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Evolutionary modification of mesenchyme cells in sand dollars in the transition from indirect to direct development

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SUMMARY *Peronella japonica*, an intermediate type of direct-developing sand dollar, forms an abbreviated pluteus, followed by metamorphosis within 3 days without feeding. In this species, ingression of mesenchyme cells starts before hatching and continues until gastrulation, but no typical secondary mesenchyme cells (SMCs) migrate from the tip of the archenteron. Here, I investigated the cell lineage of mesenchyme cells through metamorphosis in *P. japonica* and found that mesenchyme cells migrating before hatching (early mesenchyme cells [EMCs]) were exclusively derived from micromeres and became larval skeletogenic cells, whereas cells migrating after hatching (late mesenchyme cells [LMCs]) appeared to contain several nonskeletogenic cells. Thus, it is likely that EMCs are homologous to primary mesenchyme cells (PMCs) and LMCs are similar to the SMCs of typical indirect developers, suggesting that heterochrony in the timing of mesenchyme cell ingression may have occurred in this species. EMCs disappeared after metamorphosis and LMCs were involved in adult skeletogenesis. Embryos from which micromeres were removed at the 16-cell stage formed armless plutei that went on to form adult skeletons and resulted in juveniles with normal morphology. These results suggest that in *P. japonica*, LMCs form adult skeletal elements, whereas EMCs are specialized for larval spicule formation. The occurrence of evolutionary modifications in mesenchyme cells in the transition from indirect to direct development of sand dollars is discussed.

INTRODUCTION

Animals in most phyla include both direct- and indirect-developing species, which are frequently found in the same family or even in the same genus (Thorson 1950; Mileikovsky 1971). The evolutionary change from indirect to direct development has occurred independently in families in different phyla, but independently evolved direct developers often share several common features (Raff 1996). In sea urchins, direct development has evolved independently several times (Strathmann 1978), exhibiting a wide range of developmental patterns. Both direct- and indirect-developing species are readily found in the same genus, and there are even intermediate types that show the process of evolutionary modification in their development. This variability among closely related species presents an opportunity to investigate the cellular and genetic mechanisms underlying the evolutionary transition from indirect to direct development, and sea urchins and sand dollars are ideal experimental animals for examining these issues.

Modifications in ontogeny through evolution often appear to involve the temporal condensation of larval cellular fates accompanied by the early appearance of an adult program of development (Raff 1987). In typical indirect-developing sea urchins, two kinds of mesenchyme cells occur: primary mesenchyme cells (PMCs) that derive from four micromeres at the 16-cell stage embryo, ingress from the vegetal plate after hatching, and serve exclusively as skeletogenic cells (Boveri 1901; Hörstadius 1939); and secondary mesenchyme cells (SMCs) that derive from macromeres, ingress from the tip of the archenteron, and contain several types of progenitor cells such as pigment cells, blastocoelar cells, muscle cells, and coelomic pouches (Dan and Okazaki 1956; Gustafson and Kinnander 1956; Ishimoda-Takagi et al. 1984; Gibson and Burke 1985; Burke and Alvarez 1988; Tamboline and Burke 1992). As illustrated in Fig. 1, the sand dollar *Peronella japonica* typically forms a two-armed pluteus larva but undergoes metamorphosis without feeding (Mortensen 1921; Okazaki and Dan 1954; Okazaki 1975), and is thus categorized as an intermediate type of direct-developing species (Raff 1987). Although it forms micromeres at the 16-cell stage, the size and number of micromeres is irregular; mesenchyme cell ingression occurs continuously before hatching and no typical SMCs are observed in this species. Compared with the typical indirect developer, it appears that some evolutionary

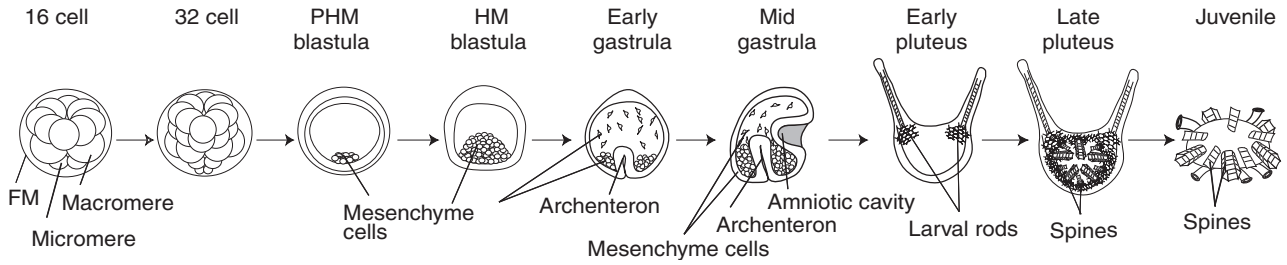

Fig. 1. Schematic views of normal development in *Peronella japonica*. PHM, prehatched mesenchyme; HM, hatched mesenchyme; FM, fertilization membrane.

modification has occurred in *P. japonica* mesenchyme cells. In this study, *P. japonica* embryos were used to demonstrate how the change in timing of ingression and the fate of mesenchyme cells occurred in the process from indirect to direct development.

Echinoderms have endoskeletons as adults, but only echinoids and ophiuroids have larval skeletons. Other classes of echinoderms such as asteroids and holothurians have an equal cleavage at the 16-cell stage and do not have PMCs or larval spicules. Thus, it has long been a mystery what kind of cells direct skeletogenesis in adult asteroids and holothurians, as they do not have PMCs, and whether PMCs contribute to adult skeletogenesis in echinoids and ophiuroids. Lineage tracing would help resolve these issues, but almost no experimental reports exist regarding the cell lineage of late development in indirect-developing echinoderms because of technical difficulties. Long-lived larval stages (a few weeks to more than a month, depending on the species) and extensive growth in larval size (generally more than 10 times larger as metamorphic larvae than as eggs) cause dilution of chemical tracers, and the methylation of exogenous genes introduced by a simple injection method results in the cessation of reporter gene expression in early larval stages (Yajima et al. 2007). *P. japonica* is a promising experimental animal with a convenient developmental style that avoids most of these difficulties. It has the abbreviated larval stage, develops without feeding or larval growth, and reaches the metamorphic stage in only 3 days. These characteristics allowed me to trace the lineage of mesenchyme cells until the juvenile stage. I found that micromere descendants were restricted to larval skeletogenesis and that nonmicromere descendants contributed to adult skeletogenesis. On the basis of these results, the evolutionary modification of mesenchyme cells in echinoids is discussed.

