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Commitment and response to inductive signals of primary mesenchyme cells of the sea urchin embryo

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In the sea urchin embryo, primary mesenchyme cells (PMC) are committed to produce the larval skeleton, although their behavior and skeleton production are influenced by signals from the embryonic environment. Results from our recent studies showed that perturbation of skeleton development, by interfering with ectoderm–extracellular matrix (ECM) interactions, is linked to a reduction in the gene expression of a transforming growth factor (TGF)-beta growth factor, *Pl-univin*, suggesting a reduction in the blastocoelic amounts of the protein and its putative involvement in signaling events. In the present study, we examined PMC competence to respond to environmental signals in a validated skeleton perturbation model in *Paracentrotus lividus*. We found that injection of blastocoelic fluid (BcF), obtained from normal embryos, into the blastocoelic cavity of skeleton-defective embryos rescues skeleton development. In addition, PMC from skeleton-defective embryos transplanted into normal or PMC-less blastula embryos are able to position in correct regions of the blastocoel and to engage spicule elongation and patterning. Taken together, these results demonstrate that PMC commitment to direct skeletogenesis is maintained in skeleton perturbed embryos and confirm the role played by inductive signals in regulating skeleton growth and shape.

Key words: growth factors, primary mesenchyme cells, sea urchin embryo, skeletogenesis, transplantation.

Introduction

Epithelial–mesenchymal interactions play an important role in the development of many organisms (Adams & Watt 1993; Yasugi 1993; Roberts *et al*. 1998; Wells & Melton 2000). In the sea urchin embryo, numerous lines of evidence indicate that primary mesenchyme cells (PMC) are influenced by ectoderm during all the steps of skeleton production, including the migration of PMC, the position and the timing of mesenchymal ring formation as well as the size and the pattern of skeleton (Ettensohn & McClay 1986; Horstadius 1957; Ettensohn 1990; Armstrong *et al*. 1993; Ettensohn & Malinda 1993; Armstrong & McClay 1994; Peterson & McClay 2003). Moreover, several experiments have demonstrated that PMC are able to elongate spicules autonomously. In fact, micromeres or PMC cultured *in vitro* in the presence of horse serum (HS) or blastocoelic fluid (BcF) can produce spicules that resemble those produced *in vivo*, although no regular patterning is found (Okazaki 1975; Kinoshita & Okazaki 1984; Kiyomoto & Tsukahara 1991).

Our recent studies have began to address the molecular nature of the cues involved in skeletogenesis by taking advantage of an experimental model of skeleton-deficient embryos (Zito *et al*. 1998; Zito *et al*. 2000; Zito *et al*. 2003) developed in our laboratory utilizing monoclonal antibodies (McAb) to an extracellular matrix (ECM) protein (Matranga *et al*. 1992). Briefly, we have demonstrated that treatment of early and mesenchyme blastula embryos with McAb to *Pl*-nectin, an outer ECM protein (Matranga *et al*. 1992), greatly affects both skeleton growth and patterning, but has no effect on ectoderm and endoderm differentiation (Zito *et al*. 1998; Zito *et al*. 2000). This result does not depend on the direct effect of McAb anti-*Pl*-nectin on PMC, because the injection of McAb into the blastocoel does not inhibit spicule elongation (Zito *et al*. 1998). Our recent results show that spicule growth inhibition, originally caused by interfering with ectoderm–ECM interactions, probably depends on decreased levels of mRNA coding for the

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TGF-β growth factor *Pl-univin*, whose product seems to be the ectoderm inductive signal to PMC (Zito *et al*. 2003).

The purpose of this study was to gain information on the competence of PMC from skeleton-defective embryos to respond to inductive signals coming from a normal embryonic environment. We examined the effects of the microinjection of BcF, prepared from normal embryos, into the blastocoel of skeleton-defective embryos. In addition, we tested the ability of PMC taken from skeleton-defective embryos to direct skeletogenesis when transplanted into normal or PMC-less embryos.

Materials and methods

Perturbation assay

Perturbation assays with McAb to *Pl*-nectin (NEVIE-11h7 clone) were performed on whole *Paracentrotus lividus* embryos according to Zito *et al*. (1998). Briefly, purified IgG were added at a concentration of 3 µg/µL to 96 microtiter well plates (Greiner Bio-One, Longwood, FL, USA) containing 100 embryos/100 µL. This concentration was shown to cause the most severe abnormalities after 24 h of culture (Zito *et al*. 2003). Control embryos were treated with unrelated IgG. Embryos were continuously cultured in the presence of the antibody from the developmental stages indicated in Results, at 16°C in Millipore filtered sea water (MFSW).

