Role of Cell Contact in the Specification Process of Pigment Founder Cells in the Sea Urchin Embryo

Authors: Takata, Hiromi, Kominami, Tetsuya, and Masui, Mizuko

Source: Zoological Science, 19(3) : 299-307

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.19.299

ZOOLOGICAL SCIENCE **19**: 299–307 (2002)

Role of Cell Contact in the Specification Process of Pigment Founder Cells in the Sea Urchin Embryo

Hiromi Takata*, Tetsuya Kominami and Mizuko Masui

Department of Biology and Earth Sciences, Faculty of Science, Ehime University, Matsuyama, 790-8577, Japan

ABSTRACT—Effects of LiCl on the specification process of pigment founder cells were examined in the sea urchin *Hemicentrotus pulcherrimus*. If embryos were treated with 30 mM LiCl during 4–7 or 7–10 hours postfertilization, pigment cells increased significantly. Aphidicolin treatment indicated that this increase was due to the increase in the pigment founder cells. Interestingly, if the embryos were treated sequentially with LiCl and Ca^{2+}-free seawater during 4–7 and 7–10 hr, respectively, they differentiated only about the same number of pigment cells as control embryos. Further, the increase was scarcely discerned when the embryos were treated with LiCl in the absence of Ca^{2+} during 7–10 hr. These results suggested that effect of LiCl would be ascribed to the increase in cell adhesiveness. In fact, LiCl-treated embryos were more difficult to be dissociated into single cells. Cell electrophoresis showed that the amount of the negative cell surface charges decreased considerably in LiCl-treated embryos. It was also found that the number of pigment cells seldom exceeded 100, even if embryos were exposed to a higher concentration of LiCl. This suggested that only a subpopulation of the descendants of veg2 blastomeres received the inductive signal emanated from the micromere progeny.

Key words: sea urchin, SMC, pigment cell, cell specification, cell contact

INTRODUCTION

Mesodermal tissues of sea urchin pluteus larvae are composed of two population of mesenchyme cells, primary and secondary mesenchyme cells (PMCs and SMCs, respectively). Processes of PMC differentiation have been well characterized owing to its unique lineage, migratory behavior and skeletogenic properties (Ettensohn, 1992; Wilt, 1999). Another population of mesenchyme cells, SMCs, differentiates into four types of cells, pigment, blastocoelar (Tamboline and Burke, 1989, 1992), circum esophageal muscle (Burke and Alvarez, 1988) and coelomic pouch cells. Several markers have been known for each lineage of SMC-derived cells, except coelomic pouch cells. A monoclonal antibody sp1/20.3.1 binds to the surface of pigment cells (Gibson and Burke, 1985). Expression of *S9*, *CyIIa* (Miller *et al*., 1996), and a homeobox containing gene *SpHmx* (Martinez and Davidson, 1997), become restricted to pigment cells. Myosin heavy chain is specifically expressed in circum esophageal muscles (Wessel *et al*., 1990). A monoclonal antibody SMC2 recognizes blastocoelar cells (Sweet *et al*., 1999).

* Corresponding author: Tel. 089-927-9653;
FAX. 089-927-9630.
E-mail: taka@sci.ehime-u.ac.jp

Ancestors of SMCs are veg2 blastomeres formed after the 6th cleavage, i.e., at the 60-cell stage (Hörstadius, 1973; Okazaki, 1975; Cameron *et al*., 1991). As has been reported, descendants of micromeres play an important role in the specification of SMC lineage (Sherwood and McClay, 1997, 1999). Transplantation experiments done in *Lytechunus variegatus* showed that SMC lineage is specified during the 8-10th cleavage stages with the inductive signal emanated from the micromere progeny (McClay *et al*., 2000). However, it is largely unknown when founder blastomeres of each lineage are specified, or how many times founder cells divide before they differentiate into each type of cells.

In *Hemicentrotus pulcherrimus*, number of pigment cells remarkably decreased when embryos were treated with Ca^{2+}-free seawater (CaFSW) during later cleavage stages (Kominami, 1998). Since this effect was stage-specific, we could know the timing of pigment cell specification. Interestingly, numbers of pigment cells were frequently multiples of 4 in the CaFSW-treated embryos in which pigment cells markedly decreased. This finding revealed the existence of pigment founder cells and unambiguously indicated that the pigment founder cells divide twice before they differentiate into pigment cells. Aphidicolin treatment elucidated the time schedule of cell divisions in pigment lineage, and indicated that the number of pigment founder cells ranged from 10 to 15 (Kominami, 2000).

It has been reported that LiCl treatment increases the number of SMC-derived cells, including pigment cells (Livingston and Wilt, 1990; Sherwood and McClay, 1999). However, quantitative data of LiCl action and the stage-specificity of LiCl treatment are unavailable. Here, we re-examine the effects of LiCl on the differentiation of pigment cells, hoping that experiments from such a point of view would show us different aspects of pigment cell specification. We sometimes experienced that LiCl-treated embryos were adhesive. Using cell electrophoresis, the amount of cell surface charges, which is known to affect cell adhesiveness, was also monitored.

MATERIALS AND METHODS

Materials

Adults of the sea urchin *H. pulcherrimus* were collected during the breeding season and kept in aquaria until use (15°C). Animals and gametes were handled as described before (Kominami and Masui, 1996). Millipore-filtered seawater (MFSW) supplemented with100 units/ml penicillin (Meiji Seika, Tokyo, Japan) and 50 μg/ml streptomycin (Meiji Seika) was used throughout experiments. All embryos were cultured at 18°C.

Treatment with lithium chloride and Ca^{2+}-free artificial seawater

LiCl (Wako Pure Chemicals, Osaka) was dissolved in distilled water (DW) at 0.5 M as stock, and diluted with MFSW before use. Embryos were collected with a hand centrifuge and transferred into MFSW containing 10–50 mM LiCl or into CaFSW. After three hours, embryos were collected with a hand centrifuge, rinsed three times with MFSW, and cultured up to the pluteus stage. In simultaneous treatment with LiCl and CaFSW, 0.5 M LiCl stock solution was diluted with CaFSW.

Counting of pigment cells

For counting of pigment cells, embryonic cells were rounded after the method of Kominami (1998). Briefly, embryos were treated with double strengthened of CaFSW for 20 min, and fixed with 10% formalin dissolved in MFSW. The fixed embryos were mounted on a glass slide with a small drop of glycerin, and covered. To ease the counting, embryos were squashed by absorbing the mounting solution using a piece of blotting paper. Pigment cells were counted directly under a microscope or on photographic prints. Usually, 15 embryos were examined at each observation point.

