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A switch in the cellular basis of skeletogenesis in late-stage sea urchin larvae☆

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Abstract

Primary mesenchyme cells (PMCs) are solely responsible for the skeletogenesis during early larval development of the sea urchin, but the cells responsible for late larval and adult skeletal formation are not clear. To investigate the origin of larval and adult skeletogenic cells, I first performed transplantation experiments in *Pseudocentrotus depressus* and *Hemicentrotus pulcherrimus*, which have different skeletal phenotypes. When *P. depressus* PMCs were transplanted into *H. pulcherrimus* embryos, the donor phenotype was observed only in the early larval stage, whereas when secondary mesenchyme cells (SMCs) were transplanted, the donor phenotype was observed in late and metamorphic larvae. Second, a reporter construct driven by the *spicule matrix protein 50* (*SM50*) promoter was introduced into fertilized eggs and their PMCs/SMCs were transplanted. In the resultant 6-armed pluteus, *green fluorescent protein* (*GFP*) expression was observed in both PMC and SMC transplantations, suggesting SMC participation in late skeletogenesis. Third, transplanted PMCs or SMCs tagged with *GFP* were analyzed by PCR in the transgenic chimeras. As a result, SMCs were detected in both larval and adult stages, but *GFP* from PMCs was undetectable after metamorphosis. Thus, it appears that SMCs participate in skeletogenesis in late development and that PMCs disappear in the adult sea urchin, suggesting that the skeletogenesis may pass from PMCs to SMCs during the late larval stage.
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Introduction

Many echinoderms develop into planktonic larvae in which the left coelomic pouch forms the adult rudiment that gives rise to the juvenile adult at metamorphosis (Hyman, 1955; Kume and Dan, 1957). In sea urchins, adult structures, including skeletal elements such as spines, discs of the primary tube foot, genital plates, and other test plates are all created in advance of metamorphosis in and around the forming adult rudiment (Gordon, 1926). At the onset of metamorphosis, a pentaradial adult structure emerges from inside the larval body. This metamorphic process is dramatic and includes drastic changes in body axis (creation of a novel body axis for the adult), body symmetry (from bilateral to pentaradial), and life style (from planktonic larva to benthic adult; MacBride, 1914; Hyman, 1955). Hence, echinoderm metamorphosis has long attracted the interest of embryologists and cell biologists (Wilt, 2002).

All adult echinoderms have elaborate calcareous skeletons. Sea urchins have two types of mesenchyme cells: primary (PMCs) and secondary (SMCs). PMCs ingress from the vegetal plate before gastrulation and form spicules in sea urchin larvae (Boveri, 1901; Hörstadius, 1939). Brittle stars, another class of echinoderms, also have PMCs and form larval spicules. In contrast, sea stars and sea cucumbers do not form larval spicules and do not have PMCs. They do, however, have mesenchyme cells that migrate from the tip of the archenteron, similar to the SMCs of sea urchins. It is thought that asteroids are the evolutionarily ancient class and echinoids are more derived (Paul and Smith, 1984, 1988). Thus, it is hypothesized that PMCs may have evolved in sea urchins and brittle stars for

☆ The lineage switch of skeletogenic cells from PMCs to SMCs during the late larval stage was investigated using transplantation experiments.

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larval skeletogenesis when they diverged from the main echinoderm lineage. However, it is still unknown whether PMCs are truly specialized for larval skeletogenesis and which cells form adult echinoderm skeletons.

In sea urchins, larval spicules and adult skeletal elements have several features in common. First, several genes and proteins related to skeletogenesis (e.g., *SM50*, *SM30*, *msp130*) have been reported in both larvae and adults (Anstrom and Raff, 1987; Drager et al., 1989; Parks and Raff, 1988; Shimizu-Nishikawa et al., 1990; Wilt, 2002; Yajima and Kiyomoto, 2006). Second, the morphological features and behavior of the larval and adult skeletogenic cells are similar. Cells migrate with filopodia and form a syncytium when they synthesize the skeleton (Yajima and Kiyomoto, 2006). Third, both larval and adult skeletal rudiments primarily start from triradiate spicules, and some juvenile test plates are derived from larval rods (Gordon, 1926; Hyman, 1955). Based on these common features, PMCs may form the adult skeleton as well as the larval spicules.

In contrast, other evidence suggests that SMCs are the likely origin of adult structures in the direct-developing sand dollar *Peronella japonica* (Yajima, 2007). SMCs develop into several kinds of cells such as muscle cells, coelomic pouch cells, pigment cells, and blastocoelar cells in the sea urchin embryo (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). Although SMCs are not known to be skeletogenic cells in normal development, they are possible candidates because they convert their lineage to skeletogenic cells when PMCs are artificially removed at the mesenchyme blastula stage (Fukushi, 1962; Ettensohn and McClay, 1988), suggesting their potential to become skeletogenic cells.

Although considerable experimental results have been reported on mechanisms of early larval skeletogenesis by PMCs, the relationship between PMCs/SMCs and late larval/adult skeletogenic cells has been rarely studied due to technical difficulties; the long larval stage and larval growth have made it difficult to trace cell lineages to adulthood using traditional chemical tracers. Here, genetically marked cells were used to conduct transplantation experiments to overcome these difficulties, and the role of PMCs/SMCs in skeletogenesis in late larvae and adult tissues was clarified.

Materials and methods

Animals, embryos, and larval culture

Adult *Hemicentrotus pulcherrimus* and *Pseudocentrotus depressus* were collected from Boso Peninsula, 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 washed several times in SW and then cultured at 19 °C. Larvae were reared after hatching (12 h after fertilization) according to the method described by Amemiya (1996) with slight modifications. Previously filtered SW was re-filtered through 0.22-mm pore membranes (membrane-filtered seawater, MFSW). At the onset of feeding (2–3 days after fertilization), approximately 20–30 pluteus larvae were cultured in a 50-ml plastic tube containing MFSW at 19 °C, while being gently stirred at 30 rpm with a rotator (TAITEC, Tokyo, Japan). Larvae were transferred to new MFSW twice weekly. From the third day after fertilization, two types of diatoms [*Chaetoceros gracilis* Schutt 1895 and *C. calcitrans* (Paulsen) Takano 1968], cultured as described by Yajima and Kiyomoto (2006), were added to the cultures twice weekly at 0.5 ml ($1–5\times10^5$/ml) per tube. Larvae reached the 4-armed stage after 3–7 days, the 6-armed stage after 7–14 days, and the 8-armed stage after 3–4 weeks, and after 4–5 weeks the competent larvae were placed in a dish containing a plastic plate with attached diatoms to metamorphose. After metamorphosis, the juvenile sea urchins were kept in a separate dish with MFSW, which was replaced twice weekly.