Blastocoelic fluid preparation

Blastocoelic fluid was prepared by a centrifugal method as described by Kiyomoto & Tsukahara (1991). Briefly, late gastrula embryos were washed threefold with ice-cold Ca^{2+}-Mg^{2+} free sea water (CMFSW), and packed by gentle centrifugation at 400 gravities (xg) for 2 min. After removal of supernatant, the packed embryos were centrifuged at 9,000 xg for 20 min to flatten the embryos and allow the release of the BcF, which was then collected and further centrifuged at 16,000 xg for 20 min to eliminate debris. All centrifugations were performed at 4°C. Protein concentration was determined according to the method of Lowry *et al*. (1951).

Microinjection

Embryos were prepared for microinjection using a method described by Zito *et al*. (1998). Briefly, embryos at the prehatching blastula stage were treated with MFSW pH 4.5 and, after washing in normal MFSW, were electrostatically fixed to 60 mm Petri-dish lids treated with 1% protamine sulfate (Sigma, St. Louis, MO, USA), using a mouth pipette. Microinjection needles (Eppendorf, Hamburg, Germany) were back-filled with BcF at the concentrations mentioned in the Results section. The blastocoel of the embryos received approximately 100 pL of BcF solution. Microinjection was performed using a Narishige IM-188 micromanipulator (Narishige, Tokyo, Japan) and a Cell Tram Oil injector (Eppendorf). After microinjection, embryos were collected with a mouth pipette and allowed to develop in 96 microtiter-plate wells (Greiner) at 16°C. Embryos were observed under a Zeiss Axioskop 2 Plus inverted microscope and recorded by digital camera system (Arese, Italy).

Micromanipulation

Primary mesenchyme cell transplantation experiments from embryos treated with McAb to *Pl*-nectin to host embryos required the loosening of the PMC clusters present in the blastocoel to facilitate both cell collection and labeling, so that they would be distinguishable from host cells when implanted in chimeric embryos. For this purpose, donor embryos were simultaneously treated with low Ca^{2+} and Mg^{2+} sea water (LCMSW) and vitally labeled by combining the procedures reported by Okazaki (1975) and Ettensohn & McClay (1986), respectively. In brief, embryos treated with McAb to *Pl*-nectin for 24 h were cultured for 1 h in rhodamine isothiocyanate (RITC, 20 µg/mL final concentration) solution diluted in LCMSW, which is a 4:1 mixture of CMFSW and MFSW. To prevent ciliary movements, both RITC-labeled donor and host embryos were treated with double strength MFSW for 1 min. After several washes in LCMSW or MFSW, embryos were loaded into microinjection chambers, as described by Ettensohn & McClay (1986) with some modifications. Briefly, we used a single assembly that had two contiguous chambers to be used for RITC-labeled donor and host embryos. Removal of PMC from host embryos was performed before the transplantation by flushing the blastocoel with MFSW as reported previously (Ettensohn & McClay 1988). PMC to be transplanted were collected from a few RITC-labeled donor embryos (40-h-old) contained in one chamber into TransferTips needles (Eppendorf) and transplanted into normal or PMC-less embryos contained in the next chamber. Transplantation was performed using a three-dimensional joystick manipulator (MO-202; Narishige) and a Cell Tram Oil injector (Eppendorf). After the transplantation, embryos were collected with the aid of a mouth

pipette in small Petri dishes and allowed to develop at 16°C.

Lectin-staining

Lectin-staining of whole mount embryos was performed according to Zito *et al.* (2000). Briefly, embryos were fixed for 2 min in ice-cold 0.1% formalin, permeabilized for 2 min in ice-cold methanol and then rinsed in MFSW. Embryos were incubated with conjugated wheat germ agglutinin–fluorescein isothiocyanate (WGA–FITC; Sigma, 1:400 in MFSW) for 30 min on ice in the dark, then washed threefold in MFSW. Embryos were observed under a Zeiss Axioskop 2 Plus inverted microscope equipped for epifluorescence and recorded by digital camera system.