Measurement of cell diameter

To measure cell diameters, embryos were incubated in 1 M glycine containing 1 mM $MgCl_2$ for 10 min (Kominami, 2000) and dissociated into single cells by several strokes of gentle pipetting. Then a small drop of cell suspension was mounted on a glass slide and covered with a cover slip using two strips of vinyl tape as spacers. Specimens were photographed under Differential Interference Contrast optics, and the diameters of ectoderm and pigment cells were measured.

Blockage of DNA synthesis and cell divisions

Aphidicolin (Wako Pure Chemicals, Osaka) was dissolved in DMSO at 2 mg/ml as stock, and diluted with MFSW at 2 μg/ml before use. This concentration of aphidicolin completely blocked cell divisions so far examined using 2-cell stage embryos (data not shown). Embryos collected at appropriate stages were transferred into the solution, and examined at the 48th hour of development. Embryos cultured in MFSW containing 0.1% DMSO did not show any morphological abnormality and delay in development.

Cell electrophoresis

To remove the fertilization envelope, eggs were inseminated in MFSW containing 1mM aminotriazole (Showman and Foerder, 1979, Nakarai chemicals, Kyoto), and fertilized eggs were passed through nylon mesh (pore size, 82 μm) after 10 min postfertilization. Embryos were rinsed three times with MFSW, and reared in MFSW up to the 7th cleavage stage. The amount of negative surface charges was measured after the methods of Ohshima (1975). The detailed condition for cell electrophoresis was described before (Masui and Kominami, 2001).

RESULTS

Stage specificity of the effects of LiCl treatment

In a previous study, we reported that the number of pigment cells was greatly decreased if embryos were treated with CaFSW from 6.5 to 9.5 hr postfertilization (roughly corresponds to the 7-9th cleavage stage), while treatments during other stages did not influence the number of pigment cells (Kominami, 1998). This suggests that close cell contact

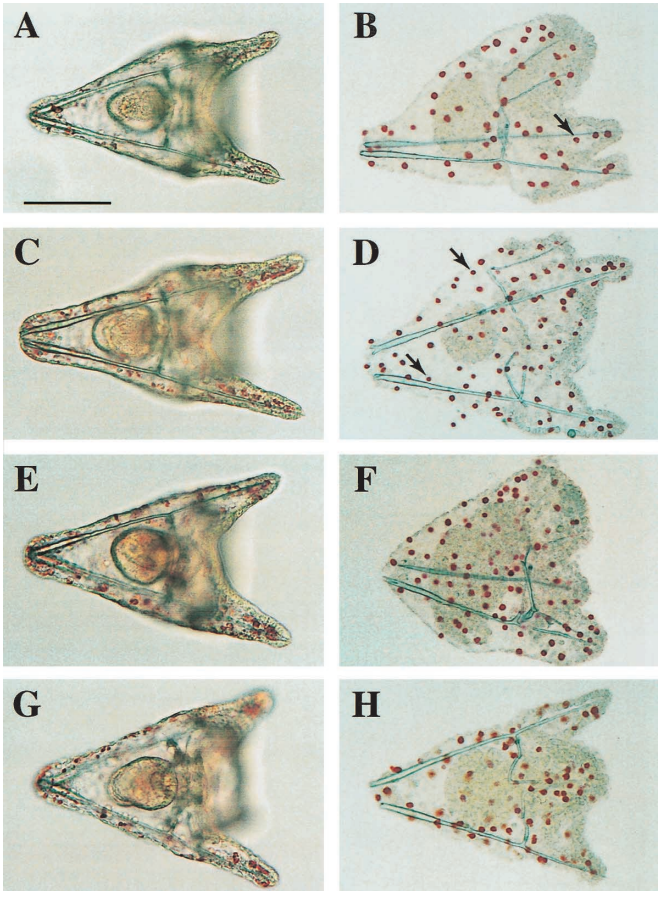

Fig. 1. Pluteus-stage embryos treated with LiCl at different timing. Embryos were treated with 30 mM LiCl every three hours from the 1st to 13th hr of development, and examined at the pluteus stage (48 hr postfertilization). A, B: Control embryos. C, D: Embryos treated from 4 to 7 hours postfertilization. E, F: 7–10 hr. G, H: 10–13 hr. B, D, F and H: squash preparations. Number of pigment cells increased remarkably when embryos were treated during 4–7 or 7–10 hr. Small arrows in B and D indicate the presumably small-sized pigment cells. Scale bar, 100 μm.

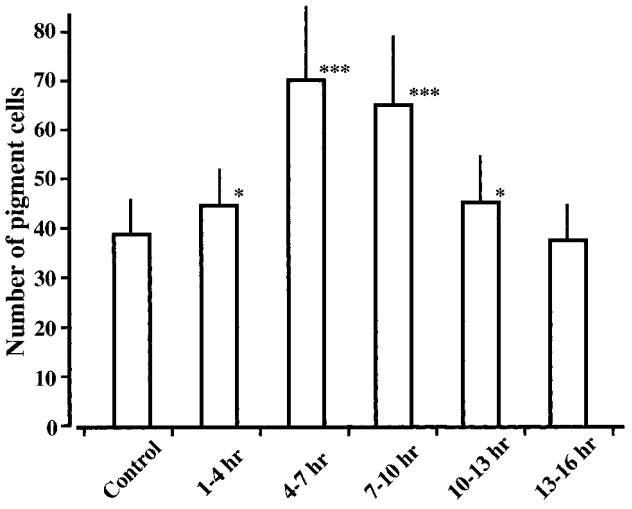

Fig. 2. Effective period of LiCl treatment. Embryos were treated with 30 mM LiCl for three hours at different timing and examined at the pluteus stage. Treatment with LiCl during 4–7 or 7–10 hours postfertilization remarkably increased the number of pigment cells. Treatment during 1–4 or 10–13 hours slightly but significantly increased pigment cells in number. Vertical thin lines indicate S.D. *: P<0.05. ***: P<0.001.

during a specific stage is necessary for the specification of pigment cell lineage. To know whether the effect of LiCl is stage-specific or not, embryos were pulse-treated for three hours from the 1st to 16th hr of development.

The external morphology of the treated embryos (Fig. 1C, E, and G) did not differ from the control embryo (Fig. 1A) at the pluteus stage (48 hours postfertilization). About 50 pigment cells were observed in control embryo (Fig. 1B). In contrast, more than 80 pigment cells differentiated in the embryo shown in Fig. 1D (4–7 hr) and F (7–10 hr). In the embryo treated during 10–13 hr, the number of pigment cells was nearly 50 (Fig. 1H). These suggested that the pulse treatment (three hours) with LiCl would cause the increment of pigment cells stage-specifically.