MATERIALS AND METHODS

Gametes and embryos

P. japonica was collected in the vicinity of the Tateyama Marine Laboratory on the Pacific coast of Japan. Gametes and sperm were obtained by injection of 10 mM acetylcholine chloride into the coelomic cavity. Eggs were washed several times with filtered natural seawater (SW). Dry sperm were kept at 4°C until use. Eggs were fertilized and rinsed several times in SW and then cultured at 26°C. Occasionally, eggs were fertilized in SW containing 1 mM aminotriazole (ATA) and then pipetted to remove the fertilization envelope. After being rinsed with SW, denuded eggs were placed in a Petri dish coated with 1.5% agar (agar dish) and filled with SW, and cultured at 26°C.

Transplantation of micromeres

Transplantation of micromeres was carried out following the methods of Wray and McClay (1988) and Amemiya and Arakawa (1996) with some modifications. In brief, at the four-cell stage, denuded eggs prepared as described above were soaked in SW containing 5 μg/ml rhodamine isothiocyanate (RITC; Sigma, St. Louis, MO, USA) for 1 h, rinsed three times with SW, and then placed in another agar dish filled with SW until the 16-cell stage. As *P. japonica* often has an irregular size and number of blastomeres, typical embryos consisting of eight mesomeres, four macromeres, and four micromeres of regular size were selected from the stained and unstained embryos at the 16-cell stage and placed in another agar dish containing Ca^{2+}-free SW. Four micromeres of unstained embryos were removed with a glass needle and placed in another agar dish filled with SW containing 1-U/ml penicillin and 100 μg/ml streptomycin. Four micromeres removed together from the rhodaminated embryos with a glass needle were collected by a mouth pipette and placed on top of macromeres of an embryo from which micromeres were previously removed. These combined embryos were incubated until the next blastomere cleavage to make them aggregate tightly. The fate of micromeres and their relation to skeletogenesis were observed under fluorescence microscopy, differential interference contrast (DIC) microscopy, and polarized light microscopy (OPTIPHOT-POL; Nikon, Tokyo, Japan).

Transplantation of early and late mesenchyme cells

Mesenchyme cell transplantation was carried out as previously described (Ettensohn and McClay 1986; Armstrong and McClay 1994; Hamada and Kiyomoto 2003), with slight modifications. For the transplantation of early mesenchyme cells (EMCs), which ingress before hatching, embryos were fertilized in SW containing 1 mM ATA and cultured until the prehatched blastula stage (5 h after fertilization at 26°C). A portion of prehatched mesenchyme blastulae were soaked in SW containing 5 mg/ml RITC for 1 h to perform vital staining and then rinsed three times with SW. At the prehatched mesenchyme blastula stage, the RITC-labeled

mesenchyme cells were transplanted into the blastocoel of unstained embryos as described previously. The number of transplanted cells was around 30, and the manipulated embryos were cultured in a Petri dish filled with SW containing 1-U/ml penicillin and 100 μg/ml streptomycin. To trace the cell fate of late mesenchyme cells (LMCs), which ingress after hatching, a mixture of EMCs and LMCs was transplanted for convenience. Embryos were fertilized in SW and cultured at 26°C until the prehatched mesenchyme blastula stage (6 h after fertilization). Donor embryos were stained with 5 μg/ml RITC as described above in advance of the transplantations and cultured until the prehatched mesenchyme blastula stage (7 h after fertilization). After more than 90% of the embryos hatched and started swimming, both unstained and rhodaminated embryos were placed in micromanipulation chambers (Ettensohn and McClay 1988). The RITC-labeled mesenchyme cells were transplanted into the blastocoel of unstained embryos. The number of transplanted cells was 30–50, and the manipulated embryos were cultured in a Petri dish filled with SW containing 1-U/ml penicillin and 100 μg/ml streptomycin. The fate of transplanted cells and their relation to skeletogenesis were observed as described above.

Immunostaining

To confirm that transplanted cells adherent to spicules were skeletogenic cells, some embryos were stained with the PMC-specific monoclonal antibody P4 (Shimizu-Nishikawa et al. 1990), which recognizes the carbohydrate epitopes of msp130, a 130-kDa membrane glycoprotein specific to PMCs (Shimizu and Matsuda 1988; Shimizu-Nishikawa et al. 1990). The immunostaining method was described previously by Yajima and Kiyomoto (2006). Briefly, the larvae were fixed with cold methanol (−30°C) in a 24-well plate for 1 h, rinsed twice at 30-min intervals in phosphate-buffered saline saturated with calcite (bleached adult tests). This calcite-saturated buffer (PBSC) was used throughout the staining procedures to minimize demineralization of spicules and to facilitate the identification of spicule regions after staining. The specimens were immunostained with the monoclonal antibody P4 (at a dilution of 1:100 to 1:1000) for 3 h at room temperature. After rinsing with PBSC under the same conditions, they were then immunostained with Alexa Fluoro 594 goat antimouse immunoglobulin G (IgG) antibody (Molecular Probes, Eugene, OR, USA) for 3 h at room temperature. After another rinse with PBSC, the stained larvae were mounted and observed under a fluorescence microscope (BX50; Olympus, Tokyo, Japan).