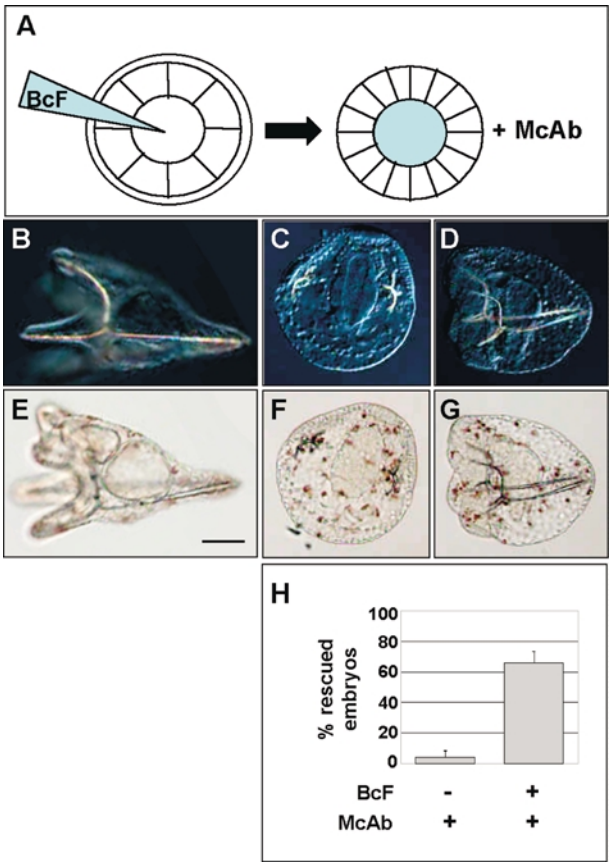

Fig. 1. Microinjection of blastocoelic fluid (BcF) and treatment with monoclonal antibodies (McAb) to *Pl*-nectin of *Paracentrotus lividus* embryos. (A) Drawing representing the steps of the experimental procedure. Microinjection of BcF into the blastocoelic cavity of prehatching blastula embryo (on the left); addition of McAb to *Pl*-nectin 2 h after microinjection (on the right). (B,E) Untreated embryos. (C,F) Control embryos cultured from the hatching blastula in the presence McAb to *Pl*-nectin. (D,G) embryos injected with BcF and then cultured with McAb to *Pl*-nectin for the same period of time of control embryos. (B), (C) and (D) are differential interference microscopies of embryos shown in (E), (F) and (G), respectively. Bar, 50 µm. (H) Histogram shows the quantitative analysis of embryos rescuing skeleton elongation for which the standard error (SE) has been calculated.

Results

BcF injection rescues skeleton-defective embryos

In vitro experiments using isolated micromeres or PMC have demonstrated that HS or BcF are needed for the production of the skeleton, calling for the presence of essential growth factors in the blastocoel of living embryos (Okazaki 1975; Harkey & Whiteley 1980; Kiyomoto & Tsukahara 1991). Our hypothesis is that the blastocoel of skeleton-deficient embryos obtained after culture in the presence of McAb to *Pl*-nectin do not contain the sufficient 'signal' for skeletogenesis to occur. If this hypothesis is correct, then microinjection of BcF obtained from normal embryos into the blastocoelic cavity of treated embryos would lead to the partial or total recovery of skeleton deficiencies. Because our recent results showed that the expression of *Pl-univin* involved in skeletogenesis reaches a peak at the late gastrula stage (Zito *et al.* 2003), we prepared BcF from this stage. BcF was microinjected into the blastocoel of prehatching blastula embryos which were then continuously cultured in the presence of McAb to *Pl*-nectin for approximately 42 h (Fig. 1A). In a first series of experiments, we tested late gastrula BcF at two different concentrations, 0.150 and 0.275 mg/mL, to find out the most effective concentration in rescuing skeletogenesis. These quantities were chosen because we estimated that the theoretical final concentration of BcF inside the blastocoel would be 0.028 and 0.052 mg/mL, respectively, assuming that blastocoel volumes of embryos after injection are approximately 523 pL, and knowing that the injected volume is always approximately 100 pL. These values match the concentrations that were used when micromeres were cultured *in vitro*, when the effective doses of BcF from *Hemicentrotus pulcherrimus* and *Toxopneustes pileolus* for the formation of spicules were 0.03–0.05 mg/mL (Kiyomoto & Tsukahara 1991). Results of experiments with an injection of higher concentration of BcF are shown in Figure 1. The lower concentration of BcF injected into the blastocoel was also effective in rescuing skeleton elongation (not shown). Control embryos cultured in the presence of McAb to *Pl*-nectin for 42 h with no BcF injection were about 96% skeleton defective (Fig. 1C,F). In contrast, injection of BcF rescued skeleton development with a