The same observation was done on 4 batches of embryos and quantified. One of the results is shown in Fig. 2. In this batch of embryos, about 40 pigment cells were observed in control embryos. LiCl treatment during 1–4 hr slightly but significantly increased pigment cells. In the embryos treated with LiCl during 4–7 or 7–10 hr, the averaged number of pigment cells was about 70. Interestingly, the effect of LiCl treatment became weak from the 10th hr onward. No effect was discerned when embryos were treated from 13 to 16th hr of development. These results show that the effective period of LiCl is largely limited to 4–7 or 7–10 hr postfertilization, which roughly corresponds to the 4- 7th or 8-9th cleavage stages, respectively.

Cell divisions in control and LiCl-treated embryos

Neither the arrest of cleavages nor the delay in development was discerned during and after LiCl treatment so far examined under an ordinary microscope. To know whether the time schedule of cell divisions was altered after the treatment, control and LiCl-treated embryos were dissociated into single cells and the diameters of the dissociated cells were measured at the pluteus stage. Pigment cells in control embryos were 8.8 μm in diameter (Fig. 3A). Diameters of pigment cells observed in the embryos treated with LiCl during 4–7 (Fig. 3B) and 7–10 hr (Fig. 3C) were 8.7 and 8.6 μm, respectively. There is no significant difference among these values. The sizes of ectoderm cells in control (Fig. 3D) and LiCl-treated embryos (Fig. 3E) were also quite the same. These data clearly indicated that LiCl did not affect the time schedule of cell divisions after LiCl treatment.

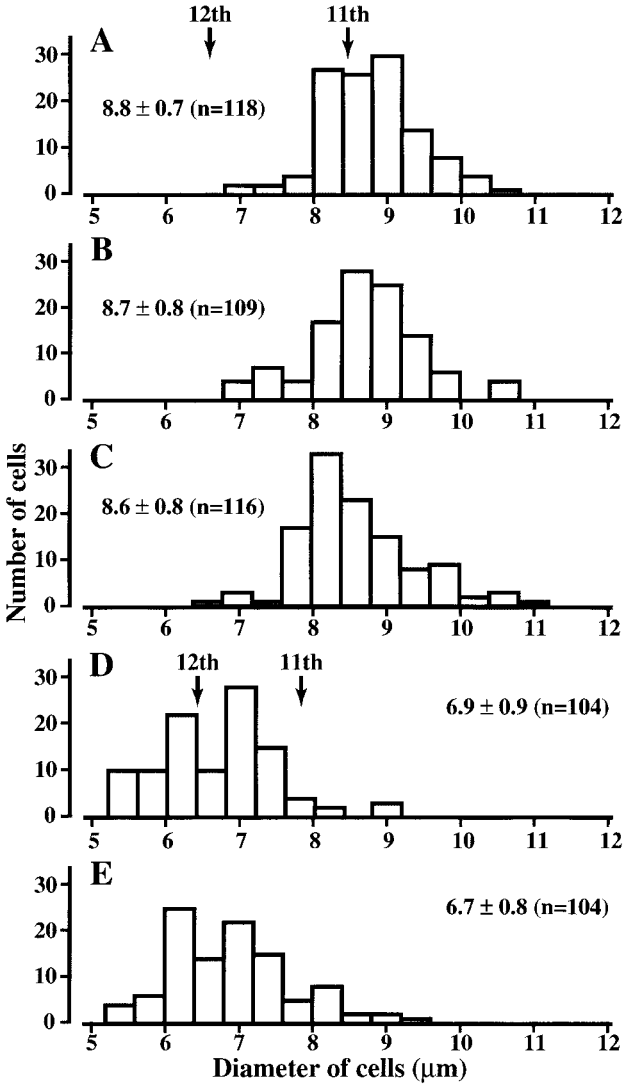

Fig. 3. Diameter of embryonic cells in control and LiCl-treated embryos. Control and LiCl-treated embryos were dissociated into single cells and the diameters of the dissociated cells were measured at the pluteus stage. A: Pigment cells in control wmbryos. B: Pigment cells in LiCl-treated (4–7 hr) embryos. C: Pigment cells in LiCl-treated (7–10 hr) embryos. D: Ectoderm cells in control embryos. E: Ectoderm cells in LiCl-treated embryos (7–10 hr). Arrows in A and D indicate the calculated diameters of pigment and ectoderm cells after the 11th and 12th cleavage.

Estimation of the number of pigment founder cells

Numbers of the pigment founder cells in control and LiCl-treated (7–10 hr) embryos were estimated after the method of Kominami (2000). If the LiCl-treated embryos were treated with aphidicolin from earlier than the 11th hour, they scarcely developed. Therefore, embryos were treated with aphidicolin from 11.5 hr postfertilization onward, and they were processed for counting of pigment cells at the 48th hr of development. About 22 pigment cells differentiated in control embryos (Fig. 4A), while 36 pigment cells were observed in the LiCl-treated embryos (Fig. 4B). Difference between the averaged numbers of pigment cells in control and LiCl-treated embryos is statistically significant ($P<0.001$, $t=8.37$, $n=119$). Thus, about 1.6 times cells in pigment lineage had been already formed in the LiCl treated embryos at 11.5 hr postfertilization. This well matched the increase in the number of pigment cells observed in LiCl-treated embryos at the pluteus stage. Together with the data described in the former section, it can be concluded that the increment of pigment cells is due to the increase in the number of founder cells specified to pigment lineage.

Counteraction of Ca^{2+}-free ASW on the effect of LiCl treatment

How does LiCl increase pigment cells? To address this question, we examined the effects of CaFSW treatment on the LiCl-treated embryos, because CaFSW treatment reduced, in contrast to the action of LiCl, the number of pigment cells (Kominami, 1998). In the squash preparation of a control embryo shown in Fig. 5B, about 50 pigment cells are observed. If the embryos were treated with CaFSW during 7–10 hr postfertilization, the number of pigment cells decreased to about one half of that observed in control embryos (Fig. 5D). As stated in the previous section, LiCl treatment during this period greatly increased pigment cells (see Fig. 1E, F). The embryos shown in Figure 5E and F were treated with 30 mM LiCl during 4–7 hr and then reared in CaFSW during 7–10 hr postfertilization (sequential treatment). About the same number of pigment cells differentiated as in control embryos (Fig. 5F). If the embryos were