Interspecific PMC and SMC transplantation

P. depressus forms skeletal organs called pedicellariae, located on the adult genital plate at the 8-armed pluteus stage (Figs. 1B, 2M), while *H. pulcherrimus* does not (Figs. 1A, 2L). PMC transplantation was carried out as described by Ettensohn and McClay (1986) and Hamada and Kiyomoto (2003), with slight modifications. The *H. pulcherrimus* and *P. depressus* embryos were fertilized at the same time in glass dishes and cultured until the mesenchyme blastula stage (15 h after fertilization). For total staining of the *P. depressus* embryos, they were transferred to MFSW containing 5 μg/ml rhodamine B isothiocyanate (RITC; Sigma, St. Louis, MO) for 1 h before transplantation. Both *H. pulcherrimus* and *P. depressus* embryos were placed in micromanipulation chambers (Ettensohn and McClay, 1988). *H. pulcherrimus* PMCs were removed (>90%) from the blastocoel with a micropipette with a continuous flow of SW, and PMCs collected from *P. depressus* embryos were transplanted into the *H. pulcherrimus* blastocoel (Fig. 2A, Table 1). The approximate number of transplanted PMCs was 0, 10, or 40, and the manipulated embryos were cultured in a 50-ml plastic tube. Transplanted donor cells were observed under a fluorescent microscope (BX50, Olympus).

SMC transplantation was conducted as described by Hamada and Kiyomoto (2003). The *H. pulcherrimus* and *P. depressus* embryos were cultured until the mid-gastrula stage, and *P. depressus* embryos were stained with RITC as described above. SMCs migrating from the tip of the archenteron were excised using a glass needle and transplanted into the blastocoel of a mid-gastrula *H. pulcherrimus* embryo. Transplanted donor cells were observed under a fluorescent microscope (BX50, Olympus) at the prism or 4-armed pluteus stage. Body-rod formation in the 4-armed pluteus stage, pedicellaria formation in the 8-armed pluteus, and other skeletal elements were observed by polarizing microscopy (OPTIPHOT-POL, Nikon).

PMC/SMC transplantation between transgenic embryos

The *Arylsulfatase* insulator (ArsI) has anti-silencing activity and maintains transgene expression longer in sea urchin embryonic and larval development (Yajima et al., 2007). Hence, in this study, I used a construct containing ArsI as a genetic tracer. The plasmid pGFP-ArsI-ArsI-*SM50* was constructed, containing a basal promoter region (−437 to +126) of the *spicule matrix protein 50* gene (*SM50*), which is well characterized (Makabe et al., 1995), tandem repeats of two ArsI elements connected to the 5′ end of the *SM50* promoter sequence, and the *green fluorescent protein* (*GFP*) reporter gene derived from the vector green lantern-1 (Gibco BRL) on the 3′ end of the *SM50* promoter. The ArsI-ArsI-

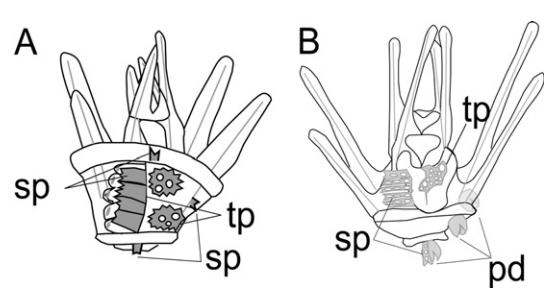

Fig. 1. Schema of normal 8-armed pluteus. (A) 8-Armed pluteus of *Hemicentrotus pulcherrimus*. (B) 8-Armed pluteus of *Pseudocentrotus depressus*. sp, Spine; pd, pedicellaria; tf, tube foot.

