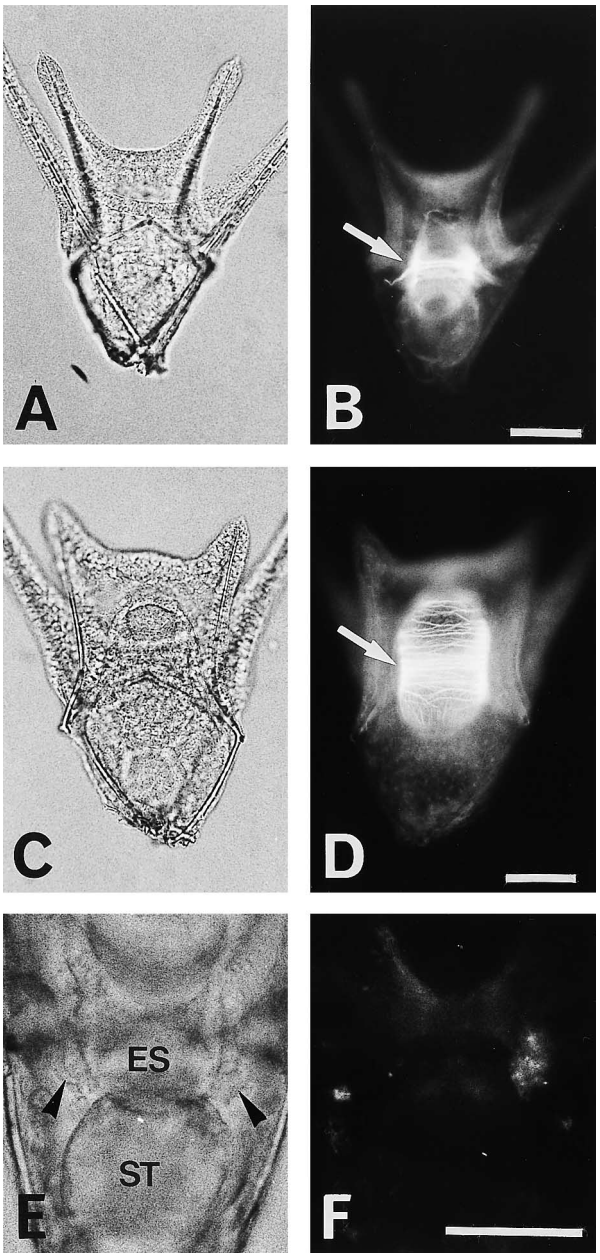

Fig. 4. Observation of pluteus larvae stained with rhodamine-phalloidin (**A**, **B**, **C**, **D**). (**A**, **B**) A pluteus derived from an animal cap recombined with a quartet of micromeres. (**A**) Light-field observation. (**B**) Epifluorescence observation. (**C**, **D**) A normal pluteus. The muscles (arrows) surrounding the esophagus of a recombined (**B**) and a normal pluteus (**D**) are stained. The muscle in the recombined pluteus is markedly smaller than that in the normal larva. There are fewer actin bands in the muscle of the recombined pluteus than in the normal larva. Light (**E**) and epifluorescence (**F**) observation of the vicinity of the esophagus in the recombined pluteus larva without rhodamine-phalloidin staining. No muscle cells was rhodaminated, indicating that the cells were derived from the animal cap mesomeres. Two coelomic pouches were observed at both sides of the esophagus (arrowheads). One of two coelomic pouches was not containing rhodaminated cells, indicating that the cells in this pouch were exclusively derived from the animal cap mesomeres. ES, esophagus; ST, stomach. Bars represent 50 μm.

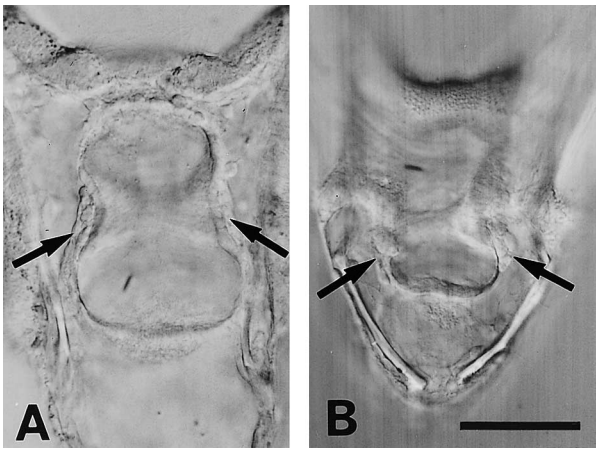

Fig. 5. Coelomic pouch cells in a normal larva (**A**) and a larva derived from an animal cap recombined with a quartet of micromeres (**B**). DIC optics observation. A pair of coelomic pouches (arrows) are formed on both sides of the gut. Bar represents 50 μm.