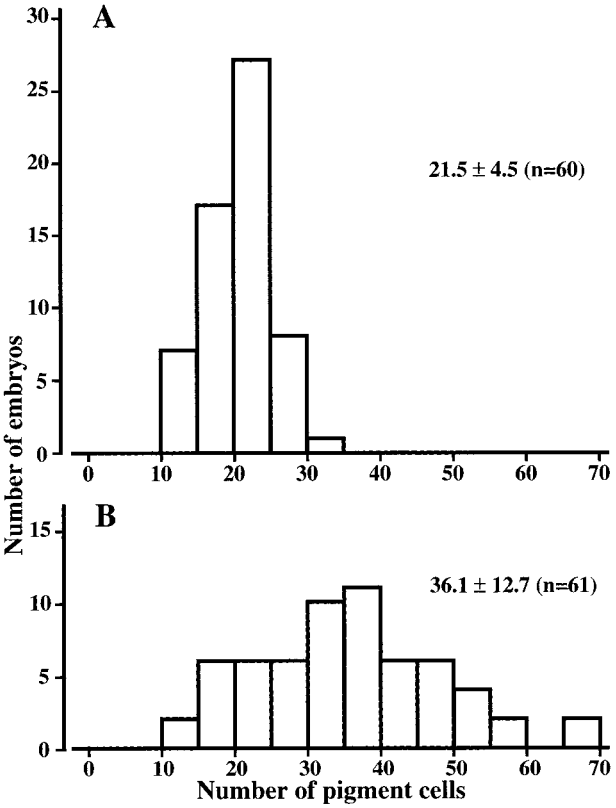

Fig. 4. Number of pigment cells in aphidicolin-treated embryos. Control and LiCl-treated (7–10 hr) embryos were transferred into MFSW containing aphidicolin from 11.5 hr postfertilization onward, and examined at the 48th hr of development. A: Control embryos. B: LiCl-treated embryos. The averaged number of pigment cells in LiCl-treated embryos is significantly larger than control embryos.

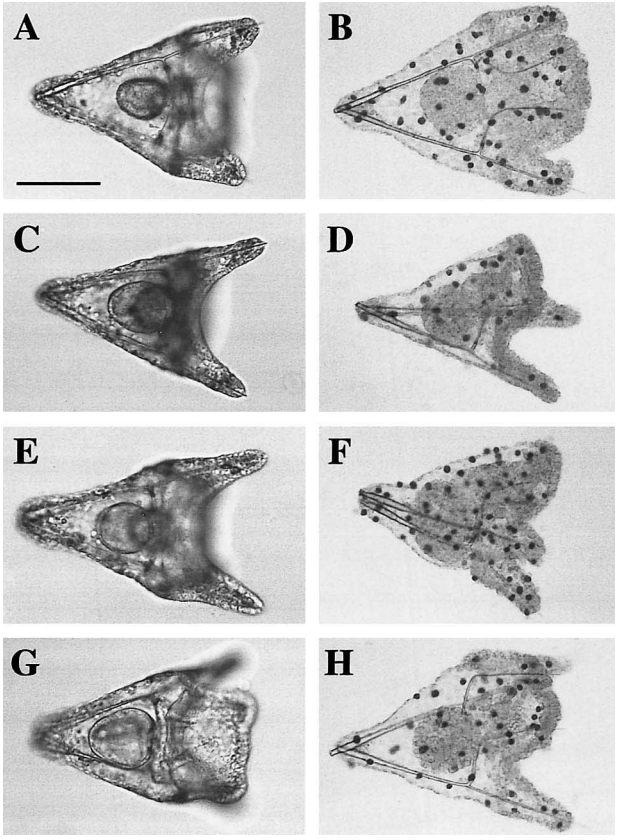

Fig. 5. Effect of Ca^{2+}-free ASW treatment upon the LiCl-treated embryos. A, B: Control embryos. In the embryo shown in B, about 50 pigment cells are observed. C, D: Embryos treated with CaFSW during 7–10 hr. The number of pigment cells greatly reduced. E, F: Embryo treated sequentially with 30 mM LiCl and CaFSW during 4–7 and 7–10 hr postfertilization, respectively. About the same number of pigment cells as in control embryos are observed. G, H: Embryos treated with 30 mM LiCl in the absence of Ca^{2+} during 7–10 hr. The number of pigment cells is not different from control embryos. B, D, F and H: squash preparations. Scale bar, 100 μm.

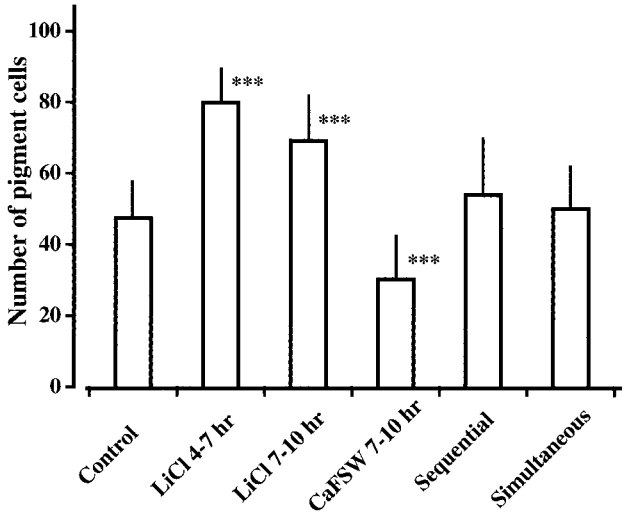

Fig. 6. Counteraction of Ca^{2+}-free ASW treatment on the effect of LiCl. Embryos were treated with LiCl or Ca^{2+}-free ASW, or with both sequentially or simultaneously, and the numbers of pigment cells were examined at the pluteus stage. Control embryos of this batch contained about 50 pigment cells. LiCl treatment during 4–7 or 7–10 hr significantly increased the number of pigment cells. Ca^{2+}-free ASW treatment during 7–10 hr greatly reduced pigment cells in number. If embryos were sequentially or simultaneously treated with LiCl and Ca^{2+}-free ASW, neither the increase nor the decrease in the number of pigment cells was observed. ***: P<0.001.

