学位論文

Studies of Endoderm and Mesoderm Induction in Echinoid Embryos

ウニ胚における内胚葉・中胚葉誘導に関する研究

平成9年6月 博士（理学）申請

東京大学大学院理学系研究科
生物科学専攻
美濃川 拓哉

Studies of Endoderm and Mesoderm Induction in Echinoid Embryos

Doctoral Thesis

Submitted to the University of Tokyo

Takuya Minokawa

Department of Biological Sciences, Graduate School of Science

The University of Tokyo

June, 1997

Contents

Contents i

General Introduction 1

Chapter 1:
"Mesodermal cell differentiation in echinoid embryos derived from the animal cap recombined with a quartet of micromeres" 5
- Abstract
- Introduction
- Materials and Methods
- Results
- Discussion

Chapter 2:
"Skeletogenic potential of induced secondary mesenchyme cells derived from the presumptive ectoderm in echinoid embryos" 26
- Abstract
- Introduction
- Materials and Methods
- Results and Discussion

Chapter 3:
"Respecification of cell fate in mesomere-descendants recombined for a limited period with micromere-descendants of echinoid embryos" 40
- Abstract
- Introduction
- Materials and Methods
- Results
- Discussion

Conclusions and Perspectives 81

Acknowledgements 85

References 87

General Introduction

One of the most essential problems in developmental biology is the way in which cell fate in embryos is specified during development. Previous studies using various kinds of animals have shown that both cytoplasmic factors and interactions between cells are important for this process (asteroids: Kiyomoto and Shirai, 1993a, 1993b; Kuraishi and Osanai, 1994; ascidians: Nishida and Satoh, 1989; Nakatani and Nishida, 1994; amphibians: Grainger, 1992; Yuge et al., 1990). In echinoids, similar to the situation in other animals, two factors have been considered important for cell fate specification (Wilt, 1987; Davidson, 1989).

Classical experiments on the development of echinoid embryos have suggested that micromeres have the potential to induce an archenteron in cells of the presumptive ectoderm. Micromeres implanted onto the animal pole of embryos at the early cleavage stage expressed the potential to induce the secondary archenteron (Hörstadius, 1935, 1973). Each animal cap recombined with a quartet of micromeres differentiated into an apparently normal pluteus with a gut (Hörstadius, 1935, 1973).

Davidson (1989) proposed a model to explain the cell fate specification of sea urchin embryos in terms of the inductive potential of micromeres suggested by the classical studies. According to this model, the micromeres and their descendants produce inductive ligands at early

cleavage stages, which become bound to the membrane surface of the micromeres in order to transmit the signal inducing neighboring cells to differentiate endo-mesoderm. This hypothesis maintains that all blastomeres except micromeres in the 16-cell stage embryo have the competence to react with the induction of micromeres. In normal embryos, macromeres are primarily specified to become endo-mesoderm, because the blastomeres make direct contact with the micromeres. The cell fate of the animal cap mesomeres is restricted to ectoderm, and the cells never express their endo-mesodermal fate during normal development. However, if the animal cap mesomeres are recombined directly with the micromeres, the animal cap respecifies their cell fate to form a small embryo with components essentially identical to those of undisturbed embryos.

Recently, several studies to examine the model have been conducted in various laboratories. Ransick and Davidson (1995) removed four micromeres or their descendants at the early (16- to 64-cell) cleavage stage to study the signaling potential of the micromeres. Gene expression of *Endo16*, an endoderm-specific marker, was examined to assess the degree of differentiation of presumptive endoderm in embryos devoid of micromeres. The results indicated that interaction between micromeres and macromeres during early cleavage was necessary for normal vegetal plate specification. The potential of embryos with micromeres implanted on the animal pole at the early cleavage stage to form a secondary archenteron has been confirmed

molecularly as well as morphologically (Ransick and Davidson, 1993). The developmental potential of the embryos derived from an animal cap recombined with a quartet of micromeres was almost identical to that of undisturbed embryos, since they metamorphosed into juveniles with pentaradiate symmetry (Amemiya, 1996). These results appeared essentially to support Davidson's model. However, some important factors essential for the model, such as the nature of the inductive ligands and their receptors, and the exact period during which micromeres transmitted their inductive signal, remained to be resolved.

The inductive interaction between micromeres and macromeres has been difficult to analyze accurately, because macromeres removed from micromeres at the 16-cell stage have the potential to differentiate into endoderm (Hörstadius, 1935; Yamaguchi et al., 1994). In contrast, the animal cap mesomeres of undisturbed embryos or those cultured in isolation exclusively differentiate into ectoderm, whereas they express the potential to differentiate endoderm when recombined with micromeres (Hörstadius, 1973; Khaner and Wilt, 1991; Ransick and Davidson, 1993; Amemiya, 1996). Therefore, embryos composed of the animal cap recombined with micromeres appear to be the ideal experimental system for analyzing the inductive interaction between micromeres and other blastomeres.

In the present study, the exact period during which micromeres express the inductive signal, and the period during which the animal cap

possesses the competence to react with the signal were examined using embryos derived from an animal cap recombined with micromeres. Moreover, the developmental potential of the animal cap subjected to the inductive influence of micromeres for a limited period was also examined using the same experimental system.

First, animal caps recombined with micromeres were examined for their potential to differentiate all types of descendants of secondary mesenchyme cell (SMC) (Chapter 1). Then, the induced SMC which differentiated in embryos derived from an animal cap recombined with micromeres were examined for their potential to produce larval skeletal elements (Chapter 2). Finally, the timing for establishment of the inductive interaction in the recombined embryos was studied (Chapter 3). In addition, the developmental potential throughout metamorphosis was examined using animal caps that had received the inductive influence of micromeres for a limited period (Chapter 3).

Chapter 1

Mesodermal cell differentiation in echinoid embryos derived from the animal cap recombined with a quartet of micromeres

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 28-cell stage embryo, and recombined with a quartet of micromeres isolated from a 16- to 28-cell stage embryo. The recombined embryos were completely depleted of the presumptive regions for 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, coelomic pouch and muscle cells- were formed in the recombined embryos. Experiments using a fluorescent probe confirmed that the pigment, blastocoelar and muscle cells in the recombined larvae were derived from the animal cap mesomeres. The larvae which were derived only from the mesomere-descendants produced from the recombined embryos by removing the micromere-descendants at blastula stage, had the potential to differentiate the coelomic pouch cells.

The results indicated that the presumptive ectoderm fraction had the potential to differentiate through cell fate regulation into four

mesodermal cell types: pigment, blastocoelar, coelomic pouch and muscle 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, 1975a; 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, coelomic pouch and muscle 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), 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 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 28-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. Each unlabeled animal cap and each 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 micromeres by moving the animal cap with a glass needle onto the micromeres lying on the agar. These recombined embryos were cultured in the same dish under dark conditions.

Micromanipulation to remove micromere-descendants

Micromanipulation to remove the micromere-descendants from the recombined embryos was modified from the method reported in the previous paper (Ettensohn and McClay, 1988). The recombined embryos were inserted into the narrow space of the Kiehart micromanipulation chamber at the desired stages (Kiehart, 1982). The glass micropipets used were beveled, back-filled with ASW and attached to a pressure microinjection

apparatus. A continuous stream of ASW from the tip of the micropipet was produced by positive pressure. The micropipet tip was inserted between the RITC-labeled and unlabeled cells in the blastula wall of an embryo, and the labeled cells were expelled from the blastula wall with a stream of ASW. All of the microsurgical process was performed under the observation with the epifluorescence microscope (Nikon Optiphot) equipped with a micromanipulator (Narishige MO-102). Only the embryos from which the rhodaminated micromere-descendants had been completely removed were subjected to the further examinations.

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

An animal cap consisting of mesomere-descendants was isolated from a 28-cell stage embryo, and recombined with a quartet of micromeres isolated from a rhodaminated 16- or 28-cell stage embryo. In most recombined embryos, PMC ingression was completed at 11-12 h after fertilization, and spicule formation started at 18 h. 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 (referred to hereafter as 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). The skeletogenic mesenchyme cells and a proportion of the coelomic pouch constituents in most recombined larvae were labeled with RITC (Fig. 1-1), 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. 1-2). These cells possessed pigment granules. The number of these cells per recombined larva varied. A few recombined larvae did not form pigment cells, although most recombined and all normal ones did so (Table 1-1). 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, 1993). These cells were morphologically distinct from PMC, which have a spherical body and possess some 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. 1-3, Table 1-1).

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. 1-4A). Coelomic pouches

morphologically identical to those in the normal larvae were found in most of the recombined larvae (Fig. 1-4B, Table 1-1). However, the coelomic pouches along the larval axis in the recombined larvae were shorter than those in the undisturbed plutei. In normal embryos, the coelomic pouches are formed by cells derived from the small micromeres and SMC. The coelomic pouches in embryos derived from the animal cap and rhodaminated micromeres appeared to be composed of rhodaminated and non-rhodaminated cells. This suggested that the pouches in the recombined embryos were formed by cells descended from the small micromeres and SMC derived from the animal cap mesomeres. However, it was unclear whether the pouches in the recombined embryos really contained cells derived from the mesomeres, since the boundary between the cells labeled and unlabeled with RITC could not be detected easily by fluorescence microscopy.

The potential of the animal cap mesomeres influenced by the inductive signal from the micromeres to differentiate the coelomic pouch cells was also confirmed by removing the micromere-descendants in the recombined embryos at blastula stage. A total of 66 embryos derived only from the animal cap mesomeres which were influenced by the inductive signal from the micromere-descendants for a limited period was produced. Among them, 63 embryos survived at 48 hr post-fertilization, and 47 embryos differentiated an archenteron. Of 47 embryos with the archenteron,

27 embryos differentiated a pair of coelomic pouches (Fig. 1-4C), indicating that the mesomere-descendants influenced by the inductive signal from the micromeres really had the potential to differentiate the coelomic pouch cells.

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 h 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. 1-5D), as in those of other echinoid species reported previously (Harris, 1986). Muscle stained with rhodamine-phalloidin was found in all but one of the recombined larvae (Table 1-1). There were considerably fewer actin bands in the muscle around the esophagus of the recombined pluteus than in undisturbed larvae (Fig. 1-5), reflecting the small size of the muscle in the recombined larvae.

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, Amemiya (1996) 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 the experiments using a fluorescent probe. A pluteus derived from a recombined embryo had the potential to differentiate pigment cells and to metamorphose into a juvenile. The study 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 considerably 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 coelomic pouch and the circumesophageal muscles 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; Khaner and Wilt, 1990). 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) 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). However, 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 64-cell stage should be important for mesomeres in respecifying their cell fate. In Chapter 3, the exact period during which animal cap cells in recombined embryos receive the inductive signal from micromere-descendants will be examined.

Table 1-1. The potential of recombined and undisturbed embryos 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)
coelomic pouch cells	recombined	19	18 (95)
	undisturbed	66	66 (100)
muscle cells	recombined	13	12 (92)
	undisturbed	24	24 (100)

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

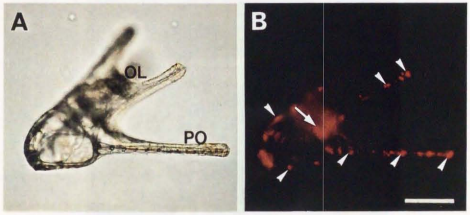


Figure 1-1. A four-armed *S. mirabilis* pluteus derived from an animal cap recombined with a quartet of rhodaminated micromeres. The skeletogenic mesenchyme cells (arrowheads) and the coelomic pouch constituents (arrow) were labeled with RITC. (A) Light field observation. (B) Epifluorescence observation.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

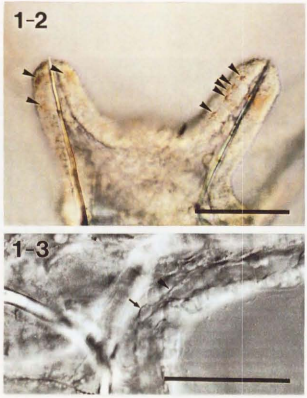


Figure 1-2. Pigment cells in a larva derived from an animal cap recombined with a quartet of micromeres. The pigment cells (arrowheads) containing pigment granules are present in the ectodermal wall. Bar represents 50 μm.