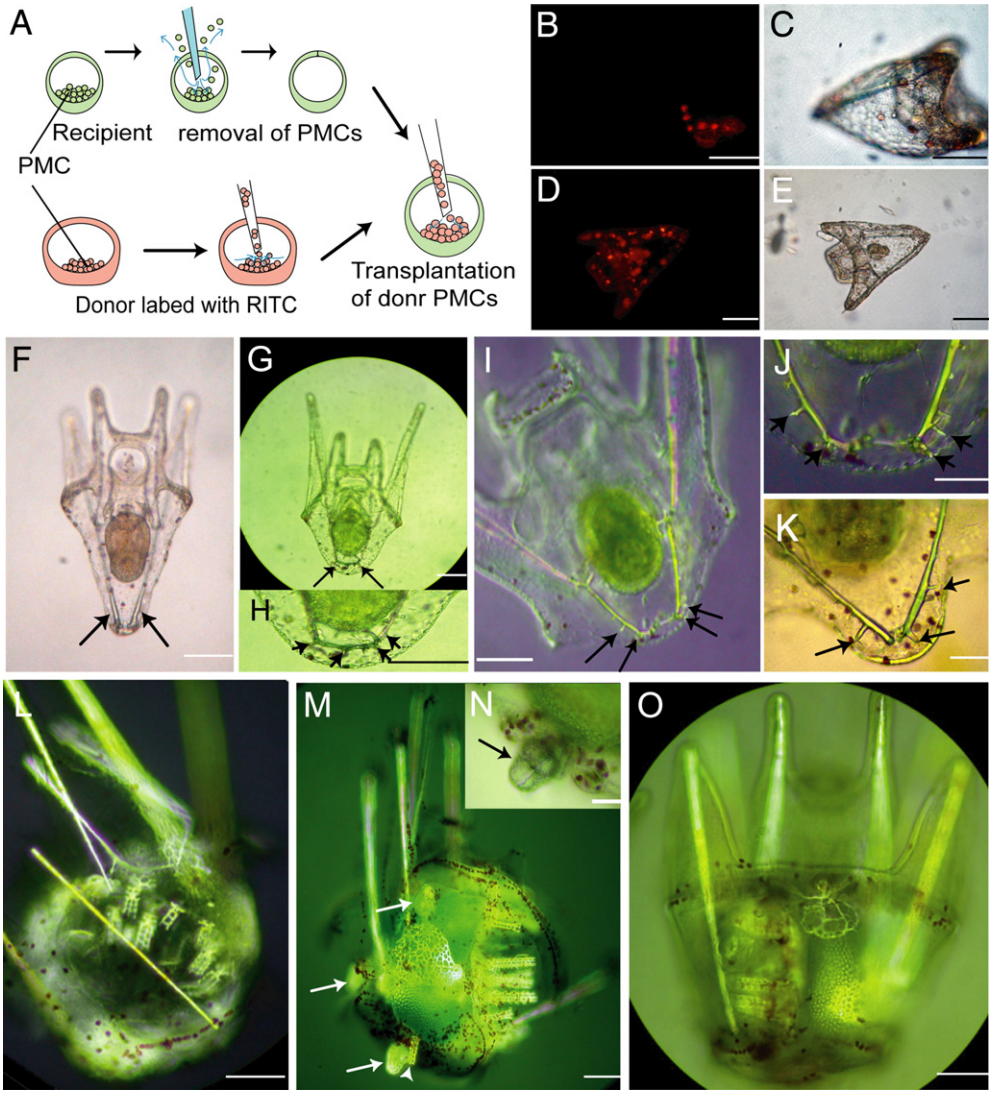

Fig. 2. Transplantation of primary mesenchyme cells (PMCs) between *Hemicentrotus pulcherrimus* and *Pseudocentrotus depressus*. (A) Experimental procedure. (B–E) Transplanted PMCs stained with RITC were observed at the 4-armed pluteus stage. RITC-positive cells were located on the larval spicules when 10 PMCs (B and C) and 40 PMCs (D and E) were transplanted. (F) Normal 4-armed *H. pulcherrimus* larva. The tip of the body rod is straight and smooth (arrows). (G and H) (magnification of G) Normal 4-armed *P. depressus* larva. The body rod is curved and spiny at the end (arrows). (I and J) (magnification of I) Chimera larvae formed a donor-type body rod (arrows). (K) Body rod (arrows) of intermediate-type chimera. (L) Normal 8-armed *H. pulcherrimus* larva. (M and N) (magnification of M) Normal 8-armed *P. depressus* larva forms three pedicellariae (white arrows; black arrow in N). (O) Chimeras did not form pedicellariae and appeared similar to normal *H. pulcherrimus* larvae. Scale bar = 100 μm.

SM50-GFP construct was introduced into fertilized eggs following the method described by Akasaka et al. (1995; Fig. 4A), using 6 ng/μl of the construct, 30 ng/μl of *H. pulcherrimus* sperm DNA as the carrier linearized with the appropriate restriction enzyme, and the fluorescent marker Alexa594-dextran (0.02%) in 40% glycerol. Approximately 6 pg of each construct was injected into fertilized eggs. PMCs expressing *GFP* at the mesenchyme blastula stage or SMCs at the mid-gastrula stage were transplanted into normal embryos as described above. Transplanted cells were observed by detecting Alexa594 under a fluorescent microscope (TE300, Nikon, Japan).

DNA extraction and polymerase chain reaction

Experimentally transplanted larvae were raised to the early 8-armed pluteus stage or to juveniles 1 week after metamorphosis, and each of ten larvae or juveniles was treated with a solution containing 200 μg/ml proteinase K, 10 mM Tris–HCl, and 1 mM EDTA (ProK solution) at 50 °C for 3 h and 96 °C for 10 min to extract genomic DNA. For PCR, 1 μl of the ProK solution containing genomic DNA was used with ExTaq polymerase (Takara, Japan) for 40 cycles (94 °C × 30 s, 50 °C × 30 s, 72 °C × 30 s). Both the forward primer (F primer: GAACTGTTCACTGGCGTGGTCCCAATTCTC) and the reverse primer (R primer: ATCGATCCAGACATGATAAGATACATTGAT) were designed within *GFP* and the following SV40 polyA′ sequence of the green lantern-1 vector (Fig. 4O). The 1.2-kb PCR product was separated on 1% agarose gel, and the DNA bands were stained with ethidium bromide.

Indirect immunostaining

To determine the location of spicule matrix protein 50 (SM50) in late-stage larvae, 6-armed plutei were indirectly immunostained with BG2, a monoclonal antibody specific to SM50. BG2 reacts with recombinant SM50 protein and specifically shows PMC localization in early development (Kitajima and Urakami, 2000). Whole-mount immunostaining was carried out as described by Yajima and Kiyomoto (2006) with slight modifications. Briefly, 6-armed plutei were fixed with cold methanol at −30 °C for 1 h and rinsed twice at 30-min

Table 1
Effect of donor *Pseudocentrotus depressus* primary mesenchyme cells (PMCs) on skeletogenesis in recipient *Hemicentrotus pulcherrimus* larvae

Number of PMCs [a]	Type of body rod [b]				Pedicellariae formation [c]		
	Donor	Intermediate	Recipient	Total no. [d]	Yes	No	Total no. [d]
≤40	35	34	25	154	0	54	170
10	4	9	81	128	0	93	128
0	0	0	23	23	0	20	30

[a] Number of donor PMCs transplanted into recipient embryos at the mesenchyme blastula stage.
[b] Number of chimeras that formed donor-, intermediate-, or recipient-type body rods at the 4-armed pluteus stage.
[c] Number of chimeras that formed pedicellariae at the 8-armed pluteus stage.
[d] Total number of PMC-transplanted chimeras created in this experiment.

intervals in phosphate-buffered saline saturated with calcite (PBSC). Washed specimens were immunostained individually in each well of a 96-well plate with BG2 (at a dilution of 1:200) for 3 h at room temperature. After rinsing with PBSC under the same conditions as before, specimens were then reacted with Alexa594 goat anti-mouse immunoglobulin G (IgG) antibody (Molecular Probes, Carlsbad, CA, USA) for 3 h in the same manner as for the first staining. After further rinsing with PBSC under the same conditions, each larva was mounted on a glass slide and a drop of mounting medium (10% 1,4-diazabicyclo [2.2.2] octane and 50% glycerin in PBSC) was added before a coverslip was placed over it. Stained larvae were observed under a fluorescent microscope (BX50, Olympus).