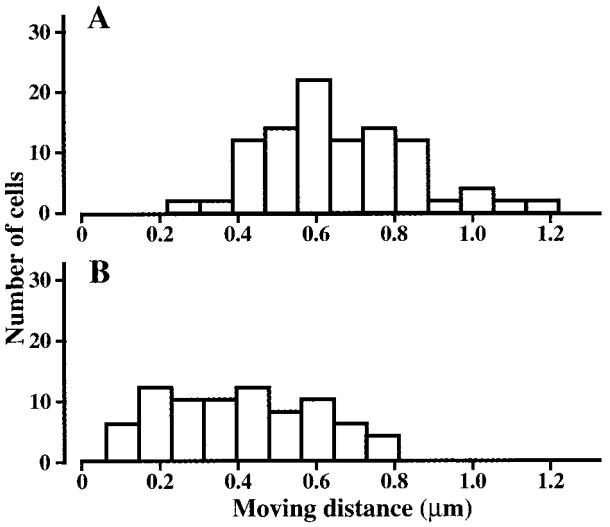

Fig. 7. Electrophoretic mobility of cells in control and LiCl-treated embryos. Control and LiCl-treated embryos (treated from the 4th hr of development) were dissociated into single cells at the 7th cleavage, and the electrophoretic mobility of them was measured with cell electrophoresis. A: control. The averaged electrophoretic mobility is about 0.65 μm/sec/V/cm. B: LiCl-treated. Blastomeres from the LiCl-treated embryos showed lower mobility than control (averaged value is about 0.39 μm/sec/V/cm).

treated with 30 mM LiCl in the absence of Ca^{2+} during 7–10 hr (simultaneous treatment), the number of pigment cells was again almost the same as in control embryos (Fig. 5H). External morphology of the embryos obtained with CaFSW (Fig. 5C), sequential (Fig. 5E) or simultaneous treatment (Fig. 5G) was not different from the control embryo (Fig. 5A).

The same experiment was done on 4 batches of embryos. Fig. 6 shows one of the quantified results. Control embryos in this batch contained about 50 pigment cells. LiCl treatment during 4–7 and 7–10 hr significantly increased pigment cells (P<0.001, t=12.05, n=28 and t =7.30, n=28, respectively). On the other hand, CaFSW treatment during 7–10 hr remarkably reduced pigment cells (P<0.001, t=6.32, n=28). If embryos were sequentially or simultaneously treated with LiCl and CaFSW, neither the increase nor the decrease in pigment cells was observed. In a batch of embryos, the number of pigment cells in the simultaneously treated embryos was significantly larger than control embryos. However, these data clearly indicate that CaFSW cancels the effects of LiCl treatment.

Negative cell surface charges in control and LiCl-treated embryos

The counteraction of CaFSW treatment on the effects of LiCl suggested that the cells would become more adhesive upon LiCl treatment. We suspected that negative cell surface charges decreased in LiCl treated embryos, since the decrease enabled closer apposition of cell membranes due to the reduction of electrostatic repulsion between them. With cell electrophoresis, the amounts of negative cell surface charges of cells in control and LiCl-treated embryos were estimated (Fig. 7). Interestingly, blastomeres from the LiCl-treated embryos showed much lower mobility than control embryos. The averaged mobility of cells in control and LiCl-treated embryos was 0.65, and 0.39 μm/sec/V/cm, respectively. The difference between the two values was statistically significant (P<0.001, t=8.86, n=177). The value of control blastomeres is well coincided those reported in previous reports (Sano, 1977, Masui and Kominami, 2001). Large deviation among observed values is probably due to difference in the mobilities between descendants of meso- and macromeres (Masui and Kominami, 2001).

Maximum number of pigment cells observed in LiCl-treated embryos

Lastly, effect of various concentration of LiCl was examined (Fig. 8). In the control embryo shown in Fig. 8A, 50 pigment cells are observed. If embryos were treated with 10 mM LiCl during 7–10 hr postfertilization, number of pigment cells increased slightly (Fig. 8B). In the embryo treated with more than 20 mM of LiCl, pigment cells considerably increased (Fig. 8C-F). In the embryos treated with 30 mM or much higher concentration of LiCl, number of pigment cells exceeded 90. It should be noted, however, that delay in development or morphological abnormalities were sometimes caused if embryos were treated with 40 or 50 mM LiCl. In this batch of embryos, majority of embryos exogastrulated when treated with 50 mM LiCl. Only a small number of embryos showed rather normal morphology.

Two representative quantified results are shown in Fig.

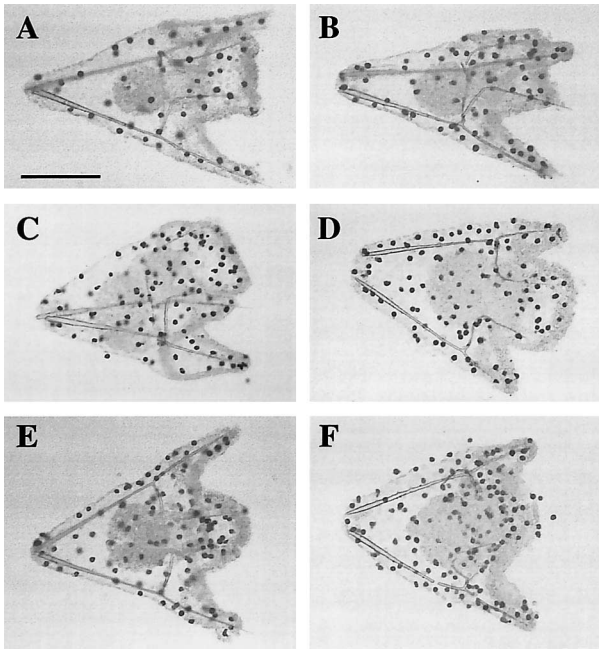

Fig. 8. Effect of different concentration of LiCl on the number of pigment cells. Embryos were treated with 10–50 mM LiCl from the 7 to 10th hr of development, and examined at the pluteus stage. A: Control embryo containing 50 pigment cells. B: Embryo treated with 10 mM LiCl. Pigment cells increased slightly. C: 20 mM. D: 30 mM. E: 40 mM. Numbers of pigment cells are nearly 90. F: 50 mM. Number of pigment cells increased considerably, but embryo was somewhat rounded. Scale bar, 100 μm.

9. In a batch of embryos shown in Fig. 9A, the number of pigment cells (about 50 in control embryos) became larger as the concentration of LiCl was raised. In this batch of embryos, exogastrulae were frequently observed when embryos were treated with LiCl at a concentration of more than 40 mM. Such embryos were excluded from counting, because some pigment cells seemed to be lost from the embryo proper. In another batch of embryos (Fig. 9B), number of pigment cells (about 40 in control embryos) was smaller than the former. Effects of LiCl treatment were not so conspicuous, although the increase in pigment cells was statistically significant. In such batches of embryos, exogastrulae were scarcely observed, even if the embryos were treated with 50 mM LiCl. In this type of embryos, the number of pigment cells reached the plateau level at 30 mM.