result of a 66% level of embryos with skeleton elongation (Fig. 1D,G). No rescue was observed in the remaining 34% of embryos. Untreated embryos cultured in MFSW for the same period of time as treated embryos were found to be at the pluteus stage (Fig. 1B,E). Figure 1(H) shows the mean of percentages of rescued embryos from two different experiments in which 82 and 17 total embryos, respectively, were scored. Significance was determined according to the Student's *T*-test ($P < 0.05$), by pooling the numbers of the two groups of rescued embryos with and without injection.

Primary mesenchyme cells from skeleton-defective embryos can engage spiculogenesis if transplanted into normal embryos

To test the hypothesis that PMC from embryos with severe skeleton defects would be able to synthesize a normal skeleton if given the appropriate microenvironment, we designed transplantation experiments in which PMC from skeleton-defective embryos were transplanted into normal embryos with or without PMC. To determine the fate of the transplanted cells, donor PMC were vitally labeled with RITC prior to each experiment.

In a first series of experiments, 10–20 PMC from 40-h-old-donor embryos, whose skeletons were severely perturbed, were transplanted into 18-h-old normal mesenchyme blastula embryos (Fig. 2A). At this point it should be recalled that in *P. lividus* embryos the total number of PMC had been estimated to be 32 ± 7 (Zito *et al*. 2003). Eight chimeric embryos were allowed to develop for 24 h with their skeletal development microscopically monitored. The skeletons of all chimeras was found to be normal regardless of the number of PMC transplanted, and most of the RITC-labeled cells were found to be closely associated with the spicules and probably cooperated with host PMC in skeleton production (Fig. 2B–D).

To determine whether transplanted PMC were able to produce skeleton, we performed experiments in which RITC-labeled PMC from skeleton-defective embryos were transplanted into blastula embryos whose own PMC had been removed (Fig. 3A). Fifteen chimeric embryos that contained 10–20 transplanted PMC were obtained. They were allowed to develop for 24 and 48 h and observed by epifluorescence. Twenty-four hours after transplantation, all chimeric embryos showed two tri-radiate spicule rudiments, correctly localized in the blastocoel with the two clusters of donor PMC closely associated with them (Fig. 3B–D). After 48 h, embryos reached the pluteus stage, showing normal skeletons with most of the RITC-labeled PMC closely associated with them (Fig. 3E–G). In these chimeric embryos, we also observed some RITC-labeled cells dispersed into the blastocoel (Fig. 3F), suggesting that some non-PMC RITC-labeled cells were mistakenly taken from the donor embryos. In addition, chimeric embryos showed few unlabeled cells also associated with skeletal rods (Fig. 3G, arrowhead). These were probably endogenous cells that had joined in spicule formation.

To detect if RITC-labeled cells found associated with the skeleton were true PMC, 24-h-old-chimeras, obtained by transplanting RITC-PMC into PMC-less embryos, were stained with FITC-conjugated WGA, which is known to label PMC specifically. A total of seven embryos were double stained and observed by epifluorescence. The cluster shown in Figure 4