Results

Interspecific PMC transplantation

The skeletal patterns produced in chimeric embryos from heterospecific transplanted PMCs depend upon the source of the PMCs (Armstrong and McClay, 1994). Thus, in the sea urchin embryo, the skeletal pattern appears to be specified autonomously by the PMCs. Based on this evidence, an interspecific transplantation study with two species that form different spicule types in the later stage was performed. The skeletal features of the resultant chimeras were examined to determine whether PMCs or SMCs contribute to late-stage skeletogenesis.

Chimeric larvae into which 10 PMCs were transplanted (Figs. 2B, C) and one larva into which 40 PMCs were transplanted (Figs. 2D, E) showed the appropriate number (i.e., 10 or 40) of RITC-positive PMCs located along their spicules. Donor PMCs appeared to be acting as skeletogenic cells in the recipient embryos, possibly in conjunction with residual recipient PMCs or mesenchyme cells derived from lineage-converged recipient SMCs (Ettensohn and McClay, 1988).

The effect of donor PMCs was observed first in the early larval stage of the chimeras. A normal *H. pulcherrimus* 4-armed pluteus has a smooth, straight body rod (Fig. 2F), whereas the body rod of *P. depressus* is spiny and curved at the posterior end (Figs. 2G, H). The chimeras formed the donor-type body rod (Figs. 2I, J), including an intermediate type that lacked either the curve or the spiny projection at the end of its body rods (Fig. 2K). When 40 PMCs were transplanted, 73% of the chimeras showed the donor phenotype (Table 1) and the donor PMCs differentiated according to their origin.

At the 8-armed pluteus stage, three pedicellariae are formed in a *P. depressus* larva (Figs. 2M, N); however, the chimeras did not form pedicellariae and looked like normal *H. pulcherrimus* larvae (Fig. 2O). No pedicellariae were formed in chimeras even when 40 PMCs were transplanted (Table 1). Transplantation of *H. pulcherrimus* PMCs did not result in any contribution to pedicellariae formation.

Interspecific SMC transplantation

To test the contribution of SMCs to skeletogenesis, labeled SMCs were transplanted heterospecifically and their contribution to late larval skeletogenesis was examined. SMCs were collected from *P. depressus* at the gastrula stage and added to the tip of the archenteron of normal *H. pulcherrimus* gastrulae (Fig. 3A). The *P. depressus* larvae were stained with RITC in advance, and the transplanted SMCs were observed under a fluorescent microscope (Figs. 3B, C). Donor cells were located in the coelomic pouches and esophagus or became pigment cells and blastocoelar cells; that is, they appeared to be acting as SMCs in the recipient embryos. At the 4-armed pluteus stage, the donor-type body rod was not formed and the chimeras looked like normal *H. pulcherrimus* (Fig. 3D).

At the 8-armed pluteus stage, however, 12% of chimeras formed a donor pedicellaria (Figs. 3E, F; Table 2). Normal *P. depressus* usually forms three pedicellariae on the third, fourth, or fifth genital plate (Figs. 1F, 2M). The chimeric larvae formed only one pedicellaria on the fourth genital plate, and some were deformed (Figs. 3G–J) compared to the normal phenotype (Fig. 2N). Some appeared to have stopped pedicellariae formation in the process of forming skeletal rudiments (Figs. 3G–I), and some lacked the connection (Fig. 3K, arrowhead) to the genital plate (Fig. 3L, arrowhead).

Besides pedicellariae formation, another donor phenotype was also found. In the 6-armed pluteus of *P. depressus*, a branch of the dorsal arch (Fig. 3P, arrow) and the dorsal transverse rods (Fig. 3P, arrowheads) elongate to connect to each other, which does not occur in *H. pulcherrimus* (Fig. 3Q). Some chimeras showed a similar phenotype to the donor, i.e., a relatively long dorsal arch and dorsal transverse rods (Fig. 3O). Furthermore, some chimeras had a number of pigment cells at the tip of the larval arm (cf. Figs. 3R and T), which is a feature of *P. depressus* (Fig. 3S). Because pigment cells are derived from SMCs, these cells are likely descendants of transplanted SMCs, suggesting that potentiality was maintained until the late larval stage. It was not easy to clarify whether the shape of the larval rods and pigment cell localization were characteristic of the donor or recipient because unusual location or malformation of spicules often occurs in manipulated larvae, and the number of pigment cells differs between individuals, probably according to their health and developmental stage. Thus, only healthy larvae were analyzed, and which resulted in a relatively low percentage of the donor phenotype in chimeric larvae (Table 2). These results suggest that SMCs are related to spicule formation in the 6-armed pluteus stage and pedicellariae formation in the metamorphic stages.