Fig. 10 shows the number of LiCl-treated embryos classified according to the number of pigment cells contained. Data obtained from 13 batches of embryos used in this study are collected. All embryos were treated with LiCl higher than 30 mM during 4–7 or 7–10 hr postfertilization. It is clear that the embryos treated with higher concentration of LiCl do not necessarily contain larger number of pigment cells. Of 521 embryos, only 23 embryos (4.4%) contained more than 100 pigment cells. Further, 18 out of 23 embryos were observed in only 2 batches of embryos. Thus, it can be concluded that the number of pigment cells does not exceed 100 even if embryos are treated with a higher concentration of LiCl.

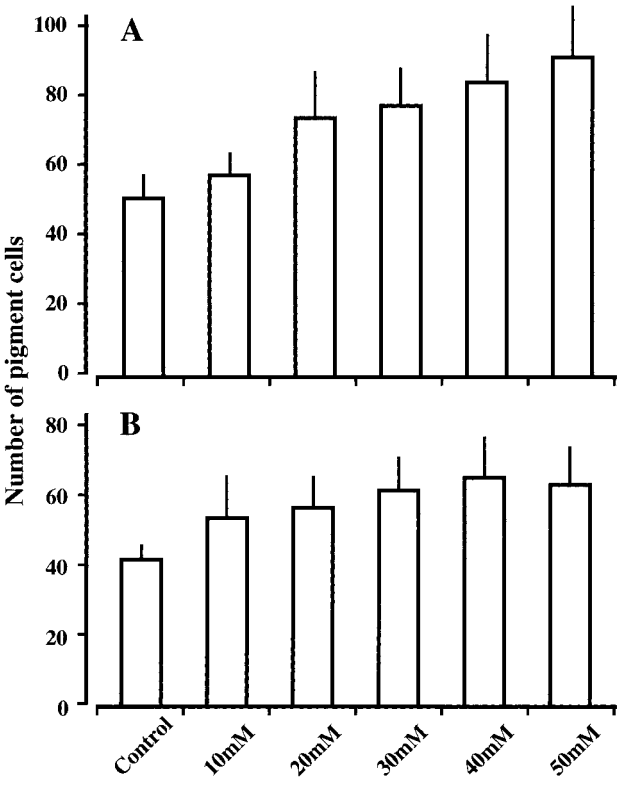

Fig. 9. Increase in the number of pigment cells in the embryos treated with different concentration of LiCl. Number of pigment cells in control or LiCl-treated embryos was obtained at the pluteus stage. Results obtained from two batches of embryos are shown. A: In control embryos, about 50 pigment cells were observed. As the concentration of LiCl rises, the number of pigment cells becomes larger. B: Number of pigment cells in this batch of embryos is rather small. Effect of LiCl treatment is not so conspicuous, although differences in the numbers of pigment cells in control and LiCl-treated embryos are statistically significant. Vertical lines indicate S.D.

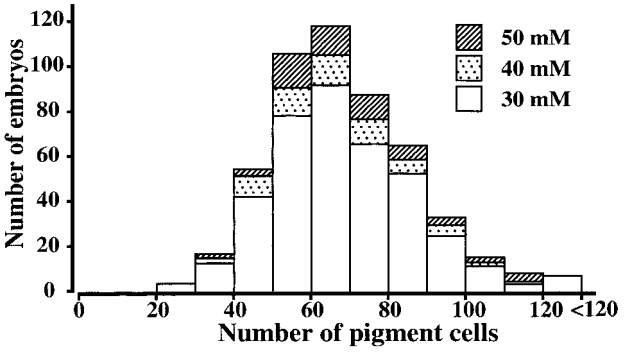

Fig. 10. Number of pigment cells in LiCl-treated embryos. Number of embryos treated with more than 30 mM LiCl during 4–7 or 7–10 hours is plotted against the number of pigment cells contained. Data obtained from 13 batches of embryos are collected. Note that only a small number of embryos contained more than 100 pigment cells. Open: embryos treated with 30 mM LiCl. Dotted: 40 mM. Hatched: 50 mM. Abscissa: Number of pigment cells. Ordinate: Number of embryos.

DISCUSSION

LiCl strengthens cell contact

LiCl has long been known as a potent vegetalizing agent in sea urchin embryos (Hörstadius, 1973). Also in other animals, LiCl exerts profound effects on a wide range of developmental phenomena. For example, *Xenopus* embryos are dorsalized or ventralized upon the application of LiCl (Kao *et al*, 1986; Regen and Steinhardt, 1988; Yamaguchi and Shinagawa, 1989). Although LiCl is suggested to affect the *Wnt* signaling system (McGrew *et al*, 1992; Brannon and Kimelman, 1996; Vonica *et al*, 2000; Akasaka and Shimada, 2001), it is still unclear why and how LiCl influences many aspects of morphogenesis and cell differentiation. Disruption of axial structure caused with LiCl in mouse embryo is not mediated through the beta-catenin/Lef-1 pathway involved in the *Wnt* signaling system (Rogers and Varmuza, 2000). Further, Huynh *et al* (1999) suggested that change in the cell adhesiveness caused with LiCl might be involved in the alteration of the axial structure in *Xenopus* embryos. Thus, physico-chemical aspects of LiCl action should be also considered.

As clearly shown in the present study, LiCl treatment increased pigment cells stage-specifically (Fig. 1, 2). During treatment, arrest or delay in cell divisions was not noticed. The diameter of pigment or ectoderm cells in the LiCl-treated embryos was almost the same as in control embryos (Fig. 3), indicating that time schedule of cell divisions was not disturbed under our experimental conditions. It was also ascertained that the increase was mainly due to the increase in the number of pigment founder cells (Fig. 4). Then, how does LiCl increase pigment cells? Although pigment cells increased in the LiCl-treated embryos (Fig. 1, 2), such increase was canceled when the embryos were followed with CaFSW treatment, which undoubtedly weaken the contact between blastomeres. When LiCl was applied to embryos in the absence of Ca^{2+}, increase in pigment cells was not observed (Fig. 5, 6). Thus, obtained results suggest that LiCl treatment strengthens cell contact.

The amount of negative cell surface charges decreased upon LiCl treatment (Fig. 7). To our knowledge, this is the first report that shows that LiCl decreases the amount of negative cell surface charges in early embryonic cells. Previously, we reported that the amount of negative cell surface charges decreased from the 5-6th cleavage stage, concomitantly with the increase in the adhesiveness of blastomeres (Masui and Kominami, 2001). It is naturally expected that the decrease in the negative cell surface charge makes the close cell apposition possible or makes the cell surface more adhesive. In fact, we sometimes experienced that LiCl-treated embryos were more difficult to be dissociated into single blastomeres. LiCl-induced close cell contact should enable the inducing signal from the micromere progeny to transfer more effectively to the descendants of veg2 blastomeres, resulting in the increase of pigment cells.