Figure 1-3. Blastocoelar cells in a larva derived from an animal cap recombined with a quartet of micromeres examined using differential interference contrast optics. A filopodium (arrowhead) extends from a blastocoelar cell (arrow). Bar represents 50 μm.

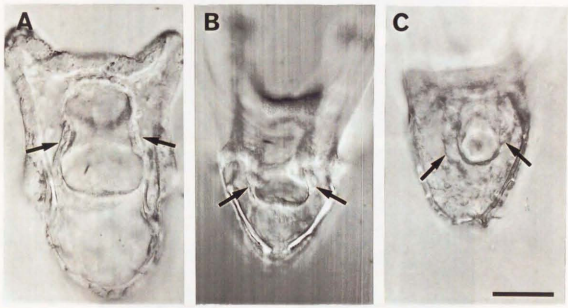


Figure 1-4. Coelomic pouch cells in a normal larva (A), a larva derived from an animal cap recombined with a quartet of micromeres (B), and a recombined larva whose micromere-descendants were completely removed at blastula stage (C). A pair of coelomic pouches (arrows) are formed on both sides of the gut in every larvae. Bar represents 50 μm.

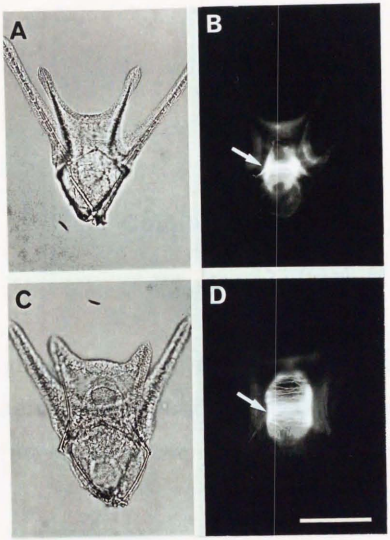


Figure 1-5. Epifluorescence observation of pluteus larvae stained with rhodamine-phalloidin. (A, B) A pluteus derived from an animal cap recombined with a quartet of micromeres. (A) Light-field observation. (B) Epifluorescence observation. (C, D) A normal pluteus. (C) Light-field observation. (D) Epifluorescence observation. 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. Bar represents 50 μm.

Chapter 2

Skeletogenic potential of induced secondary mesenchyme cells derived from the presumptive ectoderm in echinoid embryos

Abstract

During the normal development of echinoids, an animal cap consisting of 8 mesomeres in a 16-cell stage embryo differentiates exclusively into ectoderm. Micromeres in an embryo at the same stage differentiate into primary mesenchyme cells (PMC) and coelomic pouch constituents. An animal cap and a quartet of micromeres were isolated from a 16-cell stage embryo and recombined to make a chimeric embryo devoid of presumptive endoderm and secondary mesenchyme cells (SMC). The PMC in the chimeric embryo were completely removed at the mesenchyme blastula stage. The PMC-depleted chimeric embryos formed an archenteron derived from the mesomeres. Some secondary mesenchyme-like cells (induced SMC) were released from the archenteron tip. A considerable fraction of the induced SMC formed the typical mesenchyme pattern after migrating into the vegetal region, synthesized skeletogenic mesenchyme cell-surface protein (msp130), and produced the larval skeleton. These findings indicate that the induced SMC derived from the presumptive ectoderm have the same nature as natural SMC in both the timing of their release and their skeletogenic potential expressed in the absence of PMC.

Introduction

The 16-cell stage embryo of most echinoids consists of 8 mesomeres (animal cap), 4 macromeres, and 4 micromeres. The archenteron in an undisturbed embryo differentiates from the macromeres, and releases secondary mesenchyme cells (SMC) which differentiate into at least four cell types: pigment cells, coelomic pouch cells, circumesophageal muscle cells and blastocoelar cells (Ettensohn, 1992). During normal development, larval spicules are produced exclusively from primary mesenchyme cells (PMC) derived from the micromeres, and other cells never take part in skeletogenesis (Ettensohn, 1992). However, SMC can express skeletogenic phenotype by changing their natural fates if PMC are removed from the embryos at the appropriate stages (Fukushi, 1962; Ettensohn and McClay, 1988; Ettensohn, 1990; Ettensohn and Ruffins, 1993). This suggests that the inhibitory signal(s) originating from the PMC controls the skeletogenic potential of the SMC in normal embryos.

The developmental fate of animal cap mesomeres cultured in isolation, as well as in intact embryos, is restricted to the ectoderm (Hörstadius, 1973; Cameron et al., 1987). Each animal cap recombined with a quartet of micromeres forms an archenteron through the inductive influence of the micromeres, and develops into an apparently normal pluteus (Hörstadius, 1973). In a previous study, Amemiya (1996) found that the

presumptive ectoderm (animal cap) had the potential to differentiate into induced SMC under the inductive influence of micromeres. Some of the induced SMC were shown in the report to differentiate into the pigment cells. As shown in Chapter 1, the mesomere-descendants influenced by the inductive signal from the micromeres had the potential to differentiate four different mesodermal cell types: pigment, blastocoelar, muscle and coelomic pouch cells. However, the potential of the induced SMC derived from the presumptive ectoderm to differentiate into skeletogenic cells had remained to be clarified. In the present study, therefore, the skeletogenic potential of the induced SMC released from the archenteron was examined in larvae derived from the presumptive ectoderm (animal caps) which were pulse-treated with the micromeres.

Materials and Methods

Animals and embryos

Adults of the sand dollar *Scaphechinus mirabilis* were induced to shed gametes by intracoelomic injection of 0.1 M acetylcholine chloride. Eggs were washed several times with artificial seawater (ASW), then fertilized with a diluted suspension of sperm in ASW containing 1 mM aminotriazole. The fertilization envelopes were removed by shaking the eggs in a test tube. The denuded eggs were cultured in a petri dish filled with ASW at 18°C.

Manipulation to form recombined embryos and to remove PMC

The chimeric recombined embryos composed of an animal cap recombined with a quartet of micromeres were produced according to the method of Amemiya (1996). The micromeres were previously stained with a fluorescent dye (rhodamine isothiocyanate: RITC) according to the method of Wray and McClay (1988) to ensure complete removal of the PMC from the embryos.

Micromanipulation to remove the PMC from the embryos was

performed according to the method reported by Ettensohn and McClay (1988). A micropipet tip was inserted into the blastocoel of an embryo and the PMC stained with RITC were expelled from the blastocoel with a stream of ASW. After finishing the treatment, the embryos were examined using a fluorescence microscope (Nikon Optiphot). Only embryos from which rhodaminated PMC had been completely removed were subjected to further culture.

Indirect immunofluorescence

Indirect immunofluorescence staining with a skeletogenic mesenchyme cell-specific monoclonal antibody B2C2 (Anstrom et al., 1987) was performed as follows. Embryos were fixed with CFSW containing 4% formalin for 45 min at room temperature. They were rinsed with 10 mM phosphate-buffered saline (PBS) four times after fixation. The specimens were then incubated in PBS containing 1% Triton-X 100 for 1 h, rinsed with PBS four times, incubated in the primary antibody (B2C2 diluted with PBS to 1/20) for 45 min at room temperature, rinsed with PBS four times and incubated for 45 min with a secondary antibody (fluorescein-conjugated goat anti-mouse IgG, TAGO). After rinsing with PBS four times, the specimens were mounted on glass slides, and photographed.

Results and Discussion

An animal cap (consisting of eight mesomeres) and a quartet of rhodaminated micromeres were removed from different embryos at the 16-cell stage and recombined to form a chimeric embryo (Fig. 2-1A, B). In most recombined embryos, ingression of the PMC was completed at 11-12 h after fertilization, and spicule formation started at 18 h. The period of PMC ingression in the chimeric embryos was almost the same as that in normal embryos. This suggests that the timing of differentiation of the micromere-descendants is intrinsic to the cells themselves, and is uninfluenced by differences in the cell types associated with them.

The PMC of the recombined embryos were removed microsurgically between 12 (immediately after PMC-ingression) and 20 h (before archenteron invagination) after fertilization (Fig. 2-1C, D, E, F). The PMC-depleted recombined embryos invaginated to form an archenteron. Initiation of archenteron invagination in the recombined embryos lacking PMC was considerably delayed in comparison with the normal embryos. Archenteron invagination in the latter began at 14 h after fertilization. Most recombined embryos lacking PMC started archenteron invagination about 24 h after fertilization, when the normal embryos had developed to prisms or early plutei. The descendants of the small micromeres (sMic) were found to remain in the blastula wall of some embryos after PMC removal (Fig. 2-1E, F), but not in others. However, it had been reported

(Ettensohn and Ruffins, 1993) that the descendants of sMic did not contribute to the population of skeletogenic SMC. Therefore, the present experiments were carried out irrespective of whether the descendants of the sMic were retained in the blastula wall or not.

The SMC in normal embryos are distinguished from PMC by the difference in the timing of their release. The SMC are released from the archenteron tip after the start of gastrulation, whereas the PMC ingress from the vegetal plate into the blastocoel prior to archenteron invagination. In the PMC-depleted recombined embryos examined in the present study, some secondary mesenchyme-like cells (induced SMC) were released from the vicinity of the archenteron tip at about 30 h after fertilization. That is, the induced SMC in the recombined embryos were released after the start of invagination. In this respect, the induced SMC derived from the mesomeres are identical to the natural SMC derived from the macromeres. A considerable fraction of the induced SMC migrated toward the vegetal region to form a mesenchyme pattern similar to that formed by natural PMC (Fig. 2-2A). Triradiate spicules appeared in the mesenchymal aggregates derived from the induced SMC at about 45 h after fertilization. The skeletogenic potential expressed by the induced SMC in the PMC-depleted recombined embryos confirmed that the induced SMC had the same nature as natural SMC. These results are also in agreement with a report

(McClay and Logan, 1996) indicating that experimentally replaced SMC, which were probably derived from the presumptive hindgut, had the potential to replace PMC.

The PMC-depleted recombined embryos had the potential to develop into 4-armed plutei (Fig. 2-2B). The total morphology of the plutei was generally comparable to that of normal plutei. However, post-oral arms with fenestrated rods in most 4-armed plutei derived from the PMC-depleted recombined embryos were considerably shorter than those of normal plutei, as shown in Fig. 2-2B, indicating delayed arm growth. In contrast, no apparent delay was found in the growth of the antero-lateral arms. The substrata on which the post-oral and antero-lateral rods form in normal embryos are derived from macromeres and mesomeres, respectively (Hörstadius, 1973). In the PMC-depleted recombined embryos, the post-oral rods grew on the ectopic substratum produced by cells derived from the mesomeres. This might explain the delayed growth of the post-oral arms in chimeric embryos.

Expression of a skeletogenic cell-specific glycoprotein msp130 in the recombined, partial, and normal embryos was examined by indirect immunofluorescence staining using the monoclonal antibody B2C2 (Anstrom et al., 1987). The skeletogenic cells originating from the induced SMC in all of eleven plutei examined, which were derived from the PMC-depleted

recombined embryos, as well as the skeletogenic PMC in all of five normal plutei, were well stained with the antibody (Fig. 2-3). All of seven examined permanent blastulae derived from animal caps did not express the molecules at 5 days after fertilization.

A total of 42 PMC-depleted recombined embryos were produced (Table 2-1). Fifty percent of the embryos examined developed into 4-armed plutei. The remainder died or were lost during culture. In the present study the PMC were removed from the recombined embryos between 12 (immediately after PMC-ingression) and 20 h (before archenteron invagination) after fertilization. In Table 2-1, the data are shown without reference to the stages at which PMCs were removed. However, we confirmed that the developmental potential of the recombined embryos was not apparently affected by the difference in stage of PMC removal.