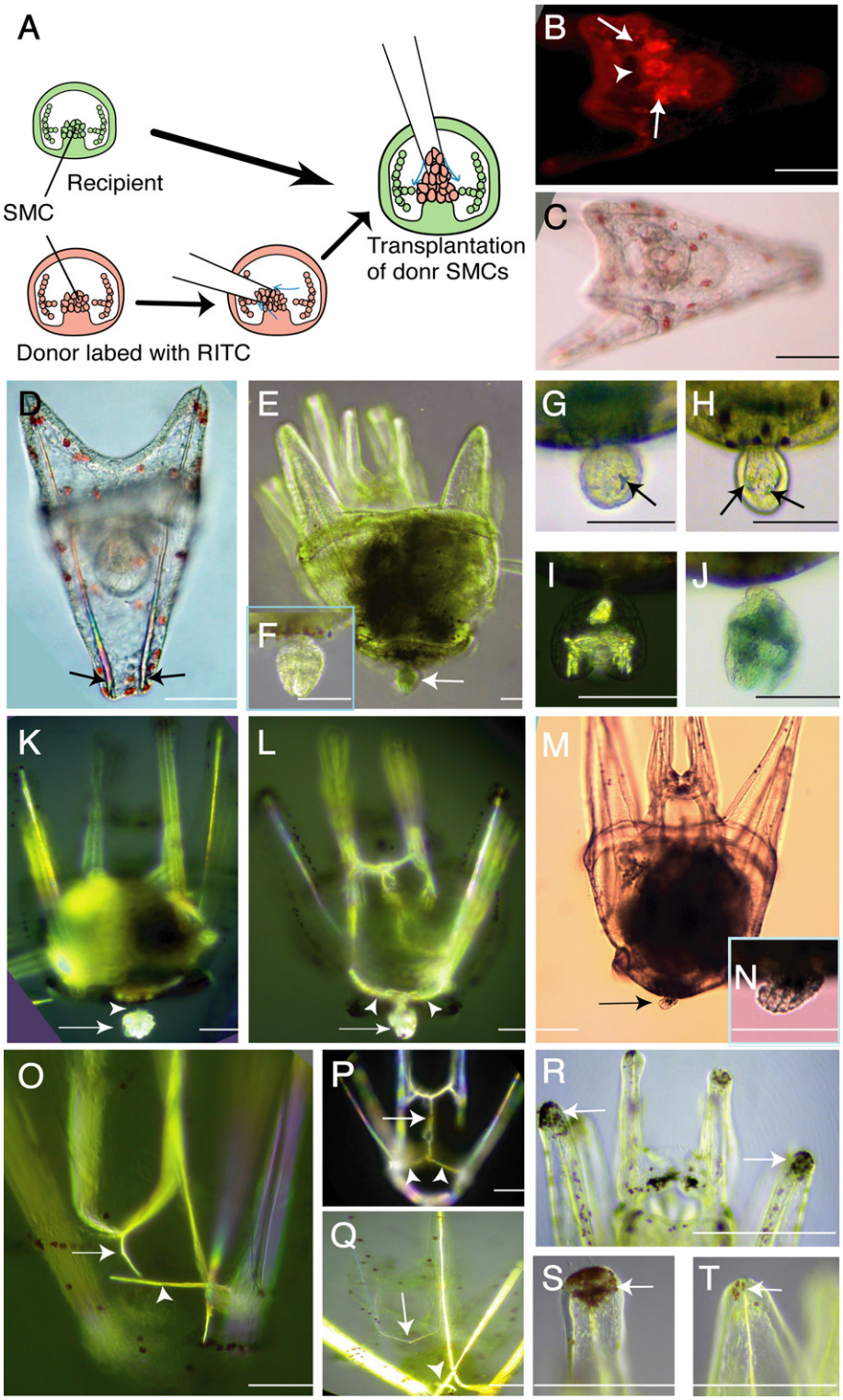

Fig. 3. Transplantation of secondary mesenchyme cells (SMCs) between *Hemicentrotus pulcherrimus* and *Pseudocentrotus depressus*. (A) Experimental procedure. (B and C) Transplanted SMCs stained with RITC were observed at the 4-armed pluteus stage. RITC-positive cells were located in the coelomic pouch (arrows) and esophagus (arrowhead). (D) Chimeras formed the recipient-type body rods (arrows). (E and F) (a magnified view of E) Chimeras formed pedicellariae (arrow). (G–J) Various types of pedicellariae formed in chimeras. (K) Pedicellaria (arrow) is lacking a junction (arrowhead) with the genital plate. (L) Pedicellaria (arrow) connected to the genital plate (arrowheads) in a normal *P. depressus* larva. (M and N) Spine (arrow) formed on the genital plate of a normal *H. pulcherrimus* larva. (O) Chimeras formed a relatively long dorsal arch (arrow) and dorsal transverse rod (arrowhead) in the 6-armed pluteus stage. (P) Dorsal arch (arrow) and dorsal transverse rods (arrowheads) of a normal *P. depressus* 6-armed pluteus are very long and almost connect to each other. (Q) The dorsal arch (arrow) and dorsal transverse rods (arrowheads) of a normal *H. pulcherrimus* 6-armed pluteus do not elongate. (R) Chimera has an accumulation of pigment cells at the tip of the larval rods at the 8-armed pluteus stage (arrows). (S) Accumulating pigment cells (arrow) at the tip of a normal *P. depressus* arm at the 8-armed pluteus stage. (T) Few pigment cells (arrow) have accumulated at the tip of a normal *H. pulcherrimus* arm at the 8-armed pluteus stage. Scale bar = 100 μm.

Table 2
Effect of donor *Pseudocentrotus depressus* secondary mesenchyme cells (SMCs) on skeletogenesis in recipient *Hemicentrotus pulcherrimus* larvae

Body part[a]	Phenotype[b] (total number[c] = 178)		
	Donor type	Recipient type	Subtotal (n)[d]
Body rod	0	165	165
Pedicellariae	8	60	68
Dorsal arch	4[e]	50	54
Pigment cells	4[e]	96	100

[a] Body rod formation was observed at the 4-armed pluteus stage, pedicellariae at the 8-armed pluteus stage, dorsal arch at the 6-armed pluteus stage, and pigment cells at the 8-armed pluteus stage.
[b] Number of chimeras that formed either donor- or recipient-type body structures.
[c] Total number of SMC-transplanted chimeras created in this experiment.
[d] Total number of chimeras surveyed at each observation point.
[e] Only larvae showing a complete donor phenotype were counted.

Expression of SM50 in the 6-armed pluteus

Both internal information from skeletogenic cells and external information from the ectoderm are necessary for skeletal formation (Armstrong et al., 1993; Armstrong and McClay, 1994). Previous interspecific chimeric experiments did not clarify whether SMCs worked directly as skeletogenic cells. To test this hypothesis, I used the construct ArsI-ArsI-*SM50-GFP* as a lineage marker. This *GFP* reporter construct is driven by the *SM50* promoter, activated specifically in skeletogenic cells throughout sea urchin development (Wilt, 2002; Yajima et al., 2007). Although reporter expression eventually ceases during larval development for unknown reasons, approximately 50% of larvae integrated with this construct express *GFP* until the 6-armed pluteus stage (Yajima et al., 2007). Using this construct, I made transgenic chimeras with *GFP*-integrated PMCs/SMCs transplanted into normal embryos (Fig. 4A). In these chimeras, only skeletogenic cells derived from transplanted PMCs/SMCs are predicted to express *GFP*. Therefore, I expected that if SMCs act directly as skeletogenic cells in later larvae, the transplanted SMCs would express *GFP* in these transgenic chimeras in 6-armed plutei.