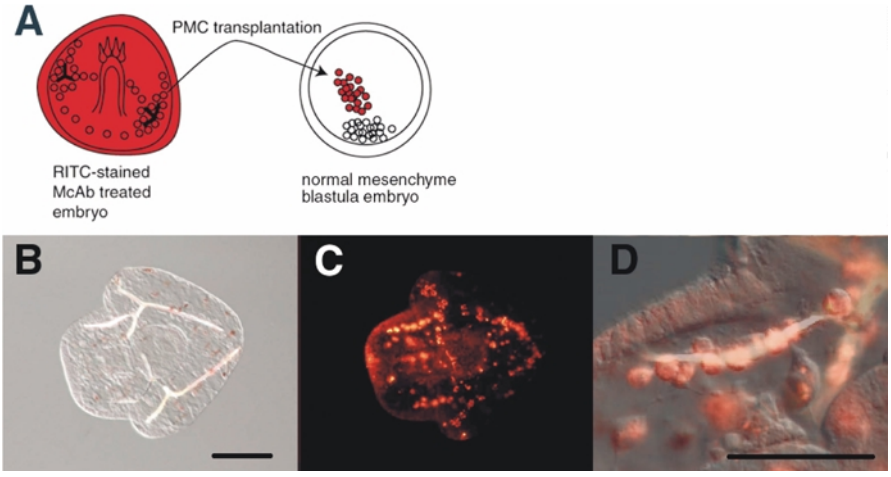

Fig. 2. Transplantation of primary mesenchyme cells (PMC) from skeleton defective embryos into normal mesenchyme blastula embryos. (A) Drawing showing the experimental procedure of transplantation experiments. (B,C) Transplanted embryo 24 h after transplantation, bright-field and epifluorescence, respectively. (D) Merged image of parts (B) and (C) at higher magnification. Bars, 50 μm.

was composed by PMC coming from donor and host embryos (Fig. 4A). All RITC-labeled cells from skeleton-defective donors were WGA-positive, indicating that only true PMC contribute to the cluster formation (Fig. 4B,C).

Discussion

Primary mesenchyme cells of the sea urchin embryo provide a good model system to study cell commitment and inductive signals. In the present study, we have focused on the competence of PMC to respond to environmental cues needed to synthesize spicules. Our results elucidated several points. First, skeleton defects observed in McAb to *Pl*-nectin treated embryos are the result of reduced or absent signal(s) in the blastocoel. Second, PMC from skeleton-defective embryos are able to synthesize a correctly patterned skeleton when they are placed in the appropriate environment. Third, PMC do not loose their competence to produce the skeleton even though they are transplanted into 24 h younger host embryos. Fourth, transplanted PMC retain their commitment to guide skeletogenesis even after a long resting period, that is, from their ingression into the blastocoel of donor embryos to their participation in skeletogenic clusters in host embryos.

These conclusions are based on a number of experimental observations. Microinjection of BcF from normal embryos, as a source of the putative 'inductive signal', can reverse the effects caused by McAb to *Pl*-nectin, promoting skeleton elongation and patterning. This result suggests that skeleton defective embryos have reduced levels of the 'inductive signal' in the blastocoel. In agreement with this hypothesis, our recent results show that the inhibition of skeleton elongation depends on decreased levels of mRNA coding for the TGF-β growth factor *Pl-univin*, whose product seems to be the 'inductive signal' to PMC (Zito *et al*. 2003). In addition, we have shown that in normal embryos *Pl-univin* has high mRNA expression levels from the early gastrula through to the pluteus stage, with a peak at the late gastrula stage (Zito *et al*. 2003). It should be recalled that BcF used for microinjection was prepared from 24-h-old late-gastrula-stage embryos of the *P. lividus* species, which further supports the hypothesis that, at this stage, embryos contain enough 'signal' to induce skeleton development. Conversely, previous studies have demonstrated that BcF promotes spicule formation and elongation in isolated micromeres cultured *in vitro* and that BcF is continuously required in the culture medium for a certain time before spicule deposition, specifically between 6–18 h of development. Furthermore, it was also demonstrated that BcF stimulating activity decreased between the mesenchyme blastula and late gastrula stages (Kiyomoto & Tsukahara 1991). The apparent discrepancy between the