In the present study, it was shown that the inductive signal from the micromeres at the period between 16-cell and mesenchyme blastula stages was sufficient for the animal cap mesomeres to obtain the potential for differentiating endo-mesoderm. The exact period when the micromeres transmit the inductive signal to respecify the cell fate of the mesomeres into endo-mesoderm will be examined in Chapter 3.

Table 2-1. Developmental potential of embryos derived from the animal cap recombined with a quartet of micromeres followed by removal of PMC.

No. embryos examined	No. embryos gastrulated	No. embryos with spicule	No. embryos developed into 4-armed plutei
42	28	26	21

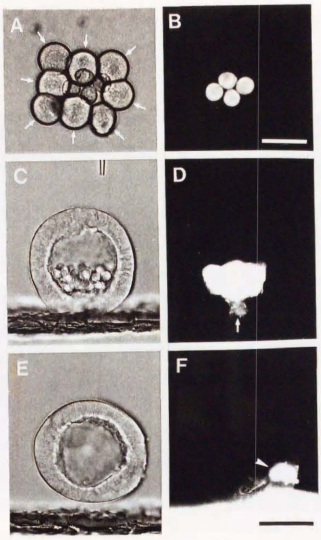


Figure 2-1. Micromanipulation procedure for removal of primary mesenchyme cells (PMC) from the chimeric mesenchyme blastula derived from the animal cap recombined with a quartet of rhodaminated micromeres. Bars represent 50 μm. (A, B) An animal cap composed of 8 mesomeres (A, arrows), immediately after recombination with a quartet of rhodaminated micromeres (B). (C, D) A chimeric mesenchyme blastula just before PMC removal. The PMC in the blastocoel (C) are rhodaminated (D). Another group of rhodaminated cells is located in the blastula wall at the vegetal pole (D, arrow). From their location, these cells seem to be the descendants of small micromeres (sMic). (E, F) The same embryo as that shown in (C, D), immediately after PMC removal. The PMC are completely removed from the blastocoel (E). A group of rhodaminated cells (arrowhead), which are possible descendants of sMic, remain located in the blastula wall (F). (A, C, E) Light field observation. (B, D, F) Epifluorescence observation.

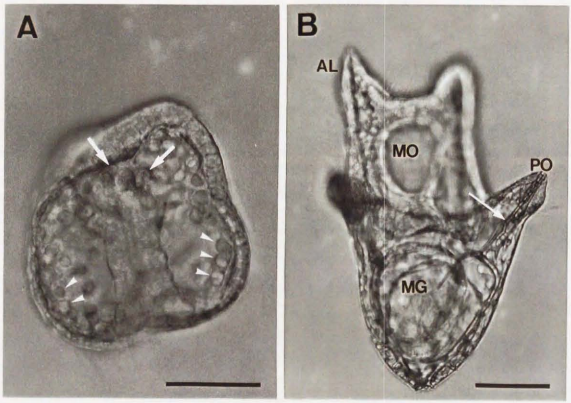


Figure 2-2. Chimeric embryos derived from an animal cap recombined with a quartet of micromeres followed by removal of PMC at the mesenchyme blastula stage. Bars represent 50 μm. (A) A late-gastrula stage embryo. Secondary mesenchyme-like cells (induced-SMC) are found in the blastocoel. Some induced SMC (arrows) are located around the archenteron tip, and others (arrowheads) in the vegetal region. (B) A 4-armed pluteus. The post-oral arms with fenestrated rods (arrow) appear to be shorter than those of the normal pluteus. MO, mouth; PO, post-oral arm; AL, antero-latelal arm; MG, mid-gut.

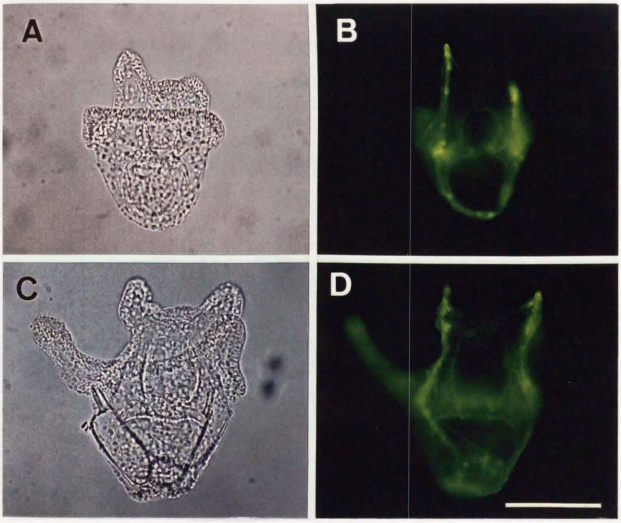


Figure 2-3. Expression of skeletogenic mesenchyme cell-specific molecules (msp130) revealed by B2C2-FITC staining of whole-mount larvae. Bar represents 100 μm. (A, B) Light (A) and epifluorescence (B) observation of a pluteus-like larva at 5 days after fertilization derived from an animal cap recombined with a quartet of micromeres followed by removal of the PMC at the mesenchyme blastula stage. The larval skeleton is well stained with the antibody (B). (C, D) Light (C) and epifluorescence (D) observation of a pluteus larva at 5 days after fertilization derived from an undisturbed control embryo.

Chapter 3

Respecification of cell fate in mesomere-descendants recombined for a limited period with micromere-descendants of echinoid embryos

Abstract

It has been reported that the micromeres of echinoid embryos have the potential to induce an archenteron in animal cap mesomeres recombined at the 16- or 32-cell stage. In the present study, experiments were performed to determine the exact period when the micromeres transmit their inductive signal to respecify the cell fate of mesomeres as endo-mesoderm. An animal cap was recombined with a quartet of micromeres, or micromere-descendants cultured in isolation, to form a recombined embryo. The micromere-descendants were completely removed at various developmental stages, resulting in an embryo composed only of mesomere-descendants which had been under the inductive influence of micromeres for a limited period. The developmental potential of the embryos was examined throughout metamorphosis. The results indicated that the signal effective for inducing an archenteron in mesomere-descendants emanated from the micromere-descendants at the early blastula stage around hatching onward. Before this stage, the micromeres and micromere-descendants showed this potential slightly or not at all. The inductive signal emanated from the micromere-descendants almost on time even when the cells were cultured in isolation. The micromere-descendants completed transmission of the signal for inducing the archenteron in the animal cap within 2 h of recombination. The animal cap acquired the competence to react with the

inductive signal from the micromere-descendants between the 16-cell stage and 2 h after the 32-cell stage. The embryos composed of only the animal cap mesomeres which had received the inductive signal from micromere-descendants for a limited period had the potential to develop into 8-armed plutei. Each pluteus formed an echinus rudiment essentially on the left side of the larval body, and metamorphosed into a juvenile with pentaradiate symmetry.

Introduction

The 16-cell stage embryo of most echinoids consists of 8 mesomeres (animal cap), 4 macromeres, and 4 micromeres. In normal embryos, the developmental fate of the animal cap mesomeres is restricted to ectoderm. The macromeres differentiate into ectoderm, endodermal gut, and mesodermal secondary mesenchyme cells (SMC). The micromeres give rise to two different cell types – large and small micromeres – at the next cleavage. The large micromeres differentiate into skeletogenic mesenchyme cells called primary mesenchyme cells (PMC) (Okazaki, 1975a; Cameron and Davidson, 1991), and the small micromeres into coelomic pouch constituents (Pehrson and Cohen, 1986; Tanaka and Dan, 1990; Cameron and Davidson, 1991; Ettensohn and Ruffins, 1993; Ruffins and Ettensohn, 1993, 1996).

Three different functions for the large micromeres in echinoid embryos have been reported. First, they differentiate autonomously into skeletogenic mesenchyme cells (Okazaki, 1975a, 1975b; Yamaguchi et al., 1994). Second, at the gastrula stage, the descendants of the large micromeres send signal(s) inhibiting the SMC to differentiate into skeletogenic mesenchyme cells (reviewed by Ettensohn, 1992).

The third function is the potential to induce an archenteron in the

neighboring cells. Classically, this function has been suggested by the following observations: (a) Micromeres implanted on the animal pole of the embryo at the early cleavage stage express the potential to induce the secondary archenteron (Hörstadius, 1935, 1973). (b) Embryos derived from an animal cap recombined with a quartet of micromeres develop into apparently normal plutei with a gut (Hörstadius, 1935, 1973).

On the basis mainly of the experimental results reported by Hörstadius, Davidson (1989) proposed a model to explain cell fate specification in echinoid embryos. In this model, it is proposed that the vegetal pole region of the egg is primordially specified. The region is completely incorporated into micromeres at the 16-cell stage, with the result that the cell fate of the micromeres is specified. The micromeres and/or their descendants express inductive activity at the early cleavage stage by producing membrane-binding ligand molecules which finally become distributed on the outer surface of the micromeres and their descendants. The receptors for the inductive ligands are distributed on the surface of every cell derived from all blastomeres except micromeres in the 16-cell stage embryo. The model further postulates that the potential of blastomeres in the upper tiers (macromeres, mesomeres and their descendants) to react with the inductive signal from the micromeres is essentially identical. In normal development, the micromeres and their descendants make direct contact only with macromeres and their descendants. Thus, the cell fate of

macromeres is primarily specified as differentiation into endo-mesoderm under the inductive influence of micromeres. After the cell fate of the macromeres has been specified, they and/or their descendants transmit an inductive signal toward the upper tier to specify the blastomeres. When the animal cap mesomeres are recombined with the micromeres, the mesomeres respecify their cell fate under the inductive influence of the micromeres, so that the recombined embryo becomes composed of tiers of blastomeres essentially identical to those in normal embryos. According to this model, the developmental potential of the recombined embryo produced by the animal cap and micromeres is the same as that of a normal embryo.

Recently, the model was examined using various experimental approaches. Differentiation of macromeres to express endodermal characteristics was markedly delayed if all micromeres were removed from the embryo at the 16- to 32-cell stage (Ransick and Davidson, 1995). The endo-mesoderm that differentiated from animal cap mesomeres recombined with micromeres was essentially identical in nature to that in the normal embryo (Amemiya, 1996; Minokawa et al., 1997 [Chapter 2 in this thesis]; Chapter 1 in this thesis). The embryo derived from an animal cap recombined with a quartet of micromeres had the potential to metamorphose into a juvenile with pentaradiate symmetry (Amemiya, 1996). The inductive influence of micromeres during the period from the 16-cell stage to the mesenchyme blastula stage was sufficient for the secondary

mesenchyme-like cells derived from the animal cap to obtain skeletogenic potential (Minokawa et al., 1997 [Chapter 2]). A secondary archenteron which was identical molecularly as well as morphologically to the native one was induced when micromeres were implanted on the animal pole of an embryo at the early cleavage stage (Ransick and Davidson, 1993). Essentially, these findings appeared to agree with the model proposed by Davidson (1989). However, there is still no direct evidence for the ligands released from the micromeres, and for the receptors that are reactive with the ligands, both of which are essential for proof of the model. It is thus very important to determine the exact period when the micromeres express their inductive signal in order to find these ligands and receptors. During normal development, the macromeres which make direct contact with micromeres in the 16-cell stage embryo differentiate into ectoderm, mesoderm and endoderm (Hörstadius, 1973; Cameron et al., 1991). Macromeres have the potential to differentiate into endodermal archenteron when they are isolated from the 16-cell stage embryo and cultured in isolation (Hörstadius, 1935; Yamaguchi et al., 1994). This property of macromeres makes it difficult to determine conclusively whether their potential to differentiate into archenteron is autonomous or conditional. In contrast, the developmental fate of mesomeres during normal development is restricted to ectoderm. Animal cap mesomeres cultured in isolation develop into permanent blastulae without any trace of endo-mesodermal

differentiation (Hörstadius, 1973; Henry et al., 1989; Amemiya, 1996). However, the mesomeres express the potential to differentiate into endo-mesoderm when they are recombined with micromeres (Hörstadius, 1973; Khaner and Wilt, 1991; Ransick and Davidson, 1993; Amemiya, 1996; Minokawa et al., 1997 [Chapter 2]; Chapter 1). These results indicate that the potential of mesomeres to differentiate into endo-mesoderm is completely conditional. Therefore, embryos consisting of animal cap mesomeres recombined with micromeres appear to be an ideal experimental system for analyzing the inductive activity of the micromeres.