Approximately 10% of transplanted larvae showed *GFP* expression along the spicules of recipient larvae at the 6-armed pluteus stage (Figs. 4G, H; Table 3), and this expression pattern was consistent with the localization of SM50 in the skeletogenic cells of the 6-armed plutei (Figs. 4M, N). In contrast, I observed no expression in these larvae at the 4-armed pluteus stage (Figs. 4B–F; Table 3) or in normal larvae at any developmental stage (data not shown), supporting the conclusion that *GFP* expression in 6-armed plutei was specific to the expression of the *SM50* promoter. I expected a certain localization pattern of GFP-expressing cells along each type of skeletal rod, but *GFP*-expressing cells were found both along the posterodorsal rods created in the 6-armed pluteus stage (Fig. 4G, arrowheads) and the post-oral rods (Figs. 4G, H, arrows) that were originally created by PMC descendants at the 4-armed pluteus stage. The *GFP* expression pattern was also surveyed in PMC-transplanted larvae, and *GFP*-expressing transplanted cells were observed in both 4-armed and 6-armed plutei (Figs. 4I–L). Localization of *GFP*-expressing donor cells in these PMC-transplanted larvae was observed along both the post-oral rods and posterodorsal rods (Table 3). From these results, it appears that SMCs differentiate into skeletogenic cells at the 6-armed pluteus stage and can then act together with PMC descendants.

Detection of transgenic PMCs/SMCs in juveniles

It is not possible to trace cellular lineages until the adult stage using *GFP* expression with the construct as described; however, the DNA sequence of the transgene is detectable even when the transgene is silent (Franks et al., 1988). Hence, I detected the *GFP* sequence integrated into the genome of transgenic chimeras with PCR to determine whether the transplanted PMCs/SMCs existed after metamorphosis. ArsI-ArsI-*SM50-GFP* was injected into fertilized eggs, the PMCs or SMCs were collected and transplanted into normal embryos, and these transgenic chimeras were cultured until the 8-armed pluteus or juvenile stage (Fig. 4A). Because the transgene is integrated in a mosaic pattern in sea urchins (Hough-Evans et al., 1988), not all PMCs/SMCs of an injected embryo contain *GFP* in their genome. I previously tested the efficiency of *SM50-GFP* expression in injected embryos (Yajima et al., 2007) and found that approximately 90% of injected embryos express *SM50-GFP* with this method. To enhance the possibility that PMCs/SMCs with an integrated *GFP* sequence in their genome were transplanted into the recipients, cells were collected from at least two donor embryos for each recipient embryo in this study.

Transgenic chimeras were collected at either the 8-armed pluteus or juvenile stage, and the integrated *GFP* sequence was detected by PCR to investigate whether PMCs or SMCs exist after metamorphosis (Fig. 4A). The experiments were done at least three times at each developmental stage. As a positive control, I used juveniles into which ArsI-ArsI-*SM50-GFP* was injected at fertilization and that were raised without transplantation. *GFP* fluorescence was not seen in these transgenic juveniles, but *GFP* sequence was detected in the genomic DNA of positive control larvae (Fig. 4P, lanes 1–4). As for the PMC-transplanted larvae, *GFP* was detected in 8-armed plutei (Fig. 4P, lanes 9–11), but not in juveniles (Fig. 4P, lanes 12–14); however, it was detected in both SMC-transplanted 8-armed plutei (Fig. 4P, lanes 15–17) and juveniles (Fig. 4P, lanes 18–20). These results imply that PMCs disappear before metamorphosis and do not exist in the adult sea urchin.

Discussion

PMCs are larval skeletogenic cells and SMCs are later skeletogenic cells

Three results obtained in this study support a difference in skeletogenic cell fate for PMC/SMC: (1) donor SMCs from *P. depressus* induced pedicellariae formation in late larvae of recipient *H. pulcherrimus* (summarized in Fig. 5), indicating the involvement of SMCs in late skeletogenesis, (2) transplanted SMCs containing ArsI-ArsI-*SM50-GFP* initiated *GFP* expression in 6-armed plutei (Figs. 4G and H), indicating that SMCs