RESULTS

Two kinds of mesenchyme cells in *P. japonica* embryos

P. japonica mesenchyme cells at the mesenchyme blastula stage (PHM/HM blastula; Fig. 1) were referred to as PMCs in Okazaki and Dan (1954), and Amemiya and Arakawa (1996) found that the micromere descendants became larval skeletogenic cells. Although *P. japonica* embryos have mesenchyme cells migrating from the vegetal plate, no mesenchyme cell ingression is observed after gastrulation (Mortensen 1921; Okazaki 1975). Furthermore, the amount of mesenchyme cells was obviously different before and after hatching; a larger number of cells were seen in the blastocoel after hatching (compare PHM blastula and HM blastula; Fig. 1). Thus, the question arises whether these mesenchyme cells were exclusively derived from micromeres and became larval skeletogenic cells.

To address this question, micromere transplantation was carried out at the 16-cell stage (Table 1, Fig. 2A). RITC-labeled micromere descendants were located in the middle of the vegetal plate at the blastula stage (Fig. 2B), and all, including small micromere descendants, ingressed at the prehatched mesenchyme blastula stage (Fig. 2C). After hatching, RITC-negative cells were observed migrating into the blastocoel, and RITC-labeled micromere descendants occupied the top of a pile of mesenchyme cells (Fig. 2, D and E). I traced the micromere descendants and confirmed that these cells were located at the bottom of the blastocoel at the gastrula stage, in the typical pattern of a PMC ring (Fig. 2F), and were exclusively associated close to the larval spicules (Fig. 2G, arrows). These results suggest that mesenchyme cells that ingress before hatching (EMCs) are exclusively derived from micromeres and contribute to larval skeletogenesis, whereas the ones that ingress after hatching (LMCs) are derived from nonmicromeres.

Fate of mesenchyme cells

Mesenchyme cells of the hatched blastula stage (HM; Fig. 1), including both EMCs and LMCs, were labeled with RITC and transplanted into the blastocoel of normal embryos (Table 2, Fig. 3A). RITC-labeled cells were observed in the blastocoel at the gastrula stage together with recipient mesenchyme cells (Fig. 3B); they then started dispersing randomly, even into the ectoderm (Fig. 3C, arrows). At the early pluteus stage, the transplanted cells were associated with larval skeletal rods in more than 95% of the larvae (Fig. 3, D and F, arrows) and also appeared in nonskeletogenic tissues (Fig. 3D, and E, arrows) in approximately 84% of the larvae. RITC-positive cells associated with the larval skeletal mesh-

Table 1. Micromere transplantation (total n^1 = 35)

Structure	% positive larvae[2]
Early mesenchyme cells	100 (n = 17)[3]
Larval rods of pluteus larva	100 (n = 17)
Adult spicules	0 (n = 15)
Juveniles	0 (n = 13)

[1]Total number of created specimens.
[2]The percentage of larvae with RITC-positive cells in each body structure was calculated using the number of larvae indicated.
[3]The number in parentheses indicate the number of larvae that survived and were surveyed at each observation event.

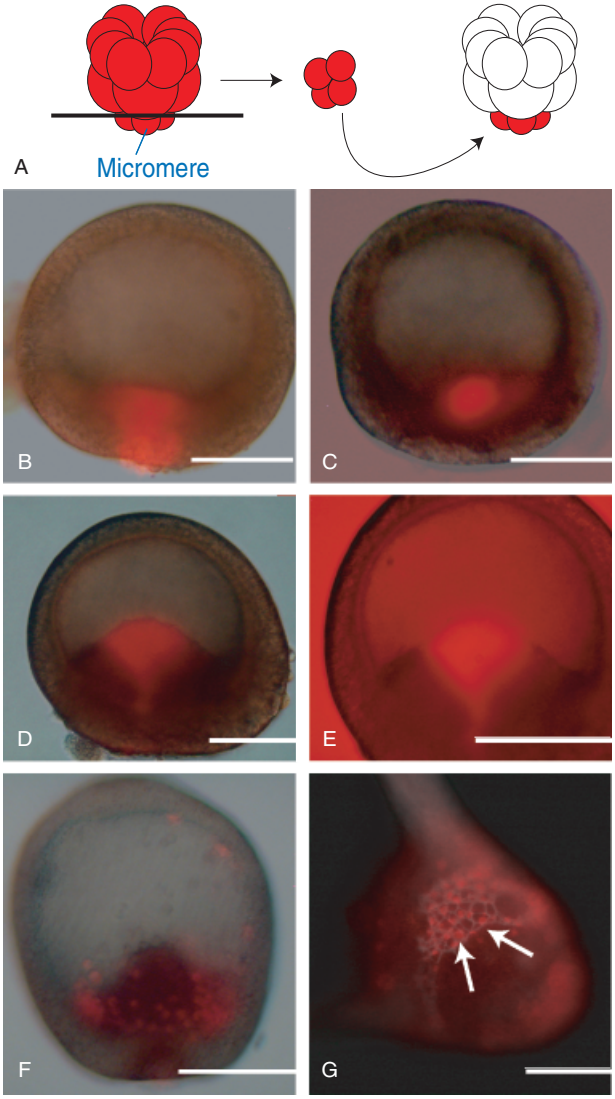

Fig. 2. The lineage trace of micromere descendants. (A) The experimental procedure. (B) Blastula stage. (C) BHM blastula stage. (D and E) HM blastula stage. RITC-negative cells are also observed in the blastocoel. (F) Mid-gastrula stage. (G) Early two-armed pluteus stage. RITC-positive cells are related to larval spicules (arrows). Scale bars = 100 μm.

work that developed from the tips of larval rods (Fig. 3, G and H, arrows) were also observed in 92% of the embryos. These results suggest that these mesenchyme cells include not only larval skeletogenic cells but also several other types of mesenchyme cells. These features are similar to typical SMCs containing several types of nonskeletogenic mesenchyme cells in typical indirect-developing species (Dan and Okazaki 1956).