In the present study, the developmental potential of embryos derived from mesomere-descendants recombined for a limited period with micromere-descendants was examined to determine the exact period when the inductive signal is transmitted from the micromeres. The period when the animal cap mesomeres had the competence to react with the inductive signal was also examined. In addition, the developmental potential throughout metamorphosis of animal cap mesomeres recombined for a limited period with micromeres was examined.

Materials and Methods

Animals and embryos

The sand dollar, *Scaphechinus mirabilis*, was mainly used. In some experiments, the sea urchin, *Hemicentrotus pulcherrimus*, was also employed. *S. mirabilis* adults were provided by Asamushi Marine Biological Station and Ushimado Marine Laboratory. *H. pulcherrimus* adults were collected in the vicinity of Misaki Marine Biological Station. 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), transferred to ASW containing 1 mM aminotriazole (ATA) to prevent hardening of the fertilization envelope (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 for *S. mirabilis* and at about 14°C for *H. pulcherrimus* in a petri dish coated with 1.2 % agar and filled with ASW.

Manipulation to form recombined embryos

Method for producing recombined embryos was described in Chapter 1.

Occasionally, a quartet of micromeres was cultured in isolation in a petri dish filled with ASW containing 100 units/ ml penicillin and 50 μg/ml streptomycin sulfate for several hours, before recombination with an animal cap.

Micromanipulation to remove micromere-descendants

The method for removing micromere-descendants was the same as that described in Chapter 1.

Histochemistry for alkaline phosphatase

The embryos and larvae fixed with 70% ethanol were placed in an 80-well plastic plate. The reaction for alkaline phosphatase (AP) activity was performed in the wells. The procedures for fixation and staining of the embryos and larvae were essentially identical to those reported by Whittaker and Meedel (1989).

Labeling of small micromeres with BrdU

The number of small micromeres was estimated by counting their nuclei stained with an anti-BrdU antibody. Staining was performed according to the method reported by Tanaka and Dan (1990) with some

modifications. Embryos were incubated in ASW containing 0.5 mM BrdU at 2 h post-fertilization, corresponding to the 8-cell stage, for 30 min. They were then rinsed with ASW twice, chased with ASW containing 300 μM thymidine for several minutes, rinsed again with ASW twice, and cultured in ASW. The embryos were fixed at various stages with CFSW containing 2% formalin and rinsed with phosphate-buffered saline (PBS). An equal volume of 4 N HCl containing 1% Triton X-100 was added to PBS containing the embryos, and the embryos were incubated in the medium for 30 min. After washing with PBS several times, the embryos were stained by indirect immunofluorescence using an anti-BrdU monoclonal antibody labeled with biotin (Becton Dickinson) and streptavidin-FITC.

Postfeeding culture of larvae

Postfeeding culture of the larvae was performed according the method reported previously (Amemiya, 1996). The larvae were transferred to 7-ml plastic bottles filled with ASW. The bottles were fixed horizontally to the tilted plate of a rotor, and rotated at 1 rpm. Seawater in the bottles was changed once every two days, and an adequate amount of the diatom *Chaetoceros gracilis* suspended in seawater was added whenever the seawater was changed.

Results

Pulsed recombination of animal cap with micromeres

It has been reported that an animal cap recombined with micromeres isolated from a 16-cell stage embryo has the potential to differentiate endoderm, even if the micromere-descendants are completely removed at the mesenchyme blastula stage (Minokawa et al., 1997 [Chapter 2]). This suggests that the inductive signal which emanates from the micromeres during the period between the 16-cell and mesenchyme blastula stages is sufficient for differentiation of the animal cap.

The following experiments (Fig. 3-1) were designed to estimate more precisely the period in which the inductive signal is transmitted from the micromeres to the animal cap in the recombined embryos. Recombined embryos, each consisting of an animal cap isolated at the 28-cell stage and a quartet of rhodaminated micromeres isolated at the 16- to 28-cell stage, were produced (Fig. 3-2). The descendants of the micromeres were completely removed from the recombined embryos at various developmental stages: 4-5 h, 5-6 h, 6-7 h, 7-8 h, 8-9 h, 9-10 h, 10-11 h, 11-12 h, and 12-18 h post-fertilization (Fig. 3-1, 3-3). The resulting embryos, consisting only of descendants of the animal cap mesomeres, were cultured and examined for their potential to differentiate endoderm at 48 h after fertilization, when the undisturbed embryos were at the pluteus stage. Hereafter, the recombined

embryos produced by an animal cap (Ani) and a quartet of micromeres (Mic) are referred to as Ani+Mic. The experimental embryos derived from an animal cap recombined with a quartet of micromeres followed by removal of the micromere-descendants (MicDes) between X and Y h after fertilization are referred to as Ani+Mic–MicDes(X-Yh). A total of 140 Ani+Mic–MicDes(X-Yh) were produced, and more than 90% of the embryos were still viable at 48 h post-fertilization.

The micromere-descendants in a fraction of the Ani+Mic embryos were completely removed at 4-5 h or 5-6 h post-fertilization. All embryos derived from the Ani+Mic–MicDes(4-5h) (n=7, the number indicating the experimental embryos alive at 48 h post-fertilization), and from the Ani+Mic–MicDes(5-6h) (n=15) developed into permanent blastulae without showing any trace of archenteron invagination (Fig. 3-4A). The alkaline phosphatase (AP) activity in some of these permanent blastulae was examined, but no embryo showed the activity.

Two and three embryos derived from the Ani+Mic–MicDes(6-7h) (n=26) and the Ani+Mic–MicDes(7-8h) (n=18), respectively, differentiated an archenteron at 48 h after fertilization (Fig. 3-4A). The remaining embryos developed into permanent blastulae (Fig. 3-5A). A few permanent blastulae differentiated some pigment cells and/or blastocoelar cells.

The blastulae derived from the Ani+Mic began to swim at 8 h after

fertilization, corresponding to timing of swimming initiation in undisturbed embryos. The undisturbed control embryos hatched out at about 9 h post-fertilization. A total of 20 Ani+Mic–MicDes(8-9h) embryos were produced. Among them, 12 embryos differentiated the archenteron by 48 h post-fertilization (Fig. 3-4A), and the AP activity in some of these embryos was examined. All of the examined embryos were positive for AP activity (Fig. 3-5B). Considerable variations were found in the developmental rate of the embryos derived from the Ani+Mic–MicDes(8-9h). Some embryos were at the early pluteus stage, while others were still late gastrulae at 48 h after fertilization.

The embryos derived from an animal cap recombined with a quartet of rhodaminated micromeres released rhodaminated PMC into the blastocoel at about 11-12 h post-fertilization. The timing for differentiation of the PMC in the recombined embryos corresponded to that in undisturbed normal embryos, suggesting that the isolation and recombination procedures did not affect the developmental schedule of the micromeres. The proportion of embryos that differentiated an archenteron among embryos derived from the animal cap recombined with micromeres whose micromere-descendants had been removed at 8 h post-fertilization onward, reached nearly a plateau within a range of about 60 - 90 %. A total of 43 Ani+Mic–MicDes were produced, whose micromere-descendants were removed during the period from 9 to 18 h post-fertilization. Among them,

35 embryos (81%) differentiated the archenteron (Fig. 3-4A, 3-6). Some variations in the ratio of embryos that differentiated the archenteron were found during the observation period, probably because of the difference in the conditions of the embryos used in each experiment.

About 87% of the embryos derived from the Ani+Mic differentiated an archenteron (Fig. 3-4B). No archenteron was formed in embryos derived only from the animal cap (Fig. 3-4B).

These results indicated that the inductive signal emanating from micromeres and micromere-descendants in the period between the 16-cell stage and 8 h post-fertilization was sufficient for the animal cap to differentiate an archenteron. In particular, the signal produced around 8 h post-fertilization appeared to be important for induction. Embryos at 8 h post-fertilization corresponded to the early blastula at about 3-4 h before PMC ingression during normal development.

The relationship between the number of divisions and the period during which the micromere-descendants express the signal for archenteron induction was then examined. The number of descendants of rhodaminated micromeres in recombined embryos at 6 and 8 h post-fertilization was counted after squashing the embryos with a cover glass. The number of small micromere-descendants (sMic) in the recombined embryos was estimated from the number of the sMic in normal embryos examined by

BrdU staining. The average numbers of micromere-descendants at 6 and 8 h post-fertilization were 17.7 (n=3, SD=4.04) and 29.3 (n=4, SD=4.50), respectively. The numbers of sMic at 6 and 8 h in undisturbed embryos were 4.0 (n=15, SD=0) and 6.6 (n=16, SD=0.81), respectively. If the microsurgical experiment did not disturb the rate of cell division, the numbers of large micromere-descendants (lMic) at 6 and 8 h were estimated to be about 14 and 23, respectively, suggesting that lMic had almost completed the 7th division at 6 h, and that some of them had begun the 8th division at 8 h post-fertilization when some of the sMic also began to divide. These results suggested that the lMic initiated to express the effective inductive signal at the 8th division.

The experiments using a regular sea urchin, *Hemicentrotus pulcherrimus*, confirmed that the signal emanating from the micromere-descendants several hours before PMC ingression was important (Fig. 3-7). The PMC in the embryos of this species ingressed at about 20 h post-fertilization. No archenteron was formed in the embryos of Ani+Mic–MicDes(7.5-9h) (n=17), while 19 of 21 Ani+Mic–MicDes(16-19h) differentiated the archenteron.

Inductive potential of micromere-descendants cultured in vitro

As shown in the previous section, recombination of micromeres with the animal cap in the period from the 28-cell to the early blastula stage is sufficient to allow the animal cap to differentiate an archenteron. This suggests that there are two different possibilities to explain the timing of micromeres in expressing their inductive activity. The first is that the entire period of recombination from the 28-cell to the early blastula stage is necessary for completion of induction. The second is that the inductive activity of micromere-descendants is expressed in a limited period around the early blastula stage.

The following experiments were therefore designed to determine the exact timing in which micromeres transmitted their inductive activity. A quartet of micromeres was cultured in isolation for various periods, and then recombined with an animal cap. The micromere-descendants in the recombined embryos were completely removed at 1-2 h after recombination, and the potential of the animal cap to differentiate the archenteron was examined after prolonged culture.

It has already been reported that micromeres cultured in isolation retain their spiculogenetic potential (Okazaki, 1975b; Kitajima and Okazaki, 1980; Yamaguchi et al., 1994). However, no report has shown that

micromeres cultured in isolation have inductive activity. Therefore, the inductive activity of micromeres cultured in isolation was examined (Fig. 3-8), before examining the exact timing of activity expression by micromeres. A quartet of micromeres was isolated from a 16- to 28-cell stage embryo at about 3 h post-fertilization, and cultured in ASW for 4-5 h. The cultured micromere-descendants were recombined with an animal cap isolated from a 28- or 32-cell stage embryo which had been fertilized about 4-5 h after fertilization of the micromere-donor embryos. Thus, the micromeres cultured in isolation were 4-5 h older than the animal cap. The recombined embryo was examined at 48 h after fertilization. These recombined embryos consisting of an animal cap and the descendants of four aged micromeres are referred to hereafter as Ani+agedMic.