we reexamined and extended these experiments to investigate further the nature of these recombined embryos. The archenteron in recombined embryos was shown to be derived from the mesomeres in experiments using a fluorescent probe (Amemiya, 1996). A pluteus derived from a recombined embryo had the potential to differentiate pigment cells and to metamorphose into a juvenile (Amemiya, 1996). The induced SMC released from the archenteron tip of the recombined embryos expressed skeletogenic potential when PMC were completely removed from the embryos (Minokawa *et al.*, 1997). These recent studies suggested that the developmental potential of embryos derived from the animal cap recombined with micromeres was qualitatively identical to that of normal embryos.

In the present study, it was shown that recombined embryos devoid of the presumptive SMC territory had the potential to differentiate all mesodermal cell types derived from the SMC in undisturbed embryos. These results confirmed that the developmental potential of recombined embryos was qualitatively identical with that of normal embryos, indicating that the cell fate in the embryos was completely regulated.

Quantitatively, however, the potential for mesodermal differentiation in recombined embryos might not be identical to that of normal embryos. In the previous paper (Amemiya 1996), it was suggested that recombined embryos had fewer pigment cells than normal embryos, and the present study confirmed this. Moreover, it was found that the circumesophageal muscles and the coelomic pouch in recombined larvae were poorly developed, suggesting that these embryos had quantitatively less potential for SMC differentiation than normal embryos.

Three possible reasons for the quantitatively lower potential of recombined embryos can be considered. The first is that the total number of mesodermal cells that differentiate in recombined embryos is lower than that in normal embryos, because the volume of recombined embryos is only about

half that of normal ones.

The second possibility is that the difference in the timing of differentiation between the ectoderm and endo-mesoderm in recombined embryos results in quantitative incompleteness of the developmental potential. The endodermal archenteron in recombined embryos differentiated markedly later than that in normal embryos, because the archenteron originated from the presumptive ectoderm. Consequently, the release of induced SMC from the archenteron tip in the recombined embryos was also delayed. The timing of ectoderm differentiation in the recombined embryos should be normal because the tissue is not of ectopic origin. Thus, the relationship between the timing of differentiation of the ectoderm and endo-mesoderm in the recombined embryos might differ from that in normal ones. If the four different mesodermal cell types differentiated from the induced SMC with normal timing under the influence of the ectoderm, they might have to differentiate before release of the normal number of induced SMC. This might result in a smaller number of the four cell types.

The third possibility is that the numbers of SMC formed by the macromeres and by the animal cap mesomeres under the inductive influence of the micromeres are different. It was suggested in the previous study that macromeres had the potential to differentiate autonomously into SMC, although the timing of macromere differentiation into SMC was delayed when the micromeres were absent (Ransick and Davidson, 1995). On the other hand, the animal cap did not have this potential, even though some mesomere pairs cultured in isolation might have been able to form SMC-descendant cells (Henry *et al*., 1989). The influence of micromeres in normal embryos might accelerate the potential of macromeres to form SMC, resulting in the formation of more SMC in normal than in recombined embryos.

In the previous (Amemiya, 1996; Minokawa *et al*., 1997) and the present studies, it was demonstrated that a signal emanating from micromeres induced endo-mesodermal cells to form from mesomere-descendants. It was also suggested that the micromere signal at an early cleavage stage, i.e. the 16- to 32-cell stage, was important for the differentiation of macromeres into endo-mesoderm at the normal time (Ransick and Davidson, 1995). It is still unclear whether the macromeres in normal embryos and the mesomeres in recombined embryos receive the same signal(s) from the micromeres. If the mesomeres in recombined embryos and the macromeres in normal embryos receive the same signal from the micromeres, then the signal from micromeres at the 16- to 32-cell stage should be important for mesomeres in respecifying their cell fate. We are now trying to determine the exact period during which animal cap mesomeres in recombined embryos receive the micromere signal for differentiation into endo-mesoderm.

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