In the late two-armed pluteus stage, about 95% of the RITC-positive cells were observed on the larval spicules, the spines located in the adult rudiment (Fig. 3I, arrows) and in nonskeletogenic tissues (Fig. 3J, arrows). In juveniles, RITC-labeled cells were observed to be associated with the spines (95%, Fig. 3, K and L, arrows) and also with other parts of the juvenile body (81%), such as tube feet (13.5%, Fig. 3M, arrow). *P. japonica* does not form a skeletal disk at the tip of the tube foot in juveniles or in adults (Fig. 3N); hence, this cell may be a nonskeletogenic cell. These results suggest that these mesenchyme cells include larval and adult skeletogenic cells and nonskeletogenic cells, and that some of them are present after metamorphosis.

Table 2. Transplantation of mesenchyme cells, including EMCs and LMCs (total $n^1 = 90$)

Structure	% positive larvae[2]
Larval rods of pluteus	96 ($n = 83$)[3]
Nonskeletogenic tissues	84 ($n = 83$)
Adult spicules	94 ($n = 54$)
Skeletal meshwork	92 ($n = 54$)
Juveniles (spine)	94 ($n = 37$)
Juveniles (tube foot)	13 ($n = 37$)
Juveniles (others)	81 ($n = 37$)

[1]Total number of created specimens.
[2]The percentage of larvae with RITC-positive cells in each body structure was calculated using the number of larvae indicated.
[3]The number in parentheses indicate the number of larvae that survived and were surveyed at each observation event.

Fate of early mesenchyme cells

P. japonica mesenchyme cells seem to be a mixture of several types of cells and include SMC-like cells. I hypothesized that a shift in timing of typical PMC and SMC ingression may have occurred in this species, and therefore surveyed the lineages of EMCs, which may be similar to typical PMCs (Table 3, Fig. 4A). Around 30 EMCs labeled with RITC were transplanted into normal embryos of the same stage. The transplanted cells were mostly located at the bottom of the blastocoel (Fig. 4, C and D, arrows) at the gastrula stage when spicule formation began (Fig. 4B, arrows); they did not disperse as seen in the previous transplantation including LMCs (Fig. 3C). At early larval stages, just as in the micromere transplantation study (Fig. 2G), the transplanted cells were associated exclusively with larval spicules in 96% of the larvae (Fig. 4E, arrows) and were stainable with the PMC-specific antibody P4 (Fig. 4F, arrows).

In the late larval stage, cells stained with RITC were observed only around the adult rudiment, not inside it (Fig. 4G, arrows); no RITC-positive cells were found along spines. After metamorphosis, RITC-positive cells disappeared, except for those that remained on the remnants of the larval structures, such as degrading larval arms (Fig. 4, H and I, arrows). Exactly the same cell behavior was observed when micro-