A total of ten Ani+agedMic embryos were produced. Among them, eight embryos formed an archenteron (Fig. 3-9). The embryos derived from the Ani+agedMic had the potential to develop into pluteus larvae with apparently normal morphology (Fig. 3-10). These results indicated that the micromere quartets cultured in isolation retained their potential to induce the archenteron in the recombined animal cap. Moreover, the inductive influence of micromere-descendants was effective in allowing the animal cap at a different developmental stage to differentiate the archenteron.

I then examined the duration of recombination needed in order for the animal cap to respecify its cell fate under the inductive influence of

micromere-descendants, as well as the exact period in which the micromere-descendants transmitted the effective signal (Fig. 3-11). Each quartet of micromeres was isolated from a 16-cell stage embryo, and cultured in isolation. Each aggregate of the micromere-descendants cultured in isolation was removed from the medium at various intervals and recombined with an animal cap freshly isolated from a 28- or 32-cell stage embryo. The micromere-descendants in each recombined embryo were completely removed at 1 to 2 h after recombination. These embryos, consisting of an animal cap recombined for a short period with cultured micromeres, are referred to hereafter as Ani+agedMic(A-Bh)–MicDes(X-Yh). In the embryos, the micromere-descendants cultured in isolation were recombined with an animal cap freshly isolated from a 28- or 32-cell embryo, and removed later. The age of the micromere-descendants at recombination was A to B h, and that at removal was X to Y h. Among the 19 Ani+agedMic(8-9h)–MicDes(10-11h) embryos that were viable at 48 h, 15 differentiated an archenteron (Fig. 3-9, 3-12). Next, the potential of Ani+agedMic(8-9h)–MicDes(9-10h) to differentiate the archenteron was examined. In this case, three of five embryos differentiated an archenteron, suggesting that even 1 h of recombination of aged micromeres was effective to a certain extent for archenteron induction, although it was insufficient in comparison with 2 h of recombination. A total of 20 Ani+agedMic(6-7h)–MicDes(8-9h) embryos were produced to examine whether the inductive signal emanated from the

cultured micromeres before 8 h post-fertilization. Only four of the 20 embryos differentiated an archenteron, indicating that the inductive activity of cultured micromeres at 6-7 h post-fertilization was considerably lower than that at 8 h (Fig. 3-9). These results indicated that 2h recombination of the cultured micromeres at 8-9 h post-fertilization was exactly sufficient to induce archenteron in the animal cap. Moreover, it was indicated that the animal cap became receptive to the inductive signal produced by the micromere-descendants in the period between the 16-cell stage and 2 h after the 32-cell stage.

The pluteus larvae derived from Ani+Mic–MicDes and Ani+agedMic–MicDes embryos were cultured by feeding to examine their developmental potential throughout metamorphosis (Table 3-1, Fig. 3-13). The micromere-descendants in these embryos were removed between 6 and 11 h after fertilization. A total of nine 8-armed plutei that formed the echinus rudiment were obtained. Among them, eight larvae formed an echinus rudiment on the normal left side of the body, and one did so on the opposite right side. This suggests that the larvae derived only from the animal cap mesomeres had the potential to express essentially normal left-right polarity. All of the nine 8-armed plutei with an echinus rudiment metamorphosed into juveniles with apparently normal morphology and pentaradiate symmetry, indicating that the embryos derived only from the animal cap mesomeres which received appropriate induction from the

micromeres had the potential to develop into juveniles.

Discussion

In the present study, an attempt was made to examine the exact period when the micromeres transmit the inductive signal that respecifies the endo-mesodermal fate of mesomeres. In addition, the timing of the mesomeres in becoming competent to react to the inductive signal from the micromeres, and the developmental potential of mesomeres that have received the inductive influence from micromeres for a limited period were also examined. The results indicated that the signal was produced by the micromeres at the early blastula stage. The competence of the mesomeres to react with the micromere signal began to be expressed in the period between the 16-cell and 2 h after the 32-cell stage. Mesomeres that received the inductive signal from the micromeres for a limited period between the 32-cell and early blastula stage had the potential to develop and metamorphose into a juvenile with pentaradiate symmetry. These results essentially agreed with the previous model of cell fate specification (Davidson, 1989). However, some modifications of the model were suggested from the present work. One was the period in which the inductive signal emanated from the micromeres, and another was that the competence of the mesomeres to react with the inductive signal differed from that of macromeres.

Two signals emanating from micromeres at different developmental stages

It has been suggested that normal interaction between macromeres and micromeres in the period from the 4th to the 6th cleavage stages is indispensable for normal specification of the vegetal plate (Ransick and Davidson, 1995). Embryos whose micromeres or micromere-descendants have been removed during these stages fail to show normal expression of a vegetal plate-specific gene, *Endo16*. Also, archenteron invagination in these embryos is considerably delayed in comparison with normal embryos. These results suggest that the inductive signal from micromeres that specifies the endodermal fate of macromeres emanates continuously between the 4th and 6th cleavage stages (Ransick and Davidson, 1995).

In the present study, the inductive signal from micromeres that respecifies the cell fate of mesomeres became effective at the early blastula stage after 8th cleavage. The signal from the micromeres in the period between the 4th and 6th cleavage stages was ineffective for inducing the archenteron in the mesomeres. In the light of the previous report (Ransick and Davidson, 1995), these results suggest that two inductive signals, early and late, emanate from micromeres at different developmental stages. The influence of the late signal on macromeres remains to be examined.

Two different possibilities about the nature of the early and late signals can be considered. First, the two signals differ qualitatively. In this regard, two different cases can further be assumed about the competence of the mesomeres and macromeres to react with the signals. In one case, the competence of mesomeres is identical to that of macromeres, i.e. both blastomeres are equipped with the same receptors for the inductive ligands and with the intracellular regulatory factors, as suggested by the previous model (Davidson, 1989). In this case, the early and late signals have different functions. For instance, the late signal directly induces differentiation of the archenteron, and the early signal is responsible for conditioning of both blastomeres. A classic experiment appears to support the function of the early signal for blastomere conditioning. Hörstadius (1936, 1939, 1973) cultured animal cap mesomeres isolated at the 16- or 32-cell stage. After various intervals in culture, an animal cap was recombined with the micromeres freshly isolated from a 16-cell stage embryo. The potential of the animal cap to differentiate the archenteron decreased when the culture period was prolonged. However, as indicated in the present study, the potential of the animal cap to differentiate the archenteron was maintained in embryos that were recombined with micromeres, suggesting that the micromeres helped the mesomeres to retain their potential. The early signal from the micromeres might exert this effect. In the other case, the competence of mesomeres to react with the early and/or late signal

differs from that of macromeres. For instance, the macromeres may be equipped with the receptor(s) for the early signal, whereas the mesomeres may not be. This may explain why the early signal did not show any distinct effect on the mesomeres.

The second possibility is that the two signals differ quantitatively, being weak at the early stage, then gradually strengthening later. The macromeres may have the potential to react even with the weak signal, whereas the mesomeres may react only with the strong signal. The micromeres may become able to express the signal sufficiently strongly to induce the archenteron in mesomere-descendants after the 8th cleavage. This possibility suggests that the competence of macromeres to react with the micromere signal is greater than that of mesomeres. Thus, a gradient of competence may exist along the animal-vegetal axis from the macromeres at the vegetal end with higher competence to the mesomeres at the animal end with lower competence. These two possibilities still remain to be examined.

Developmental potential of the animal cap mesomeres

The developmental fate of the animal cap mesomeres in undisturbed sea urchin embryos is restricted only to ectoderm (Hörstadius, 1973;

Cameron and Davidson, 1991). Animal caps isolated at the 16- to 64-cell stage by microsurgically dissecting embryos along the equatorial plane differentiate exclusively into ectoderm to form permanent blastulae (Hörstadius, 1935, 1973; Henry et al., 1989; Amemiya, 1996). Thus, the animal cap mesomeres normally differentiate only ectoderm. However, the mesomeres express pluripotency to differentiate various cell types of endo-mesoderm, when the blastomeres are isolated under specific conditions and/or treated with certain factors. Embryos derived from the animal cap recombined with micromeres form a completely differentiated gut (Hörstadius, 1935, 1973), and a second gut is induced by implantation of micromeres onto the animal pole of embryos at the early cleavage stage (Hörstadius, 1973; Ransick and Davidson, 1993). The embryoids derived from a pair of mesomeres recombined with a micromere form a gut-like structure and express gut-specific molecules (Livingston and Wilt, 1990), and an animal cap treated with LiCl has been reported to develop into a pluteus-like larva with a gut (von Ubisch, 1921). Similarly, a dissociated mesomere pair treated with LiCl differentiated a gut (Wikramanayake et al., 1995; Yoshikawa, 1997), and expressed gut-specific molecules (Livingston and Wilt, 1989). The developmental potential of mesomeres increases when they are dissociated into a mesomere pair (Henry et al., 1989; Raff, 1996), or are reaggregated after dissociation (Henry et al., 1989; Khaner and Wilt, 1990). These previous findings indicating the developmental

plasticity of mesomeres have been obtained only in early larval development. Recently, however, it was reported that an embryo derived from an animal cap recombined with a quartet of micromeres had the potential to develop and metamorphose into a juvenile (Amemiya, 1996). This suggests that mesomeres differentiate a considerable proportion of the adult tissues under the inductive influence of micromeres. However, the experimental system employed could not eliminate the possibility that most of the adult tissues had differentiated from the micromeres in the recombined embryos. In the present study, it was found that the embryos derived only from the animal cap mesomeres had the potential to metamorphose into juveniles with pentaradiate symmetry if they received the inductive influence of micromeres for a limited period. I am now trying to obtain adult sea urchins with germ cells derived only from animal cap mesomeres that have received an inductive pulse from micromeres.

Table 3-1. Formation of echinus rudiment and metamorphosis of larvae derived from recombined embryos followed by removal of micromere-descendants.

	8-pl	8-pl with ER	Handedness in formation of ER		
	total	total	Left	Right	Juvenile
Ani+Mic–Mic*	10	9	8**	1***	9
Ani+Mic****	8	8	7 *****	1	5 ******

*: The recombined embryos indicated as Ani+Mic–Mic consisted of embryos of two different origins, Ani+Mic–MicDes and Ani+agedMic–MicDes. The experiment was started using about 50 recombined embryos.

**: The origins of the larvae were two Ani+Mic–MicDes(8-9hr), five Ani+Mic–MicDes(12-18hr) and an Ani+agedMic(8-9hr)–MicDes(10-11hr).

***: The origin of the larva was Ani+Mic–MicDes(12-18hr).

****: The recombined embryos indicated as Ani+Mic consisted of embryos of two different origins, Ani+Mic and Ani+aged Mic. The experiment was started using about 40 recombined embryos

*****: The origins of the larvae were five Ani+Mic and two Ani+agedMic.

******: The origins of the larvae were four Ani+Mic and an Ani+agedMic.

8-pl: 8-armed pluteus

ER: echinus rudiment

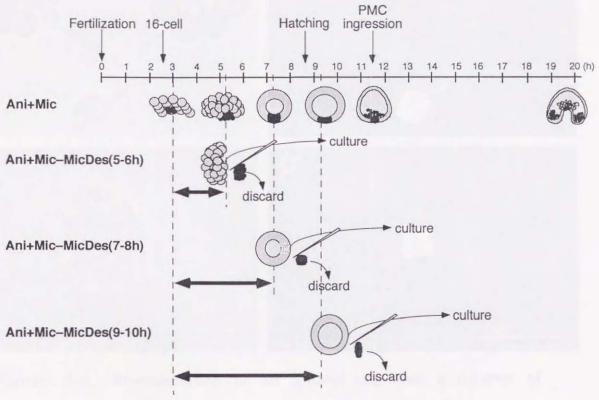


Figure 3-1. Experimental protocol for isolation of micromere-descendants from embryos derived from an animal cap recombined with a quartet of micromeres. Uppermost scale represents time (h) after fertilizaiton and developmental events in normal *S. mirabilis* embryos (Fertilization, 16-cell, Hatching and PMC ingression). Ani+Mic: Schematic representation of early development through gastrula stage of an embryo derived from an animal cap (Ani) recombined with a quartet of micromeres (Mic). Ani+Mic–MicDes(X-Yh): Schematic representation of the procedure used to isolate the micromere-descendants (MicDes) at X to Y h after fertilization from the recombined embryos. Dark arrows show the periods of recombination between the animal cap cells and the micromere descendants.