Stage-specific effect of LiCl treatment

Further consideration is necessary for explaining the obtained results in detail. In *H. pulcherrimus*, founder blastomeres of pigment lineage are specified after occurrence of the 9th cleavage (Kominami, 1998). Also in *L. variegatus*, SMC lineage is specified around the 9th cleavage (McClay *et al*., 2000). The treatment during 7–10 hr, which included the period of pigment cell specification, increased the number of pigment cells remarkably. However, the most effective period of LiCl treatment spanned from the 4th to 7th hr of development (Fig. 2). It is naturally supposed that effect of LiCl is retained in the treated embryos for a few hours after treatment.

Differentiation processes of SMC-derived cells seem to be divided into three phases. The first phase spans from 16- to 60-cell stage. Interaction between micro- and macromeres during this period is indispensable for the differentiation of meso-endodermal tissues. Deprivation of micromeres from the embryo at these stages causes decrease in the total amount of the meso-endodermal tissues (Ransick and Davidson, 1993, 1995). This decrease is no more rescued by the recombination with micromere progeny during later stages (Sweet *et al*., 1999). Treatment with LiCl during this period may enhance the competence or susceptibility of veg2 blastomeres to the induction signal that is emanated from the micromere progeny. Probably, this enhancement is brought about with the LiCl-induced close cell contact between the descendants of macromeres and micromeres (Livingston and Wilt, 1990; Khaner and Wilt, 1991). Slight increase of pigment cells observed in the embryos treated with LiCl during 1–4 hr (corresponds 1st to 4th cleavage) will be explained with the remaining effect of LiCl treatment, which would enhance the competence of veg2 blastomeres during 4–7th cleavage.

The second phase is defined as the phase when SMC lineage segregates from presumptive meso-endodermal blastomeres. This phase seems to span the later cleavage stages, probably from the 7 to 9th cleavage stage. In the embryo treated with LiCl during 4–7 hr, effect of LiCl seemed to be retained as discussed above. Further, the competence of veg2 blastomeres to the induction signal had been enhanced. This synergistic effect of LiCl would explain why the treatment during 4–7 hr increased pigment cells most effectively (Fig. 2). It is of note that endodermal cells acquire the ability to differentiate autonomously also during this phase (Chen and Wessel, 1996).

Regulation of division cycle in pigment lineage

Once the second phase ends, no cell seems to be added to mesodermal lineage. The third phase will be defined as the period when some types of mesodermal cells lineage retain the ability to change their fate according to the disturbance of normal developmental environment. The third phase seems to span from the 10th cleavage to late gastrula stage. It should be reminded that the specification to the endodermal tissues lasts up to the early prism stage (Logan

and McClay, 1997; Martins *et al*, 1998; Ransick and Davidson, 1998, Sherwood and McClay, 2001). As is well known, subpopulation of SMCs converts their fate into skeletogenetic phenotype if PMCs are removed from the blastocoel (Ettensohn and McClay, 1988; Ettensohn and Ruffins, 1993).

Treatment during 10–13 hr (beginning of the third phase) slightly but significantly increased the number of pigment cells. Before this period, however, the specification of pigment founder cells had already completed. Then, why does LiCl treatment during this stage cause increment of pigment cells? As mentioned above, most of the pigment founder cells divide twice before they differentiate into pigment cells. In fact, pigment cells were larger than the ectodermal cells that had undergone 12 rounds of cell cleavage (Fig.3). This suggests that the division cycle in pigment lineage is under a control mechanism different from that regulates the division cycle of other type of cells. It is interesting that CaFSW treatment around the 10th cleavage stage increased the number of pigment cells (Kominami, 1998). This increase was mainly due to the change in the number of cell cycles in the pigment founder cells after specification; most of the founder blastomeres of pigment lineage divided three times in CaFSW-treated embryos. Thus the small-sized pigment cells were frequently observed.

The averaged diameters of pigment cells that differentiated in control and LiCl-treated embryos are not statistically different (Fig. 3). During the course of this study, however, we noticed that some pigment cells that differentiated in the LiCl-treated embryos were smaller than the normal-sized pigment cells (Fig. 1, arrows). Although sizes of pigment cells could not be compared strictly on squash preparations, LiCl seemed to disturb the control mechanism that regulates the division cycle of pigment lineage, as well as CaFSW treatment. Disturbance in such control mechanism would explain the slight increase in the number of pigment cells observed in the embryos treated with LiCl during 10–13 hr.

Number of pigment cells does not exceed one hundred

Except a few cases, the number of pigment cells did not exceed 100 even if the embryos were exposed to a higher concentration of LiCl than usually used (Fig. 10). This means that the number of pigment founder cells is 25 at most even in the LiCl-treated embryos. Taking the effect of LiCl treatment described in the previous section into consideration, the number of pigment founder cells would be smaller than 25. If veg2 blastomeres strictly repeat horizontal and vertical cleavages mutually, 32 cells align in a circle after the 9th cleavage, contacting directly with micromere progeny. However, such alignment of blastomeres seems to be topologically unstable. From the 7th cleavage onward, some cells should divide horizontally, but the other cells might divide vertically or obliquely. It is likely that the number of blastomeres contacting directly with the micromere progeny is around 20, and only such blastomeres are specified to pigment lineage, although McClay *et al*. (2000) suggested that blastomeres one-cell apart from the micromere progeny are also specified to pigment and blastocoelar cells. Examination of the arrangement of blastomeres near the vegetal pole during later cleavage stages will show us new aspects of SMC specification.

ACKNOWLEDGMENTS

The authors are very grateful to the staff of Tateyama Marine Laboratory for their hospitality and help in collecting animals.