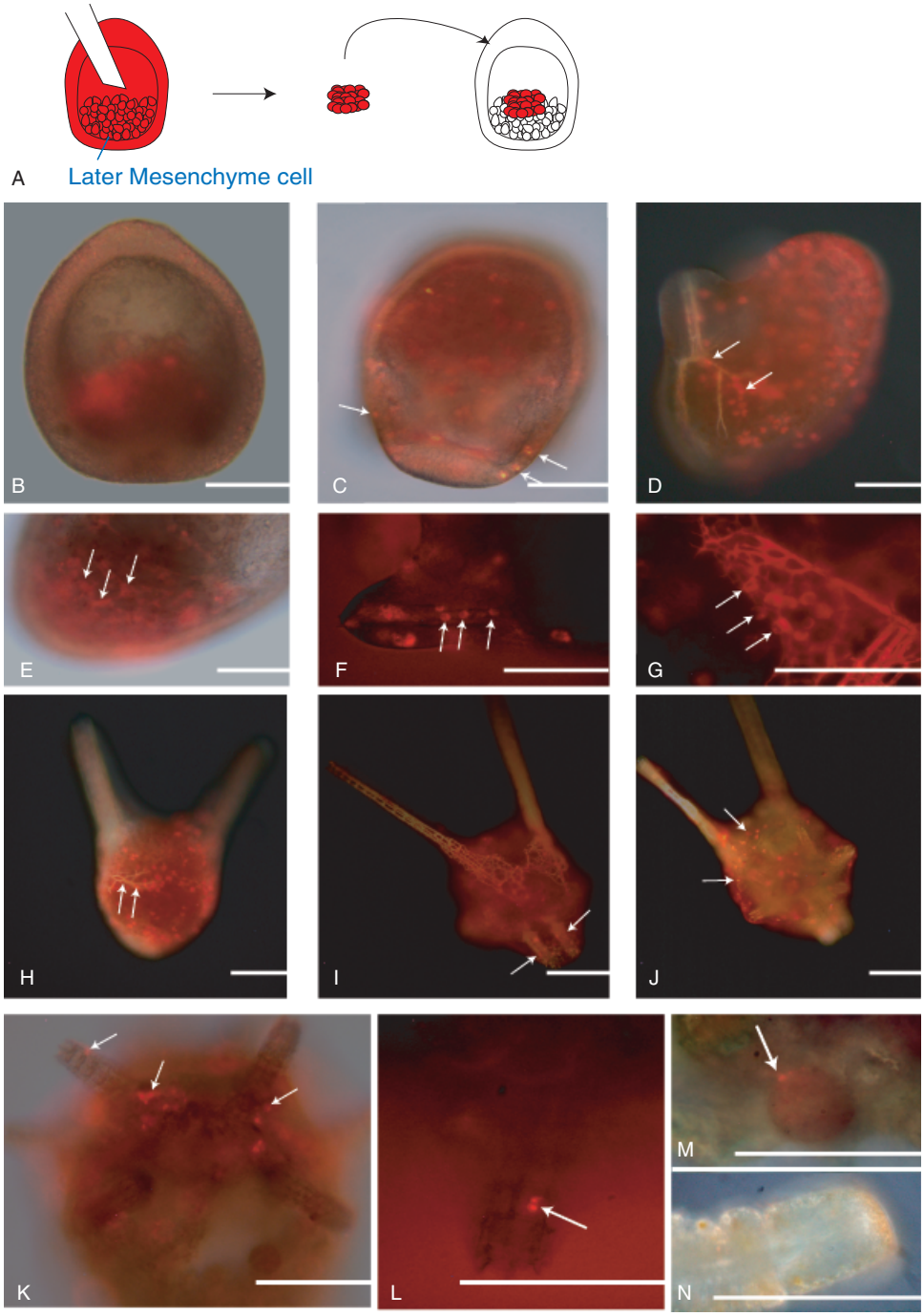

Fig. 3. The lineage trace of mesenchyme cells. (A) The experimental procedure. (B) HM blastula stage. (C) Mid-gastrula stage. Arrow indicates RITC-positive cells located in the ectoderm. (D) Prism stage. RITC-positive cells are located in the various portions of blastocoels and blastoderm. Arrows indicate cells related to the larval spicules. (E) A part of an early pluteus larva focused on the ectoderm. The RITC-positive cells do not seem to be associated with spicules (arrows). (F–H) Early pluteus stage. RITC-positive cells seem to be located on the larval rod (F, arrows) and skeletal meshworks (G and H, arrows). (I and J) Late larval stage. RITC-positive cells are related to spines (I, arrows) and in nonskeletogenic tissues (J, arrows). (K–M) Juvenile sand dollar viewed from the aboral side (K and L), and tube foot viewed from the distal end (M). RITC-positive cells are observed on the spines (K and L, arrows) and on the test plates (K, arrowheads), and in some cases, in the tip of the tube foot. (N) Tube foot of sexually mature adult *Peronella japonica*. Lateral view. No skeletal disk is observed in the tube foot. Scale bars = 100 μm.

meres were transplanted (Table 1). Several hundred RITC-labeled cells were located on larval rods, skeletal meshworks, and around the adult rudiment at the larval stage, but not inside it. However, the number of cells decreased significantly toward metamorphosis and none, except on the degrading larval structures, were observed in juveniles.

These results suggest that EMCs exclusively become skeletogenic cells dedicated to larval skeletogenesis and do not appear to persist after metamorphosis. From these and previous results, it is likely that LMCs include non-skeletogenic cells and adult skeletogenic cells.

Skeletogenesis of micromere-deprived embryos

In typical indirect-developing sea urchins, some cell–cell interactions occur between micromeres and macromeres,

Table 3. EMC transplantation (total $n^1 = 77$)

Structure	% positive larvae[2]
Larval rods of pluteus larva	97 ($n = 70$)[3]
Nonskeletogenic tissues	9 ($n = 70$)
Adult spicules	0 ($n = 57$)
Skeletal meshwork	29 ($n = 57$)
Juveniles	0 ($n = 25$)

[1]Total number of created specimens.
[2]The percentage of larvae with RITC-positive cells in each body structure was calculated using the number of larvae indicated.
[3]The number in parentheses indicate the number of larvae that survived and were surveyed at each observation event.

and between PMCs and SMCs. When PMCs are removed from indirect-developing sea urchins, cell lineage conversion occurs and SMCs are involved in larval skeletogenesis instead of PMCs (Ettensohn and McClay 1988; Hamada and Kiyomoto 2003). In these species, micromeres also induce gastrulation. When micromeres are removed at the 16-cell stage, gastrulation is severely delayed and embryos sometimes fail to complete gastrulation (Ransick and Davidson 1995; Hamada and Kiyomoto 2003).

It seems, however, that *P. japonica* micromeres have less inductive ability than those of typical indirect developers. Some *P. japonica* embryos that do not form micromeres under natural conditions do develop normally (Amemiya and Arakawa 1996), and no delay in gastrulation is observed in embryos from which micromeres are removed at the 16-cell stage (Iijima et al. 2000). To study the micromere features of *P. japonica* in more detail, micromeres were removed at the 16-cell stage (Table 4, Fig. 5A). At the prehatched mesenchyme cell stage, micromere-less embryos began to rotate for hatching, but no ingressed mesenchyme cells were observed (Fig. 5B). This result confirms that the EMCs are derived from micromeres. The micromere-deprived embryos hatched on time and no delay was detected on their subsequent development. Surprisingly, they developed into armless larvae at the early larval stage (Fig. 5, C and D, arrows) but formed adult spicules at the late larval stage (Fig. 5, E–H, arrowheads) and metamorphosed normally to juvenile sand dollars (Fig. 5, I and J). No severe delay in gastrulation or lineage conversion in mesenchyme cells was observed. These results suggest that micromeres appear to exist only for larval skeletogenesis in *P. japonica*.