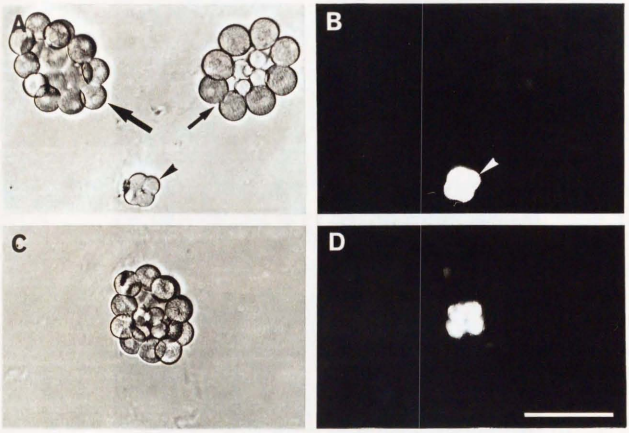


Figure 3-2. Recombination of an animal cap and a quartet of rhodaminated micromeres isolated from two different embryos of the sand dollar, *Schaphechinus milabilis*. Bar, 100 μm. (A, B) An animal cap (large arrow) and a vegetal half (small arrow) isolated from a 28-cell stage embryo. A quartet of micromeres (arrow heads) isolated from another rhodaminated embryo is located in the lower region of the field. (A) Light-field observation. (B) Epifluorescence observaton. (C, D) An embryo just after recombination of an animal cap and the quartet of rhodaminated micromeres shown in Fig. 3-2A, B. (C) Light-field observation. (D) Epifluorescence observation.

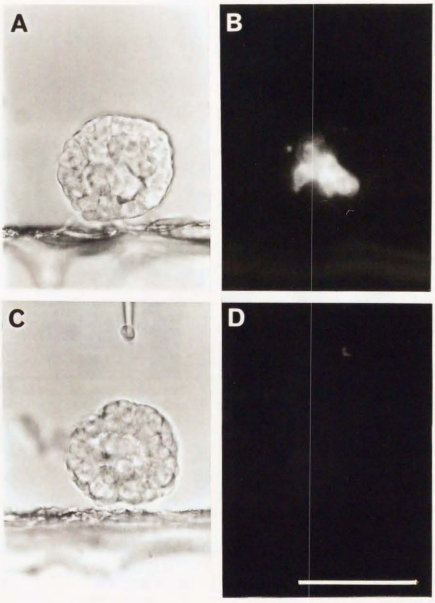
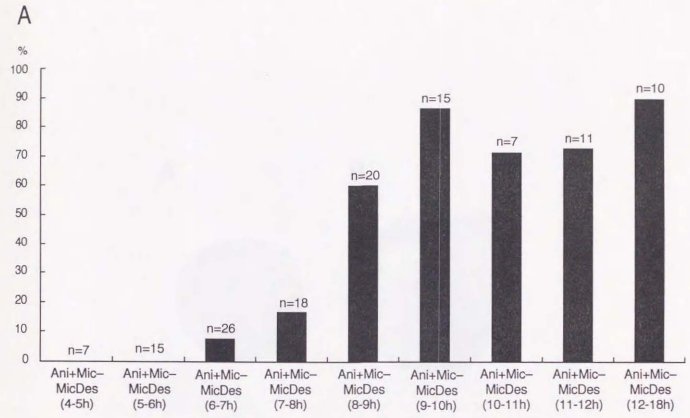


Figure 3-3. Micromanipulation procedure for removal of micromere-descendants. Bar, 100 μm. (A, B) A blastula at about 6 h post-fertilization derived from an animal cap recombined with a quartet of rhodaminated micromeres. (A) Light-field observation. (B) Epifluorescence observation showing the descendants of micromeres labeled with RITC. (C, D) The same blastula shown in Fig. 3-3A, B just after removal of the micromere-descendants. (C) Light-field observation. (D) Epifluorescence observation.



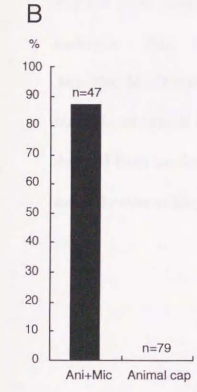


Figure 3-4. Histogram representing percentage of individuals differentiating an archenteron in *S. mirabilis* embryos of various origins. The embryos were examined at 48 h post-fertilization. (A) The ratio of embryos that differentiated on archenteron among embryos derived from an animal cap recombined for a limited period with a quartet of micromeres [Ani+Mic–MicDes(X-Yh)]. The micromere-descendants were completely removed from the recombined embryos during the periods shown in parentheses on the abscissa. (B) The ratio of embryos that differentiated an archenteron among embryos derived from an animal cap recombined with a quartet of micromeres, and those derived only from the animal cap. Abscissa, origin of embryos; ordinate, ratio of embryos that differentiated an archenteron; n, number of experimental embryos that were viable at 48 h post-fertilization.

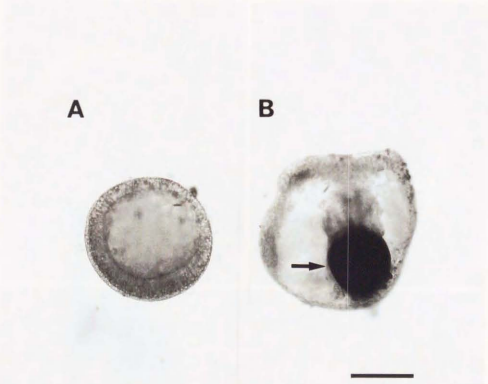


Figure 3-5. Alkaline phosphatase (AP) activity in Ani+Mic–MicDes(X-Yh) embryos. Bar, 100 μm. (A) A permanent blastula derived from an Ani+Mic–MicDes(6-7h). AP activity is not detectable in the permanent blastula in which no archenteron has formed. (B) A gastrula-like embryo derived from an Ani+Mic–MicDes(8-9hr). Strong AP activity is detectable in an archenteron-like structure (arrow) within the embryo.

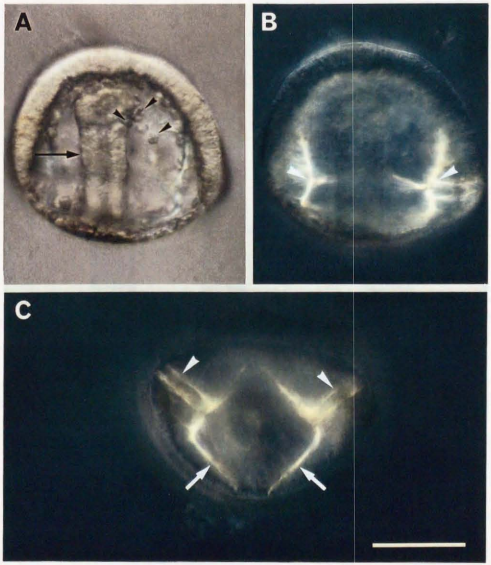


Figure 3-6. A prism larva at 48 h after fertilization derived from an Ani+Mic–MicDes(9-10h) embryo observed using a microscope equipped with differential interference optics. Bar, 100 μm. (A) A larva viewed from the oral side. An archenteron (arrow) and some secondary mesencyme cells (arrowheads) have formed. (B) The same larva shown in (A) viewed from the oral side, focusing on the larval spicules. A pair of larval spicules (arrowheads) are located bilaterally in the blastocoel. (C) The same larva shown in (A) viewed from the vegetal side. The spicules are composed of a pair of fenestrated post-oral rods (arrowheads) and simple body rods (arrows).

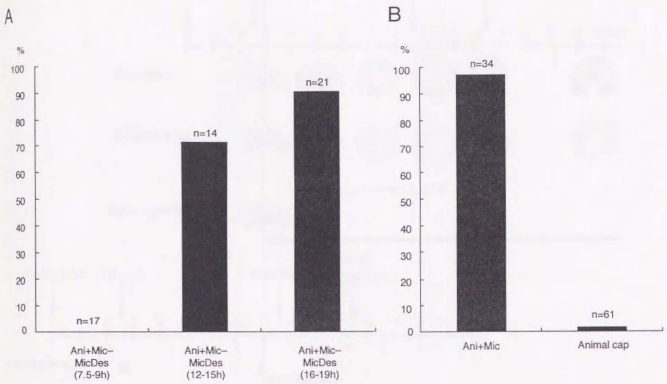


Figure 3-7. Histogram representing the percentage of individuals differentiating an archenteron among *H. pulcherrimus* embryos of various origins. The embryos were examined at 72 h post-fertilization. (A) The ratio of embryos that differentiated an archenteron among embryos derived from an animal cap recombined for a limited period with a quartet of micromeres [Ani+Mic–MicDes(X-Yh)]. The micromere-descendants were completely removed from the recombined embryos at the times shown in parentheses on the abscisssa. (B) The ratio of embryos that differentiated an archenteron among embryos derived from an animal cap recombined with a quartet of micromeres, and those derived only from an animal cap. Abscissa, origin of embryos; ordinate, ratio of embryos that differentiated an archenteron; n, number of experimental embryos that were viable at 72 h post-fertilization.

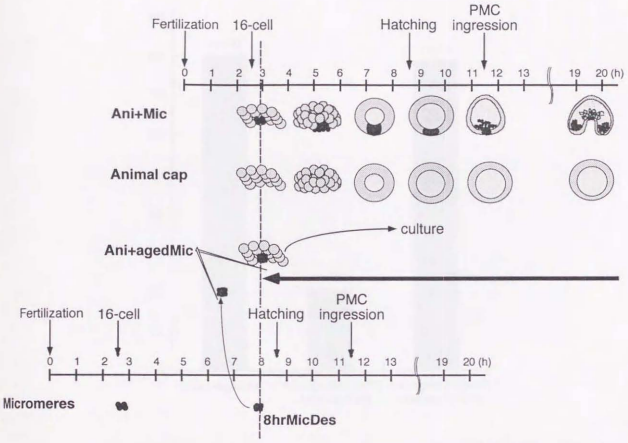


Figure 3-8. Experimental protocol for formation of embryos derived from an animal cap isolated from a 32-cell stage embryo recombined with micromere-descendants cultured in isolation for 5 h. Scales show time (h) post-fertilization and developmental events in normal *S. mirabilis* embryos (Fertilization, 16-cell, Hatching and PMC ingression). Ani+Mic; Schematic representation of early development through gastrula of an embryo derived from an animal cap (Ani) recombined with a quartet of micromeres (Mic). Animal cap; Schematic representation of early development of an embryo derived from an animal cap. Ani+agedMic; Schematic representation showing the formation of an embryo derived from an animal cap freshly isolated from a 32-cell stage embryo recombined with micromere-descendants cultured in isolation (agedMic) for 5 h. The age of the micromere-descendants at recombination was 8 h post-fertilization. A dark arrow shows the period during which the animal cap cells were recombined with the micromere-descendants.