REFERENCES

Akasaka K, Shimada H (2001) Body plan of sea urchin embryo: an ancestral type animal. Zool Sci 18: 757–770

Brannon M, Kimelman D (1996) Activation of *Siamois* by the Wnt pathway. Dev Biol 180: 344–347

Burke RD, Alvarez CM (1988) Development of the esophageal muscles in embryos of the sea urchin *Strongylocentrotus purpuratus*. Cell Tissue Res 252: 411–417

Cameron RA, Fraser SE, Britten RJ, Davidson EH (1991) Macromere cell fates during sea urchin development. Development 113: 1085–1091

Chen SW, Wessel GM (1996) Endoderm differentiation in vitro identifies a transitional period for endoderm ontogeny in the sea urchin embryo. Dev Biol 175: 57–65

Ettensohn CA, McClay DR (1988) Cell lineage conversion in the sea urchin embryo. Dev Biol 125: 396–409

Ettensohn CA (1992) Cell interactions and mesodermal cell fates in the sea urchin embryo. Development (Suppl): 43–51

Ettensohn CA, Ruffins SW (1993). Mesodermal cell interactions in the sea urchin embryo: properties of skeletogenic secondary mesenchyme cells. Development 117: 1275–1285

Gibson AW, Burke RD (1985) The origin of pigment cells in embryos of the sea urchin *Strongylocentrotus purpuratus*. Dev Biol 107: 414–419

Hörstadius S (1973). "*Experimental embryology of echinoderms*", Oxford University Press (Clarendon), London

Huynh MH, Sage EH, Ringuette M (1999) A calcium-binding motif in SPARC/osteonectin inhibits chordomesoderm cell migration during *Xenopus laevis* gastrulation: evidence of counter-adhesive activity in vivo. Dev Growth Differ 41: 407–418

Kao KR, Masui Y, Elinson PR (1986) Lithium-induced respecification of pattern in *Xenopus laevis* embryos. Nature 322: 371–373

Khaner O, Wilt F (1991) Interactions of different vegetal cells with mesomeres during early stages of sea urchin development. Development 112: 881–890

Kominami T (1998) Role of cell adhesion in the specification of pigment cell lineage in embryos of the sea urchin, *Hemicentrotus pulcherrimus*. Dev Growth Differ 40: 609–618

Kominami T (2000) Establishment of pigment cell lineage in embryos of the sea urchin, *Hemicentrotus pulcherrimus*. Dev Growth Differ 42: 41–51

Kominami T, Masui M (1996) A cyto-embryological study of gastrulation in the sand dollar, *Scaphechinus mirabilis*. Dev Growth Differ 38: 129–139

Livingston BT, Wilt FH (1990). Range and stability of cell fate determination in isolated sea urchin blastomeres. Development 108: 403–410

Logan CY, McClay DR (1997) The allocation of early blastomeres to the ectoderm and endoderm is variable in the sea urchin embryo. Development 124: 2213–2223

Martins GG, Summers RG, Morrill JB (1998) Cells are added to the archenteron during and following secondary invagination in the

sea urchin *Lytechinus variegatus*. Dev Biol 198: 330–342
Martinez P, Davidson EH (1997) *SpHmx*, a sea urchin homeobox gene expressed in embryonic pigment cells. Dev Biol 181: 213–222
Masui M, Kominami T (2001) Change in the adhesive properties of blastomeres during early cleavage stage in sea urchin embryo. Dev Growth Differ 43: 43–53
McClay DR, Peterson RE, Range RC, Winter-Vann AM, and Ferkowwicz MJ (2000) A micromere induction signal is activated by beta-catenin and acts through Notch to initiate specification of secondary mesenchyme cells in the sea urchin embryo. Development 127: 5113–5122
McGrew LL, Otte AP, Moon RT (1992) Analysis of *Xwnt-4* in embryos of *Xenopus laevis*: a Wnt family member expressed in the brain and floor plate. Development 115: 463–473
Miller RN, Dalamagas DG, Kingsley PD, Ettensohn CA (1996) Expression of *S9* and actin *CyIIa* mRNAs reveal dorso-ventral polarity and mesodermal sublineages in the vegetal plate of the sea urchin embryo. Mech Dev 60: 3–12
Okazaki K (1975) Normal development to metamorphosis. In "*The Sea Urchin Embryos*" Ed by G. Czihak ed, pp 175–232 Springer-Verlag, Berlin
Oshima N (1975) Electrophoretic mobility of se urchin eggs during the division cycle. Dev Growth Differ 17: 19–25
Ransick A, Davidson EH (1993) A complete second gut induced by transplanted micromeres in the sea urchin embryo. Science 259: 1134–1138
Ransick A, Davidson EH (1995) Micromeres are required for normal vegetal plate specification in sea urchin embryos. Development 121: 3215–3222
Ransick A, Davidson EH (1998) Late specification of veg1 lineage to endodermal fate in the sea urchin embryo. Dev Biol 195: 38–48
Regen CM, Steinhardt RA (1988) Lithium dorsalizes but also mechanically disrupts gastrulation of *Xenopus laevis*. Development 102, 677–686
Rogers I, Varmuza S (2000) LiCl disrupts axial development in mouse but does not act through the beta-catenin/Lef-1 pathway. Mol Reprod Dev 55: 387–392
Sano K (1977) Change in cell surface charge during differentiation of isolated micromeres and mesomeres from sea urchin embryos. Dev Biol 60: 404–415
Sherwood DR, McClay DR (1997) Identification and localization of a sea urchin Notch homologue: insights into vegetal plate regionalization and Notch receptor regulation. Development 124: 3363–3374
Sherwood DR, McClay DR (1999) LvNotch signaling mediates secondary mesenchyme specification in the sea urchin embryo. Development 126: 1703–1713
Sherwood DR, McClay DR (2001) LvNotch signaling plays a dual role in regulating the position of the ectoderm-endoderm boundary in the sea urchin embryo. Development 128: 2221–2232
Showman RM, Foerder CA (1979) Removal of the fertilization membrane of sea urchin embryos employing aminotriazole. Exp Cell Res 120: 253–255
Sweet HC, Hodor PG, Ettensohn CA (1999) The role of micromere signaling in Notch activation and mesoderm specification during sea urchin embryogenesis. Development 126: 5255–5265
Tamboline CR, Burke RD (1989) Ontogeny and characterization of mesenchyme antigens of the sea urchin embryo. Dev Biol 136: 75–86
Tamboline CR, Burke RD (1992) Secondary mesenchyme of the sea urchin embryo: ontogeny of blastocoelar cell. J Exp Zool 262: 51–60
Vonica A, Weng W, Gumbiner BM, Venuti JM (2000) TCF is the nuclear effector of the beta-catenin signal that patterns the sea urchin animal-vegetal axis. Dev Biol 217: 230–243
Wessel GM, Zhang W, Klein H (1990) Myosin heavy chain accumulate in dissimilar cell types of the macromere lineage in the sea urchin embryo. Dev Biol 140: 447–454
Wilt FH (1999) Matrix and mineral in the sea urchin larval skeleton. J Struct Biol 126: 216–226
Yamaguchi Y, Shinagawa A (1989) Marked alteration at midblastula transition in the effect of lithium on formation of the larval body pattern of *Xenopus laevis*. Dev Growth Differ 31: 531–541

(Received October 1, 2001 / Accepted November 22, 2001)