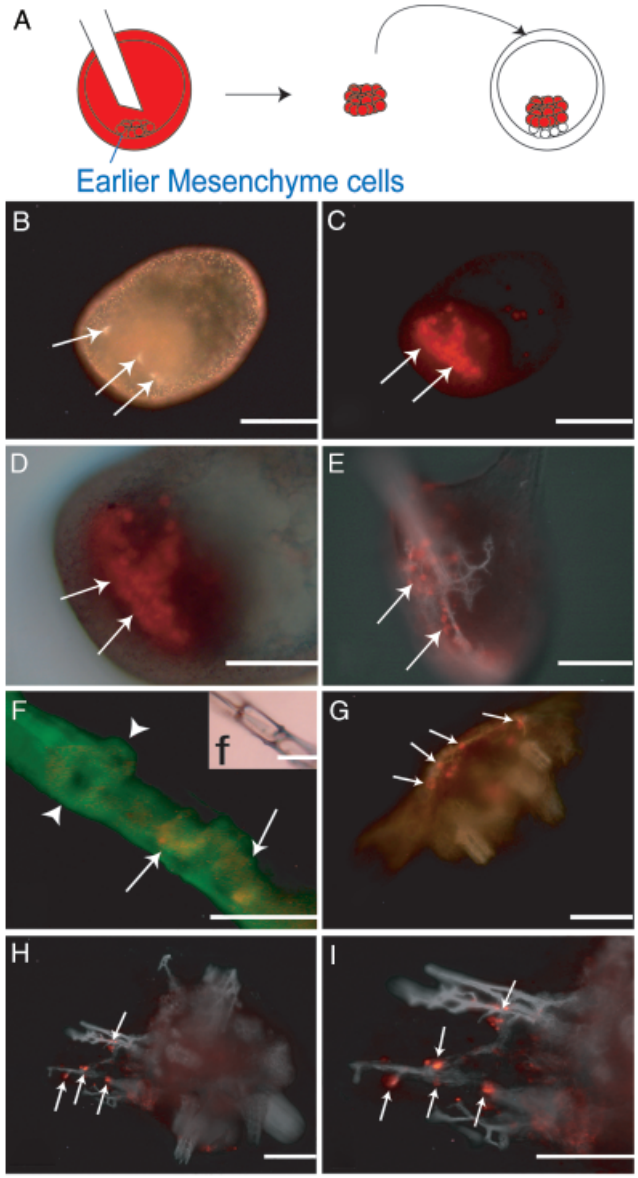

Fig. 4. The lineage trace of early mesenchyme cells (EMC). (A) The experimental procedure. (B–D) Mid-gastrula stage. The rudiments of spicules are created (B, arrows), RITC-positive cells are located at the bottom of the blastocoel along the skeletal rudiments (C and D, arrows). (E) Early pluteus stage. RITC-positive cells are exclusively located on the larval spicules (arrows), F and f (the bright view of F), double staining of RITC and P4 antibody. P4 antibody stains both skeletogenic cells and spicules. Some cells located in the larval rod (arrows) were double-stained both with RITC (red) and P4 antibody (green). The others were stained only with P4 antibody (green). Ectoderm surrounding the skeletal rod is not stained either with P4 or with RITC. (G) Late larval stage. RITC-positive cells are observed only on the test plates around the adult rudiment (arrows); they were not found on the spines. (H and I [enlarged view of H]) juvenile sand dollar. RITC-positive cells are observed only on the degrading larval rods (arrows), and not on the adult structures such as spines and test plates. Scale bars = 100 μm. Scale bars of F and f = 20 μm.

DISCUSSION

EMC and LMC in *P. japonica*

EMCs, ingressed from the vegetal plate at the prehatched blastula stage, formed a cellular ring similar to the typical

Table 4. Micromere-deprived embryos (total n^1 = 38)

Event	% positive larvae[2]
Mesenchyme ingression before hatching	0 (n = 20)[3]
Larval rods of pluteus	0 (n = 18)
Formation of adult spicules	100 (n = 16)
Metamorphosed normally	100 (n = 15)

[1]Total number of created specimens.
[2]The percentage of larvae with RITC-positive cells was calculated using the number of larvae indicated.
[3]The number in parentheses indicate the number of larvae that survived and were surveyed at each observation event.

PMC ring at the onset of spicule formation (Fig. 4, B and C), contained only micromere descendants (Fig. 2, B–E), contributed exclusively to the larval skeleton (Fig. 4E), and were stained with the PMC-specific antibody P4 (Fig. 4, F and f).

According to the criteria proposed by Raff (1987), cells in two different species are considered homologous if similarities are found in fate, time and place of origin, morphology and behavior, cell-lineage-restricted gene expression, and cell lineage. The similarities found between EMCs and typical PMCs satisfy four of the five criteria. The criterion not satisfied is the time of appearance. Differences in the relative timing of PMC ingression can be observed in other cases, such as in *Eucidaris tribuloides* (Wray and McClay 1988) and in several other species of echinoids (Gustafson and Wolpert 1967; Gibson and Burke 1985; Takata and Kominami 2004). Thus, the shift in timing of developmental events is considered a feature of evolutionary change (Gould 1970; Raff and Kaufman 1983; Wray and McClay 1988). Both EMCs and LMCs in *P. japonica* ingress earlier than in most echinoids, but strong evidence exists that the EMCs are similar, and perhaps homologous, to the PMCs of other species.