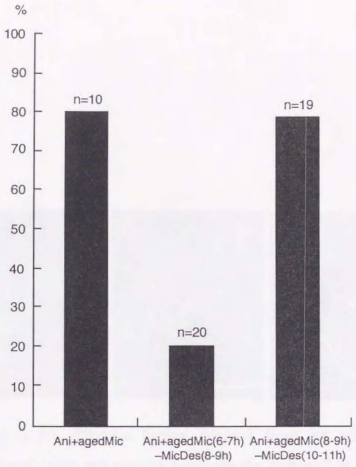


Figure 3-9. Histogram representing the proportion of individuals that differentiated an archenteron among *S. mirabilis* embryos derived from an animal cap isolated at the 32-cell stage recombined with micromere-descendants that had been cultured in isolation. Ani+agedMic are embryos derived from an animal cap (Ani) recombined with micromere-descendants cultured in isolation for 4 to 5 h (agedMic). The age of the micromere-descendants at recombination was 7 to 8 h post-fertilization. Ani+agedMic(6-7h)–MicDes(8-9h) are embryos derived from an animal cap recombined with micromere-descendants cultured in isolation for 3 h. The age of the micromere-descendants at recombination was 6 to 7 h post-fertilization, and that at removal was 8 to 9 h post-fertilization. Ani+agedMic(8-9h)–MicDes(10-11h) are embryos derived from an animal cap recombined with micromere-descendants cultured in isolation for 5 h. The age of the micromere-descendants at recombination was 8 to 9 h post-fertilization, and that at removal was 10 to 11 h post-fertilization. Abscissa; origin of embryos, ordinate; embryos that differentiated an archenteron.

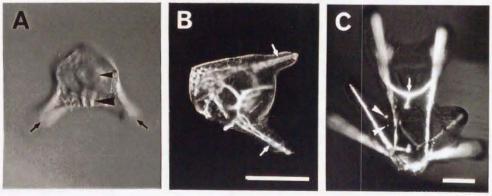


Figure 3-10. Pluteus larvae derived from an animal cap recombined with micromere-descendants cultured in isolation (Ani+agedMic). An animal cap freshly isolated from a 32-cell stage embryo was recombined with micromere-descendants cultured in isolation for 5 h. The age of the micromere-descendants at recombination was 8 h post-fertilization. Bars represent 100 μm. (A) An early pluteus derived from an Ani+agedMic embryo viewed from the oral side. Two post-oral arms (arrows), a midgut (small arrowhead) and a hindgut (large arrowhead) are evident. (B) Dark field microscopy of a squashed early pluteus derived from an Ani+agedMic embryo, showing fenestrated post-oral rods (arrows). (C) Dark field microscopy of a late pluteus larva derived from an Ani+agedMic embryo, showing a dorsal arch (arrow), and post-oral and antero-lateral arms. Rudiments of the juvenile skeleton (arrowheads) have formed in the larva.

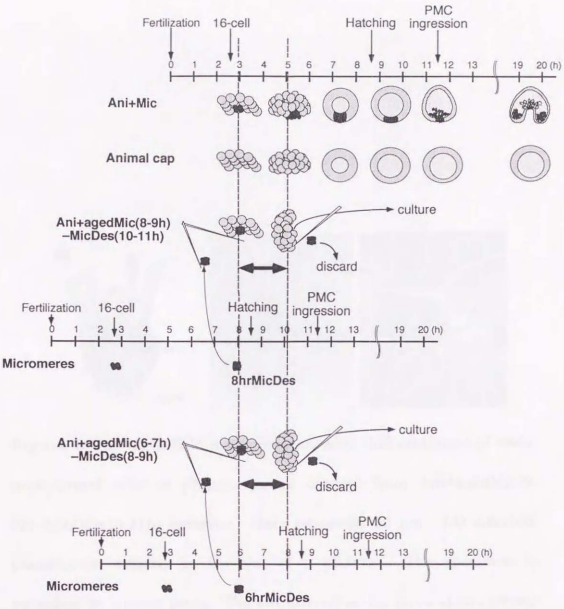


Figure 3-11. Experimental protocol employed for formation of embryos derived from an animal cap isolated from a 32-cell stage embryo recombined for 2 h with micromere-descendants cultured in isolation for various periods. Scales represent time (h) after fertilization and developmental events in normal *S. mirabilis* embryos (Fertilization, 16-cell, Hatching and PMC ingression). Ani+Mic; Schematic representation of early development through gastrula of an embryo derived from an animal cap (Ani) recombined with a quartet of micromeres (Mic). Animal cap; Schematic representation of early development of an embryo derived from an animal cap. Ani+agedMic(A-Bh)–MicDes(X-Yh); Schematic representation for formation of embryos derived from an animal cap isolated from a 32-cell stage embryo recombined with micromere-descendants. The age of the micromere-descendants at recombination was A to B h post-fertilization. The micromere-descendants were removed from the recombined embryo at 2 h after recombination. The age of the micromere-descendants at removal was X to Y h. Dark arrows show the duration for which the animal cap cells were recombined with the micromere-descendants.

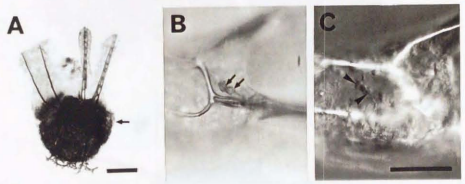


Figure 3-12. Light-field microscopy showing differentiation of endo-mesodermal cells in pluteus larvae derived from Ani+agedMic(8-9h)–MicDes(10-11h) embryos. Bars represent 50 μm. (A) Alkaline phosphatase activity in the gut of a pluteus. The specimen is squashed by a cover glass. The gut (arrow) of the larva shows strong AP activity. A pair of fenestrated post-oral and a pair of simple antero-lateral rods are evident. (B) Pigment cells (arrows) in a larva. (C) Blastocoelar cells with filopodia (arrowheads) in a larva.

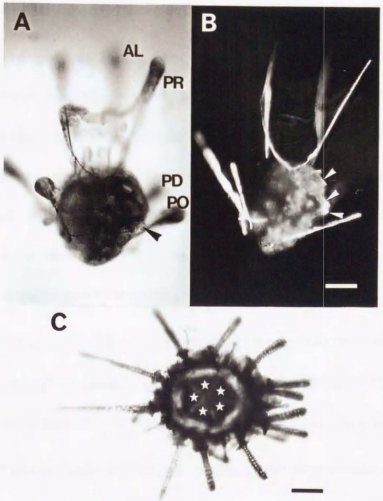


Figure 3-13. An 8-armed pluteus and a metamorphosed juvenile derived from embryos produced from an animal cap recombined with a quartet of micromeres, followed by removal of the micromere-descendants at the early blastula stage at 8 h post-fertilization. Bars represent 100 μm. (A) Light-field observation of an 8-armed pluteus with an echinus rudiment, viewed from the ventral side with the larval mouth uppermost. The echinus rudiment (arrowhead) in this larva has formed on the left side of the body. PO, post-oral arm; AL; antero-lateral arm; PD, postero-dorsal arm; PR, pre-oral arm. (B) Dark-field observation of the same pluteus as that shown in (A). Some juvenile spines (arrowheads) as well as larval skeleton elements are evident. (C) Light-field observation of a metamorphosed juvenile, viewed from the aboral side. The specimen shows complete pentaradiate symmetry with five radial teeth (asterisks).

Conclusion and Perspectives

In the present study, the exact period during which micromeres transmit their inductive signal, and the period for the animal cap is receptive to the signal were examined using embryos derived from an animal cap recombined with the micromeres. In addition, the developmental potential of animal caps that had received the inductive influence of micromeres for various periods was also examined. On the basis of the results obtained, I was able to make the following conclusions:

1: Induced SMC derived from the presumptive ectoderm in embryos derived from the animal cap recombined with micromeres have a differentiation capacity completely identical with that of normal SMC.

2: The developmental stage at which micromere-descendants express the signal responsible for inducing an archenteron in the recombined mesomere-descendants is the early blastula, around hatching. The period necessary for sufficient signal to be transmitted from the micromere-descendants to the recombined mesomere-descendants is less than 2 h.

3: The inductive activity of the micromere-descendants is generally expressed on time, even in cells cultured in isolation.

4: The inductive signal pulse from the micromere-descendants between the early developmental stage and the blastula stage is sufficient to allow an animal cap to develop and metamorphose into a juvenile with

pentaradiate symmetry.

5: The animal cap becomes receptive to the inductive signal from the micromere-descendants between the 16-cell stage and 2 h after the 32-cell stage.

Some problems still remain to be clarified. It appears that micromeres produce two different signals. One (late inductive signal) was found in the present study to be effective at the blastula stage, and the other (early inductive signal) has been reported by Ransick and Davidson (1995) to be expressed at the 16- to 64-cell stage. The early inductive signal is apparently ineffective for inducing an archenteron in animal cap mesomeres, whereas it is effective for specifying the vegetal plate derived from the macromeres into the endodermal fate. Two different possibilities can be postulated to explain the negative effect of the early signal on the animal cap mesomeres. One is a difference in receptiveness to the early signal between mesomeres and macromeres. In this case, the mesomeres are unreceptive to the early signal, whereas the macromeres are receptive. The other possibility is that the early signal has a conditioning effect on the blastomeres. The receptiveness of mesomeres to the micromere signal has been reported to decrease when they are cultured in isolation (Hörstadius, 1936). In contrast, the receptiveness of the animal cap mesomeres appears

to be maintained when they are recombined with micromeres at the 16- to 32-cell stage, as found in the present study. These results suggest that the early signal functions to maintain the competence of mesomeres during the early developmental stages through to the blastula stage.

In order to examine these two possibilities, it is important to clarify whether the competence of mesomeres to react with the inductive signal from micromeres is identical with that of macromeres. In other words, it is necessary to examine the components, such as cell membrane receptors and intracellular regulatory factors, which are necessary for mediation of the inductive signal from micromeres, both quantitatively and qualitatively, between mesomeres and macromeres.

The next problem is the function of the late inductive signal during normal development. The late signal is effective in inducing the animal cap mesomeres to differentiate the archenteron. It appears that the signal is also involved in the normal differentiation of macromeres, although this remains to be examined. Recently, some genes, such as *Endo 16* (Nocente-McGrath et al., 1989), *HpTa* (a sea urchin orthologue of *brachyury*: Harada et al., 1995) and *Hphnf3* (a sea urchin orthologue of *forkhead*: Harada et al., 1996), were found to be expressed in the presumptive vegetal plate region at the swimming blastula stage. The period in which the inductive signal emanates from the micromere-descendants corresponds to the time when the

genes are expressed, or just before. I consider that the signal which emanates from micromere-descendants at the blastula stage functions to trigger the genes in the vegetal plate for the endodermal differentiation of normal embryos. The possibility could be examined by molecular biological approaches, such as *in situ* hybridization and northern blot analysis, for assessing the expression of the genes in the recombined embryos.

Acknowledgements

I would like to express my sincere gratitude to Prof. Shonan Amemiya, University of Tokyo, for his instructions, criticisms, and continuous advice on the whole part of the study.

I am grateful to Prof. Yukihisa Hamaguchi, Tokyo Institute of Technology, for his instructions on the micromanipulation procedures and continuing guidance throughout the course of this study. I would like to thank Dr. Masaaki Yamaguchi of Kanazawa University, Dr. Ritsu Kuraishi of Tohoku University and Dr. Hiroki Nishida of Tokyo Institute of Technology for their valuable advice. I also thank Prof. Ryoichi Arai, Prof. Koichiro Shiokawa, Prof. Fumio Tajima of University of Tokyo and Prof. Takashi Suyemitsu of Saitama University for critical reading of the manuscript. I am very grateful to Prof. Masaaki Morisawa, all the staff members and the students of the Misaki Marine Biological Station, University of Tokyo, for their encouragement during my stay in the station. I am also grateful to all the staff members of the Noto Marine Laboratory of Kanazawa University, Asamushi Marine Biological Station of Tohoku University and Ushimado Marine Laboratory of Okayama University for supplying materials. Thanks are due to Prof. Rudolf A. Raff, Indiana University, for providing B2C2. Thanks are also extended to Messrs. Mizuki Aihara, Minoru Iijima, Shungo Kano, Taku Hibino, Haruyuki Nishigohri and Miss Chisato

Kitazawa for their help and encouragement.