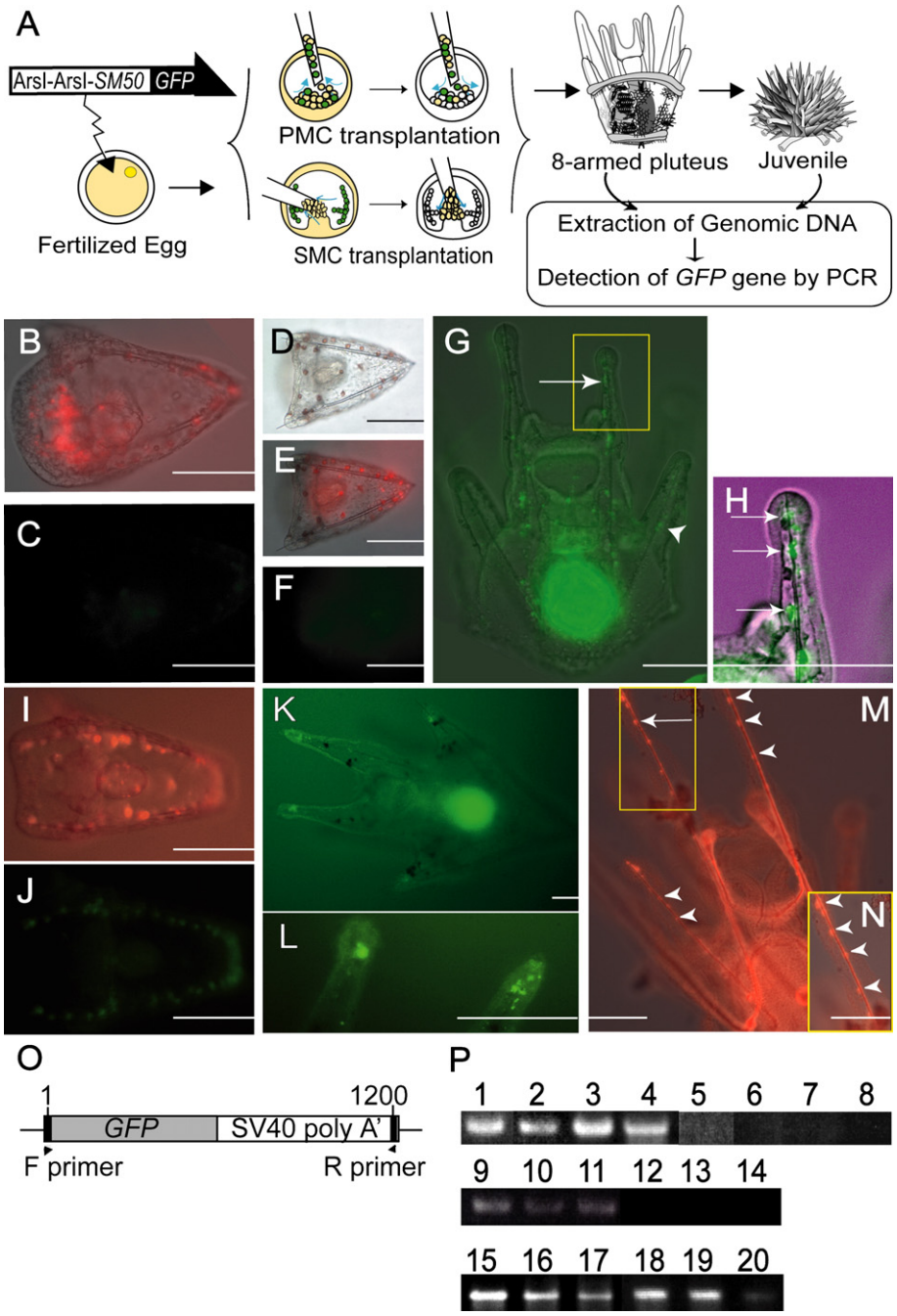

Fig. 4. PMC/SMC transplantation between transgenic and normal *Hemicentrotus pulcherrimus* embryos. (A) Experimental procedure. (B–H) SMC-transplanted larvae. At the 4-armed pluteus stage, transplanted SMCs are located in the coelomic pouch and muscle (B) or pigment cells (D and E) according to Alexa594 fluorescence; however, they do not show GFP fluorescence (C and F). At the 6-armed pluteus stage, GFP fluorescence is observed in the cells along the post-oral rods (G and H, arrows) and posterodorsal rods (G, arrowhead). Fluorescence in the stomach is auto-fluorescence caused by diatoms. (I–L) PMC-transplanted larvae. At the 4-armed pluteus stage, Alexa594-positive cells are located along the spicules (I) and express GFP (J). At the 6-armed pluteus stage, GFP-expressing cells are observed on the post-oral and posterodorsal rods (K, arrow, and L). Fluorescence in the stomach is auto-fluorescence caused by diatoms. (M and N) (magnification of M) Localization of SM50 protein in the later stage is observed by immunostaining of 6-armed pluteus larvae with SM50 antibody. SM50-positive cells are found on all larval rods (M and N, arrows) and their localization pattern is consistent with that of GFP-expressing cells in PMC/SMC-transplanted larvae (G and K). (O) Primer binding sites for detecting *GFP* and the following SV40 polyA′ fragments by PCR are indicated. F primer: forward primer. R primer: reverse primer. (P) *GFP* fragment detected by PCR. Each lane indicates an independent experiment. Lanes 1–4, positive control. *GFP* is detected in ArsI-ArsI-*SM50-GFP* injected larvae at all developmental stages: 4-armed pluteus (lane 1), 6-armed pluteus (lane 2), 8-armed pluteus (lane 3), and juvenile (lane 4). Lanes 5–8, negative control. No visible bands were obtained from non-injected larvae, 4-armed pluteus (lane 5), 6-armed pluteus (lane 6), 8-armed pluteus (lane 7), or juvenile (lane 8). Lanes 9–14, PMC-transplanted larvae. GFP was detected in 8-armed plutei (lanes 9–11), but not in juveniles (lanes 12–14). Lanes 15–20, SMC-transplanted larvae. *GFP* was detected in both 8-armed plutei (lanes 15–17) and juveniles (lanes 18–20). Scale bar = 100 μm. GFP, green fluorescent protein; PMC, primary mesenchyme cells; SMC, secondary mesenchyme cells.

Table 3
SM50-GFP expression of PMC/SMC descendants in pluteus-stage larvae

Transplanted cells[a]	Number of GFP-expressing larvae[b]			Total number[c]
	4-Armed pluteus	6-Armed pluteus		
		Post-oral rods	Posterodorsal rods	
PMCs	38	5	6	45
SMCs	0	8	8	78

SM50, spicule matrix protein 50.
[a] Primary (PMCs) or secondary mesenchyme cells (SMCs) containing the *green fluorescent protein* (*GFP*) gene were transplanted into normal embryos, and the transplanted larvae were cultured to the 6-armed pluteus stage.
[b] Number of transplanted larvae expressing *GFP* at the 4- and 6-armed pluteus stages.
[c] Total number of transplanted larvae surveyed in this experiment.

differentiate into skeletogenic cells at the 6-armed pluteus stage, and (3) *GFP* was undetectable after metamorphosis of larvae into which *GFP*-integrated PMCs had been transplanted (Fig. 4P), suggesting that PMCs disappear after metamorphosis. Although there is no direct evidence that SMCs are involved in adult sea urchin skeletogenesis, these results together indicate that it is likely that PMCs act only in embryos and larvae and that SMCs are the skeletogenic cells in late larvae and adult sea urchins. Of course, it is possible that novel cells derived from an ectoderm or endoderm lineage could also be involved that was not detectable herein.

The SMC population comprises at least four cell types at the embryonic stage (Burke and Alvarez, 1988; Dan and Okazaki, 1956; Gustafson and Kinnander, 1956; Ishimoda-Takagi et al., 1984; Gibson and Burke, 1985; Tamboline and Burke, 1992; Tokuoka et al., 2002; Katow et al., 2004). So, an important question is which cells of SMC population will become skeletogenic cells in later stages. It is thought that coelomic pouch cells, one type of SMC descendant, form adult structures, including the skeleton. However, the SMC involvement in late-stage skeletogenesis observed here occurred at the 6-armed pluteus stage, which is well before adult rudiment formation at the 8-armed pluteus stage. SMCs appear to receive suppressive signals from PMCs at the gastrula stage, and a major reduction is seen in the number of pigment cells following SMC's conversion when PMCs are removed (Ettensohn, 1990; Ettensohn and Ruffins, 1993). It is possible that these skeletogenic SMCs are released from the suppressive signals of PMCs in later stages, allowing them to become skeletogenic cells at the 6-armed pluteus stage.