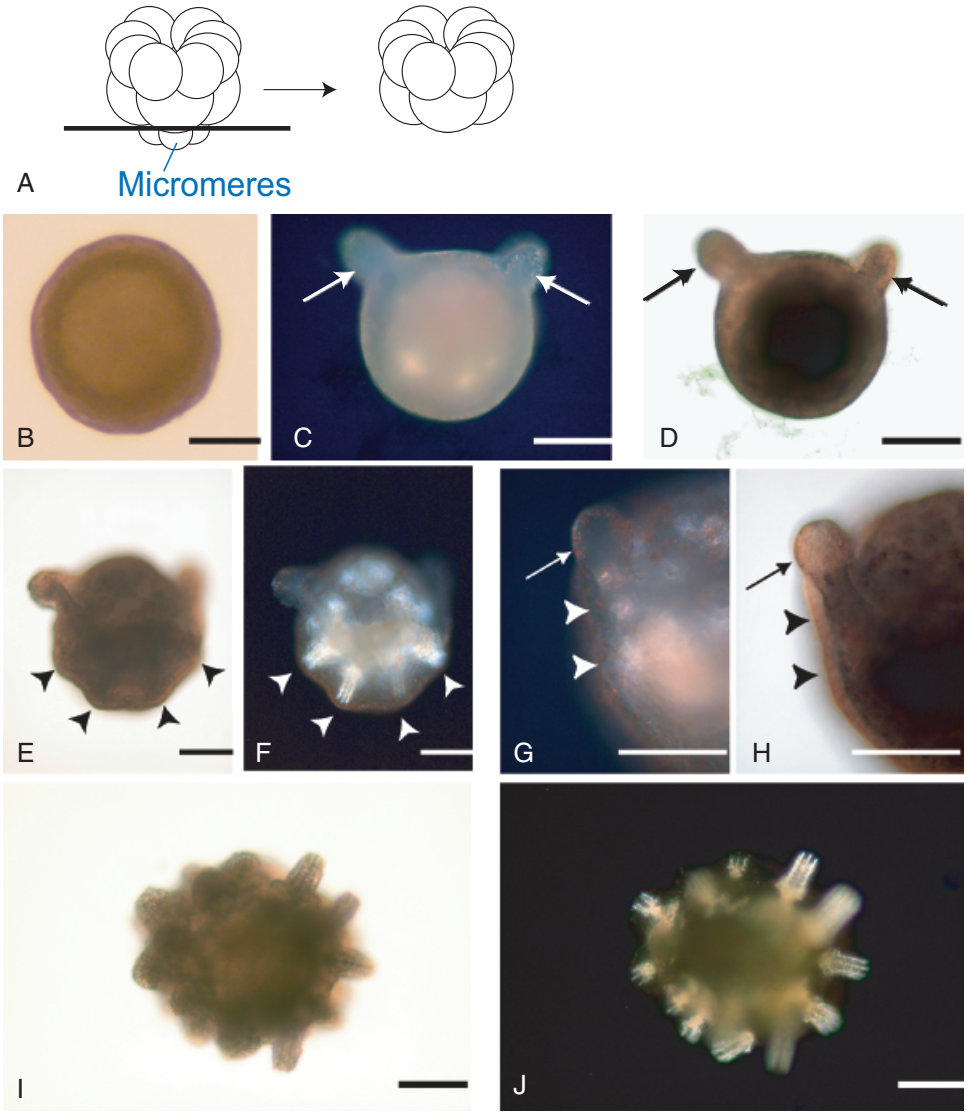

Fig. 5. Micromere-less embryo lacks larval spicules but forms an adult skeleton. (A) The experimental procedure. (B) BHM blastula stage. No mesenchyme ingression is observed. (C and D) Early larval stage. Larval arms do not elongate due to the lack of larval rods (arrows). (E–H) Late pluteus stage. Larvae lack larval rods (arrows) but form adult skeletons (arrowheads). (I and J) Juvenile sand dollar. Micromere-less larva metamorphoses normally. Scale bars = 100 μm.

It is not clear whether the nonmicromere descendants are derived from macromeres and typical SMCs because no SMC-specific marker is available for this species. However, LMCs do appear in embryos without micromeres, and it is clear that this LMC population can give rise to non-skeletogenic cells in the ectoderm and blastocoel. This LMC population is similar to SMCs; they are likely similar to the SMCs of sea urchins and derived from macromeres according to the regular lineage of sand dollar development. SMCs are derived from macromeres in typical indirect developers (Hörstadius 1939), and mesenchyme cells are derived from vegetal blastomeres in direct developers (Wray and Raff 1990).

Involvement of nonmicromere descendants in adult skeletogenesis

Larval and adult skeletogenic cells share several common features, such as the expression of proteins related to the skeletogenesis (reviewed by Wilt 2002) and the morphological and behavioral similarities of spicule-forming cells (Yajima and Kiyomoto 2006). However, it is not known whether PMCs direct adult skeletogenesis in addition to larval spicule formation. Some reports have described adult test plates and pedicellariae directly developed from larval spicules (Gordon 1926; Burke 1979; Wilt 1999; Yajima and Kiyomoto 2006). I found that some skeletal meshworks, which will become a juvenile test plate, appear to be physically connected to larval skeletal rods (Fig. 3H), and RITC-positive micromere descendants were also observed on that same skeletal meshwork (Fig. 4E). However, the micromere descendants/EMCs were never found inside the adult rudiment or on the spines. Furthermore, the number of EMCs seemed to decrease during the progress to metamorphosis, and no RITC-positive cells were observed on the test plates after metamorphosis. In juvenile sand dollars, no micromere descendants were detected except on the remnants of larval structures such as regressing larval rods (Fig. 4, H and I). This suggests that the adult test plates derived from larval rods are formed by EMCs, but the micromere descendants do not persist after metamorphosis. They decrease in number and disappear after metamorphosis (Fig. 4, G and H).

LMCs derived from nonmicromeres, however, appear to be related to adult skeletogenesis. RITC-positive cells, which are a mixture of EMCs and LMCs, were observed on the larval rod and also in the adult rudiment and on the spines (Fig. 3I, arrows). They did not disappear upon metamorphosis but were continuously observed on the spines or tests of juvenile sand dollars (Fig. 3, K–M), showing that LMCs include adult skeletogenic cells. The micromere removal experiment (Fig. 5) also clearly indicates that LMCs contribute to adult skeletogenesis. Even though transplantation of LMCs alone has not been performed, it is likely that LMCs contribute only to skeletal meshworks and to adult skeletons, and not to larval rods, because embryos lacking EMCs developed skeletal meshwork and adult spines although their larval rods were completely absent (Fig. 5, G and H). Other interpretations are possible; for example, the LMCs of micromere-deprived embryos may not have received signals from micromeres and thus could not differentiate into larval skeletogenic cells. However, micromere signals seem to be absent or unnecessary in *P. japonica* development (Iijima et al. 2000). These results and facts suggest that in *P. japonica*, micromere descendants contribute to larval skeletogenesis, nonmicromere descendants direct adult skeletogenesis, and that both contribute to the creation of skeletal meshworks (summarized in Fig. 6).

Evolutionary pathway of mesenchyme cells in the transition from indirect to direct development

It is thought that direct-developing sea urchins evolved from indirect developers because nonfeeding sea urchin larval forms are more morphologically diverse compared with the broad similarity in the larval feeding modes of indirect developers (Strathmann 1978; Raff 1996). Diverse direct devel-