References

Amemiya, S. (1996). Complete regulation of development throughout metamorphosis of sea urchin embryos devoid of macromeres. *Develop. Growth Differ.* 38, 465-476.

Anstrom, J. A., Chin, J. E., Leaf, D. S., Parks, A. L. and Raff, R. A. (1987). Localization and expression of msp 130, a primary mesenchyme lineage-specific cell surface protein of the sea urchin embryo. *Development* 101, 255-265.

Cameron, R. A. and Davidson, E. H. (1991). Cell type specification during sea urchin development. *Trends Genet.* 7, 212-218.

Cameron, R. A., Fraser, S. E., Britten, R. J. and Davidson, E. H. (1991). Macromere cell fates during sea urchin development. *Development* 113, 1085-1091.

Cameron, R. A., Hough-Evans, B. R., Britten, R. J. and Davidson, E. H. (1987). Lineage and fate of each blastomere of the eight-cell sea urchin embryo. *Genes Dev.* 1, 75-84.

Davidson, E. H. (1989). Lineage-specific gene expression and the regulative capacities of the sea urchin embryo: a proposed mechanism. *Development* 105, 421-445.

Ettensohn, C. A. (1990). Cell interactions in the sea urchin embryo studied by fluorescence photoablation. *Science* 248, 1115-1118.

Ettensohn, C. A. (1992). Cell interactions and mesodermal cell fate in the sea urchin embryo. *Development* 1992 Supplement, 43-51.

Ettensohn, C. A. and McClay, D. R. (1988). Cell lineage conversion in the sea urchin embryo. *Dev. Biol.* 125, 396-409.

Ettensohn, C. A. and Ruffins, S. W. (1993). Mesodermal cell interactions in the sea urchin embryo: properties of skeletogenic secondary mesenchyme cells. *Development* 117, 1275-1285.

Fukushi, T. (1962). The fates of isolated blastoderm cells of sea urchin blastulae and gastrulae inserted into the blastocoel. *Bull. Mar. Biol. Stn. Asamushi, Tohoku Univ.* 6, 21-30.

Grainger, R. M. (1992). Embryonic lens induction: shedding light on vertebrate tissue determination. *Trends Genet.* 8, 349-355.

Gustafson, T. and Wolpert, L. (1963). Studies on the cellular basis of morphogenesis in the sea urchin embryo. *Exp. Cell Res.* 29, 561-582.

Harada, Y., Akasaka, K., Shimada, H., Peterson, K. J., Davidson, E. H. and Satoh, N. (1996). Spatial expression of a *forkhead* homologue in the sea urchin embryo. *Mech. Dev.* 60, 163-173.

Harada, Y., Yasuo, H. and Satoh, N. (1995). A sea urchin homologue of the chordate *Brachyury* (*T*) gene is expressed in the secondary mesenchyme founder cells. *Development* 121, 2747-2754.

Harris, P. J. (1986). Cytology and immunocytochemistry. *Methods Cell Biol.* 27, 243-262.

Henry, J. J., Amemiya, S., Wray, G. A. and Raff, R. A. (1989). Early inductive interactions are involved in restricting cell fates of mesomeres in sea urchin embryos. *Dev. Biol.* 136, 140-153.

Hörstadius, S. (1935). Über die Determination im Verlaufe der Eiachse bei Seeigeln. *Pubbl. Stn. Zool. Napoli* 14, 251-479.

Hörstadius, S. (1936). Weitere Studien über die Determination im Keim von *Paracentrotus lividus* Lk. *Roux Arch. Entw. Mech. Organ.* 135, 1-39.

Hörstadius, S. (1939). The mechanics of sea urchin development, studied by operative methods. *Biol. Rev.* 14, 132-179.

Hörstadius, S. (1973). *Experimental Embryology of Echinoderms.* Oxford: Clarendon Press.

Ishimoda-Takagi, T., Chino, I. and Sato, H. (1984). Evidence for the involvement of muscle tropomyosin in the contractile elements of the coelom-esophagus complex in sea urchin embryos. *Dev. Biol.* 105, 365-376.

Katow, H. and Solursh, M. (1980). Ultrastructure of primary mesenchyme cell ingression in the sea urchin *Lytechinus pictus*. *J. Exp. Zool.* 213, 231-246.

Khaner, O. and Wilt, F. (1990). The influence of cell interactions and tissue mass on differentiation of sea urchin mesomeres. *Development* 109, 625-634.

Khaner, O. and Wilt, F. (1991). Interactions of different vegetal cells with

mesomeres during early stages of sea urchin development. *Development* 112, 881-890.

Kiehart, D. P. (1982). Microinjection of echinoderm eggs: apparatus and procedures. *Methods Cell Biol.* 25, 13-31.

Kitajima, T. and Okazaki, K. (1980). Spicule formation *in vitro* by the descendants of precocious micromere formed at the 8-cell stage of sea urchin embryo. *Develop. Growth Differ.* 22, 265-279.

Kiyomoto, M. and Shirai, H. (1993a). The determinant for archenteron formation in starfish: co-culture of an animal egg fragment-derived cell cluster and a selected blastomere. *Develop. Growth Differ.* 35, 99-105.

Kiyomoto, M. and Shirai, H. (1993b). Reconstruction of starfish eggs by electric cell fusion: a new method of detect the cytoplasmic determinant for archenteron formation. *Develop. Growth Differ.* 35, 107-114.

Kuraishi, R. and Osanai, K. (1994). Contribution of maternal factors and cellular interaction to determination of archenteron in the starfish embryo. *Development* 120, 2619-2628.

Livingston, B. T. and Wilt, F. H. (1989). Lithium evokes expression of vegetal-specific molecules in the animal blastomeres of sea urchin embryos. *Proc. Natl. Acad. Sci. USA* 86, 3669-3673.

Livingston, B. T. and Wilt, F. H. (1990). Range and stability of cell fate determination in isolated sea urchin blastomeres. *Development* 108, 403-410.

McClay, D. R. and Logan, C. Y. (1996). Regulative capacity of the archenteron during gastrulation in the sea urchin. *Development* 122, 607-616.

Minokawa, T., Hamaguchi, Y. and Amemiya, S. (1997). Skeletogenic potential of induced secondary mesenchyme cells derived from the presumptive ectoderm in echinoid embryos. *Dev. Genes Evol.* 206, 472-476.

Nakatani, Y. and Nishida, H. (1994). Induction of notochord during ascidian embryo genesis. *Dev. Biol.* 166, 289-299.

Nishida, H. and Satoh, N. (1989). Determination and regulation in the pigment cell lineage of the ascidian embryo. *Dev. Biol.* 132, 355-367.

Nocente-McGrath, C., Brenner, C. and Ernst, S. G. (1989). Endo 16, a lineage-specific protein of the sea urchin embryo, is first expressed just prior to gastrulation. *Dev. Biol.* 136,264-272.

Okazaki K. (1975a). Normal development to metamorphosis. In *The sea urchin embryo.* (ed. G. Czihak). pp.177-232. Berlin: Springer-Verlag.

Okazaki, K. (1975b). Spicule formation by isolated micromeres of the sea urchin embryo. *Am. Zool.* 15, 567-581.

Pehrson, J. R. and Cohen, L. H. (1986). The fate of the small micromeres in sea urchin development. *Dev. Biol.* 113, 522-526.

Raff, R. A. (1996). *The Shape of Life.* Chicago: Univ. of Chicago Press.

Ransick, A. and Davidson, E. H. (1993). A complete second gut induced by transplanted micromeres in the sea urchin embryo. *Science* 259, 1134-1138.

Ransick, A. and Davidson, E. H. (1995). Micromeres are required for normal vegetal plate specification in sea urchin embryos. *Development* 121, 3215-3222.

Ruffins, S. W. and Ettensohn, C. A. (1993). A clonal analysis of secondary mesenchyme cell fates in the sea urchin embryo. *Dev. Biol.* 160, 285-288.

Ruffins, S. W. and Ettensohn, C. A. (1996). A fate map of the vegetal plate of the sea urchin (*Lytechinus variegatus*) mesenchyme blastula. *Development* 122, 253-263.

Showman, R. M. and Foerder, C. A. (1979). Removal of the fertilization membrane of sea urchin embryos employing aminotriazole. *Exp. Cell Res.* 120, 253-255.

Tamboline, C. R. and Burke, R. D. (1992). Secondary mesenchyme of the sea urchin embryo: ontogeny of blastocoelar cells. *J. Exp. Zool.* 262, 51-60.

Tanaka, S. and Dan, K. (1990). Study of the lineage and cell cycle of small micromeres in embryos of the sea urchin, *Hemicentrotus pulcherrimus*. *Develop. Growth Differ.* 32, 145-156.

Ubisch, Lv. (1929). Über die Determination der Larvalen Organe und der Imaginalanlage bei Seeigeln. *Wilhelm Roux' Arch. Entwicklungsmech. Org.* 117, 80-122.

Whittaker, J. R. and Meedel, T. H. (1989). Two histospecific enzyme expressions in the same cleavage-arrested one-celled ascidian embryos. *J. Exp. Zool.* 250,168-175.

Wikramanayake, A. H., Brandhorst, B. P. and Klein, W. H. (1995). Autonomous and non-autonomous differentiation of ectoderm in different sea urchin species. *Development* 121, 1497-1505.

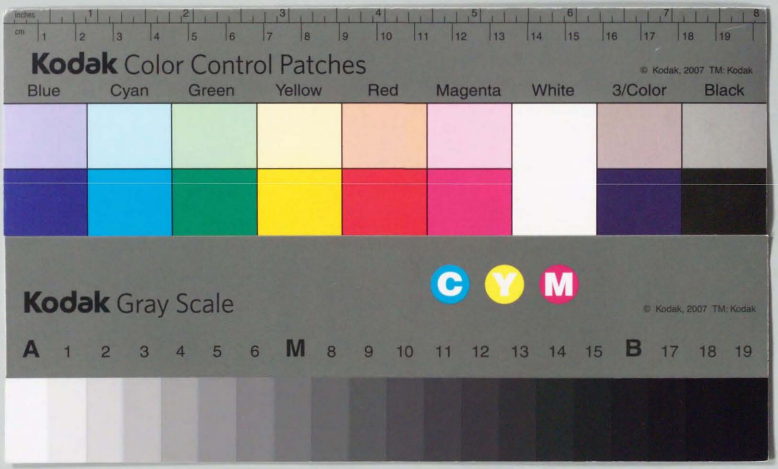
Wilt, F. H. (1987). Determination and morphogenesis in the sea urchin embryo. *Development* 100, 559-575.

Wray, G. A. and McClay, D. R. (1988). The origin of spicule-forming cells in a 'primitive' sea urchin (*Eucidaris tribuloides*) which appears to lack primary mesenchyme cells. *Development* 103, 305-315.

Yamaguchi, M., Kinoshita, T. and Ohba, Y. (1994). Fractionation of micromeres, mesomeres, and macromeres of 16-cell stage sea urchin embryos by elutriation. *Develop. Growth Differ.* 36, 381-387.

Yoshikawa, S.-I. (1997). Oral/aboral ectoderm differentiation of the sea urchin embryo depends on a planar or secretory signal from the vegetal hemisphere. *Develop. Growth Differ.* 39, 319-327.

Yuge, M., Kobayakawa, Y., Fujisue, M. and Yamana, K. (1990). A cytoplasmic determinant for dorsal axis formation in an early embryo of *Xenopus laevis*. *Development* 110, 1051-1056.


inches
cm
Kodak Color Control Patches
© Kodak, 2007 TM: Kodak
Blue
Cyan
Green
Yellow
Red
Magenta
White
3/Color
Black
Kodak Gray Scale
C
Y
M
© Kodak, 2007 TM: Kodak
A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19