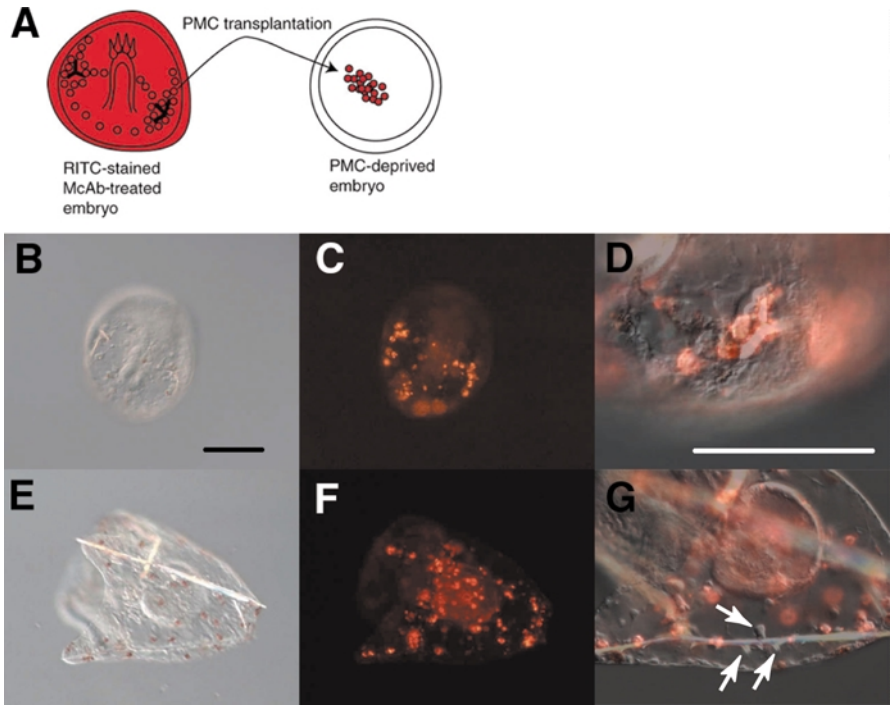

Fig. 3. Transplantation of PMC from skeleton-defective embryos into PMC-deprived embryos. (A) Drawing showing the experimental procedure of transplantation experiments. Transplanted embryo at 24 h (B–D) and 48 h (E–G) after transplantation. Bright-field (B,E) and epifluorescence (C,F) of the same embryo. (D,G) Merged images of parts (B) and (C) and (E) and (F), respectively, at higher magnification. Arrows in (G) indicate unlabeled endogenous cells associated with skeletal rods. Bars, 50 μm.

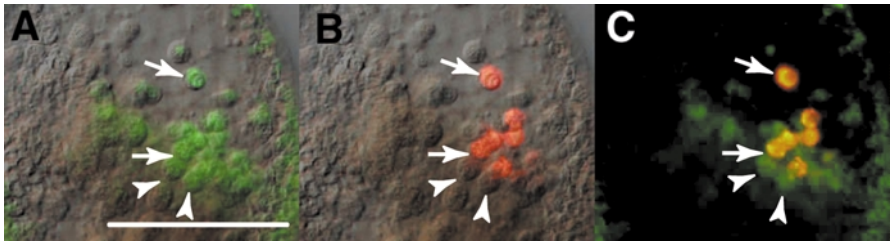

Fig. 4. Transplantation of PMC from skeleton defective embryos into PMC-deprived embryos. (A) Transplanted embryo 24 h after transplantation stained with WGA-FITC. (B) RITC labeled PMC in the same embryo. (C) A merged picture of figures (A) and (B). Arrows and arrow heads indicate RITC-labeled and unlabeled skeletogenic cells, respectively. Bar, 50 µm.

highest levels of skeleton promoting activity found at different stages in different sea urchin embryo species could be explained by the fact that *in vivo* PMC display a different behavior from PMC cultured *in vitro* in the presence of BcF. Another possibility is that the different sea urchin species used have distinct time schedules for induction of skeleton development.

By transplantation experimentation, we demonstrated that PMC from skeleton defective embryos are competent to respond to environmental stimuli found in the blastocoel of host embryos. Considering that transplanted PMC are taken from 40-h-old skeleton-defective embryos, their competence lasts for a considerable period of time if compared to their normal time schedule of skeleton production. Accordingly, Ettensohn & McClay (1986) demonstrated that PMC transplanted from an older to a younger embryo settle on the floor of the blastocoel and then migrate on the temporal schedule of the host, indicating for the first time that PMC are not distributed passively, but that they are competent to respond to some directional cues in the blastocoel. Recently, it has been suggested that signals underlying PMC migration, soon after their ingression into the blastocoel, could be different from signals providing spatial cues needed at the time PMC begin to make the skeleton (Peterson & McClay 2003).

Finally, we found that chimeric embryos, originating from PMC-less embryos and rhodamine labeled PMC explanted from skeleton defective embryos, have a few unlabeled cells adjacent to skeletal rods and probably participating in skeleton elongation. This could be explained by the conversion of the host embryos' SMC to a skeletogenic fate. This finding is not new because it has been already demonstrated that PMC presence normally prevent SMC from becoming skeletogenic but, in their absence, some SMC change their commitment and become skeleton-synthesizing cells (Ettensohn & McClay 1988; Ettensohn & Ruffins 1993).

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