It is still not clear whether the skeletogenic cells divide and proliferate in later stages. In this study, it was impossible to count the number of transplanted PMCs/SMCs with GFP fluorescence because they formed a syncytium (Giudice, 1973; Okazaki, 1975), and the GFP fluorescence diffused into adjacent cells. However, the number of skeletogenic cells in larvae was obviously greater (Yajima and Kiyomoto, 2006) than the number of PMCs (Hamada and Kiyomoto, 2003) and skeletogenic SMCs, each about 60 (Ettensohn and McClay, 1988). If hundreds of these late skeletogenic cells are derived exclusively from SMCs, it indicates that an increase in the number occurred in their lineage. Adult echinoderm organization may derive from pluripotent stem cells that are set aside until the late developmental stages (Davidson et al., 1995), and analysis of the SMC lineage in later development may provide new insights into this hypothesis.

Chimera experiments revealed donor-dependent phenotypes

Pedicellariae are adult sea urchin skeletal organs that are used for catching food such as algae. Some species also have pedicellariae in the late larval and juvenile stages (Hyman, 1955). The development of pedicellariae in late larvae has been described, but the cellular origins have been obscure (Burke, 1980; Dubois and Ameye, 2001). Pedicellariae in late larvae are formed on the genital plate outside the adult rudiment, and some of these plates are derived from larval rods at the late larval stage. Therefore, PMCs were considered a potential cellular origin of genital plates and of pedicellariae. In this study, however, donor SMCs contributed to pedicellariae formation in chimeric larvae, while donor PMCs did not (Fig. 3). These results lead to the following two possibilities: SMCs work directly as skeletogenic cells to form the pedicellariae or SMCs only provide cues to other skeletogenic cell lineages. SMC involvement was not clarified in this study. However, the former possibility is more likely because another experiment using *GFP* constructs indicated that SMCs become skeletogenic cells at late larval stages (Fig. 4G).

It is also not clear from this study how donor SMCs form the pedicellaria in heterospecific recipients. On the genital plate where pedicellariae are formed, spines are also formed in both *P. depressus* (Figs. 1B, 2M) and *H. pulcherrimus* (Figs. 1A, 3M, N). Pedicellariae are composed of three skeletal rudiments that are morphologically similar to spine rudiments. Therefore, the early process of pedicellariae formation appears exactly like the formation of three spines (Gordon, 1926). Thus, the signals for spine and pedicellariae formation are likely similar, and *H. pulcherrimus* signals can likely be received by *P. depressus*

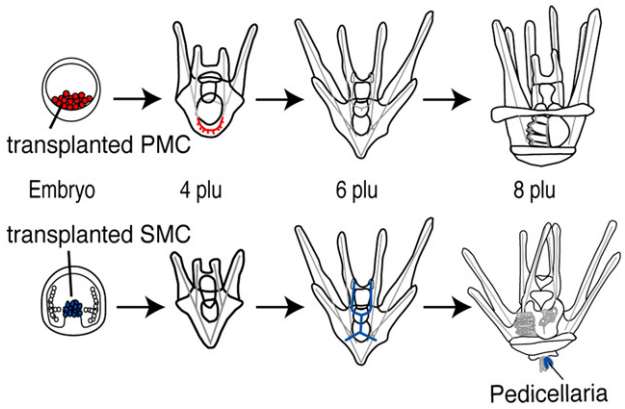

Fig. 5. Summary of the chimera experiment using *Pseudocentrotus depressus* and *Hemicentrotus pulcherrimus*. When PMCs were transplanted, donor-type body rods were formed in 4-armed plutei (shown in red), whereas donor phenotype spicules were formed in 6-armed and 8-armed plutei when SMCs were transplanted (shown in blue). PMC, primary mesenchyme cells; SMC, secondary mesenchyme cells.

SMCs, thereby enabling *P. depressus* SMCs to form pedicellariae instead of spines in the chimeras.

Some chimeras also formed donor-type spicules in the 6-armed pluteus stage (Figs. 3O–Q) or had many pigment cells at the tip of larval arms in the 8-armed pluteus stage (Figs. 3R–T), suggesting that donor SMCs could sustain their cell fate until later stages and act at the appropriate time and location in heterospecific recipients. Proper skeletogenesis or cell localization in chimeras indicates that donor PMCs/SMCs were able to receive ectodermal cues from the recipients. The conservation of cues provided by the ectoderm seems to depend on the phylogenic distance between the species (Von Ubisch, 1933, 1939; Armstrong et al., 1993; Armstrong and McClay, 1994). *P. depressus* is the most basal member of the family Strongylocentrotidae, and *H. pulcherrimus* has been suggested to be included in the genus *Strongylocentrotus* (Biermann et al., 2003). These results support the contention that in sea urchins, developmental systems could be highly conserved within the family.

Evolutionary insight to skeletogenic mesenchyme cells in echinoderms

Basic classes of echinoderms, such as sea stars (Paul and Smith, 1984, 1988), only have mesenchymal cells migrating from the tip of the archenteron during gastrulation and thus do not have PMCs, but they do possess an endoskeleton in the adult stage. Therefore, the origin of the unusual timing of mesenchyme migration in sea urchins is a mystery. Furthermore, the cellular basis of skeleton formation in late larvae and adults in other echinoderms is unknown. The results obtained here and in a previous study of the direct-developing species *P. japonica* (Yajima, 2007) lead me to hypothesize that SMCs may be the ancestral skeletogenic cells of echinoderms. *Eucidaris*, an evolutionarily primitive sea urchin, does not have PMCs and mesenchymal cells migrating from the archenteron form the larval spicules (Schroeder, 1981; Wray and McClay, 1988). Thus, when echinoids diverged from the basal echinoderms, a shift in timing may have occurred in a subpopulation of mesenchymal cells to produce larval spicules earlier in development, resulting in PMCs. Further study of the SMC lineage in the late larval and juvenile stages of sea urchins and other echinoderms will be useful in evaluating this hypothesis.

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