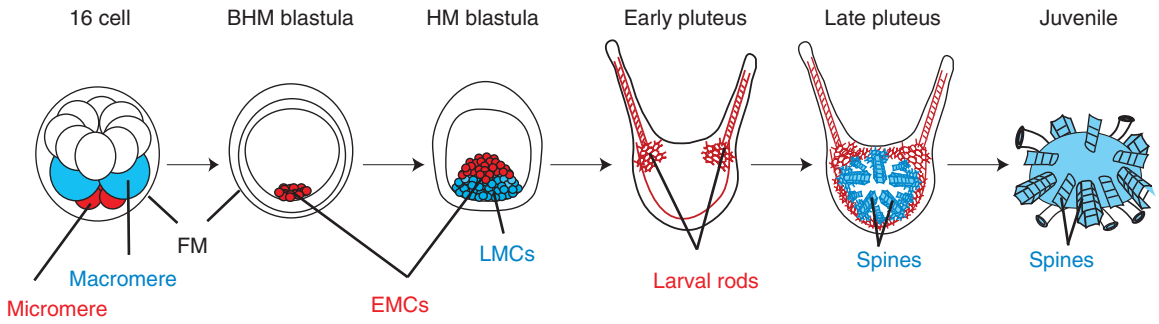

Fig. 6. A brief fate map of skeletogenic cells in *Peronella japonica*. The fate of micromere descendants is colored red and the fate of nonmicromere descendants is colored blue. In this illustration, it is assumed that LMCs are derived from macromeres according to the regular lineage of macromeres or vegetal blastomeres in indirect or direct developers, respectively.

opers, however, have several common features in their early development, such as the absence of typical SMCs, equal cleavage at the 16-cell stage that results in the loss of micromeres, and a number of precocious ingressions of mesenchyme cells from the vegetal plate. *P. japonica* shows several intermediate features between indirect and direct developers, such as intermediate egg size (average 276 µm; Amemiya and Arakawa 1996), unusual cleavage patterns, such as irregular number and size of micromeres, and timing of cellular ingression of mesenchyme cells. Therefore, it can provide insight into how direct developers arose from indirect developers in echinoid evolution.

Although evolution is generally not substantively reversible (Dollo's law, reviewed by Gould 1970), the probability of successfully reactivating silenced genes or "lost" developmental programs over evolutionary timescales of 0.5–6 million years has been discussed (Marshall et al. 1994). Thus, it is possible that intermediate forms of *P. japonica* are showing the process of moving back from direct development. In any case, the programs "regained" in that evolution would represent programs that were previously "lost" in the process from indirect to direct development. Therefore, the intermediate features obtained from *P. japonica* could directly or indirectly demonstrate the process of echinoid evolution from indirect to direct development.

Because typical direct developers have an equal cleavage at the 16-cell stage, both larval and adult skeletogenic cells are derived from the same vegetal blastomeres and ingress continuously until/during gastrulation (Williams and Anderson 1975; Raff 1987; Parks et al. 1989; Wray and Raff 1989, 1990). Thus, it is almost impossible to find a difference between larval and adult skeletogenic cells in these species, and it is controversial whether these mesenchyme cells can be referred to as PMCs/SMCs. *P. japonica*, however, forms micromeres at the 16-cell stage. Therefore, it was possible to distinguish EMCs (PMCs) and LMCs (SMCs) from their origins, and I was able to reveal the ones responsible for larval and adult skeletogenesis.

The current data from *P. japonica* indicate the most likely scenario that in the process toward direct development, besides the egg volume becoming larger, the timing of SMC ingression shifted to an earlier stage, which resulted in a large population of mesenchyme cells ingressing from the vegetal plate in direct developers (summarized in Fig. 7). Therefore, mesenchyme cells of direct developers could include both PMCs and SMCs. It is possible that the skeletogenic cells for larval spicules are derived from PMCs (or EMCs) and those for adult skeletons are derived from SMCs (or LMCs) in species that partially form larval spicules as remnants (e.g., *Heliocidaris erythrogramma*). In those that have lost all larval structures (e.g., *Phyllacanthus parvispinus*), it is possible that the PMC lineage was deleted and only SMCs were left as adult skeletogenic cells.

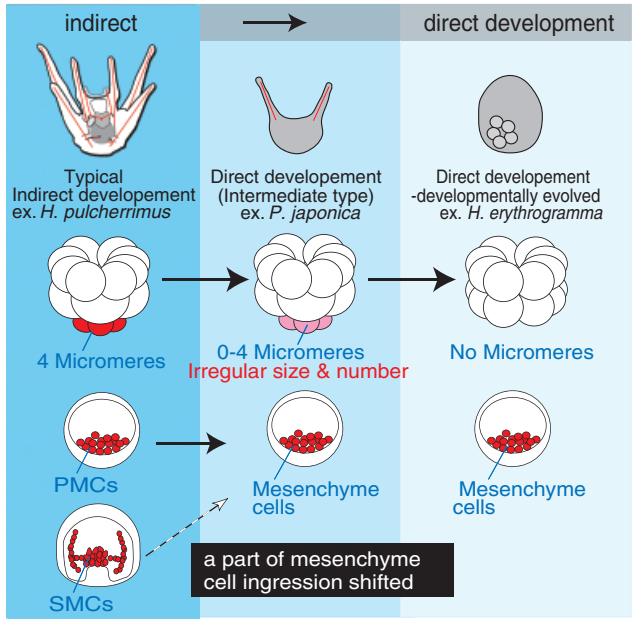

Fig. 7. Hypothetical model of the evolutionary modification of mesenchyme cell ingression in sand dollars. A typical indirect developer has four micromeres, with PMCs and SMCs, and forms a feeding eight-armed pluteus larva. An intermediate direct developer has an irregular size and number of micromeres and no typical SMCs, but has a larger number of mesenchyme cells in early development and forms an abbreviated nonfeeding pluteus larva. A direct developer has no micromeres and no typical SMCs, but has a larger number of mesenchyme cells in early development and lacks a larval stage. In the process toward direct development, a shift in the timing of SMC ingression may have resulted in the loss of micromeres and PMCs in direct developers.

To examine these hypotheses, it would be helpful to investigate, in several direct developers that form remnant larval spicules, whether remnants of PMCs/SMCs occur in the timing of mesenchyme ingression, a localized pattern of precursors is seen in the vegetal plate, or a spatiotemporal patterning in the expression of certain genes exists. Furthermore, the different origins of larval and adult skeletogenic cells observed in *P. japonica* may reflect the developmental style of indirect developers. Data on SMC lineages in typical indirect developers are essential to clarify the origin of adult skeletogenic cells in echinoids and to provide an overview of mesenchyme evolution.

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