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Skeletogenesis by transfated secondary mesenchyme cells is dependent on extracellular matrix–ectoderm interactions in *Paracentrotus lividus* sea urchin embryos

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In the sea urchin embryo, primary mesenchyme cells (PMCs) are committed early in development to direct skeletogenesis, provided that a permissive signal is conveyed from adjacent ectoderm cells. We showed that inhibition of extracellular matrix (ECM)–ectoderm cells interaction, by monoclonal antibodies (mAb) to *Pl*-nectin, causes an impairment of skeletogenesis and reduced expression of *Pl*-SM30, a spicule-specific matrix protein. When PMCs are experimentally removed, some secondary mesenchyme cells (SMCs) switch to skeletogenic fate. Here, for the first time we studied SMC transfating in PMC-less embryos of *Paracentrotus lividus*. We observed the appearance of skeletogenic cells within 10 h of PMCs removal, as shown by binding of wheat germ agglutinin (WGA) to cell surface molecules unique to PMCs. Interestingly, the number of WGA-positive cells, expressing also msp130, another PMC-specific marker, doubled with respect to that of PMCs present in normal embryos, though the number of SM30-expressing cells remained constant. In addition, we investigated the ability of SMCs to direct skeletogenesis in embryos exposed to mAbs to *Pl*-nectin after removal of PMCs. We found that, although phenotypic SMC transfating occurred, spicule development, as well as *Pl*-SM30-expression was strongly inhibited. These results demonstrate that ectoderm inductive signals are necessary for transfated SMCs to express genes needed for skeletogenesis.

Key words: *in situ* hybridization, mAb to *Pl*-nectin, mesenchyme cells, pigment cells, RT–PCR.

Introduction

Indirect developing sea urchin embryos have two distinct populations of mesodermal cells, primary and secondary mesenchyme cells, PMCs and SMCs, respectively. Although both cell populations delaminate from the vegetal epithelium into the blastocoel where they migrate actively, they diverge with respect to their time of ingression, lineage and developmental fates. PMCs are a homogeneous population of cells, rigidly specified early in development to differentiate exclusively as skeletogenic cells and are the first to ingress into the blastocoel at the blastula stage (Okazaki 1975; Decker & Lennarz 1988). A great number of experimental observations suggest that PMCs require axial, temporal and scalar information provided by the overlying ectoderm to synthesize a normal sized and patterned skeleton (Ettensohn & Malinda 1993; Armstrong & McClay 1994; Peterson & McClay 2003). PMCs give rise to about 32 or 64 cells per embryo depending on the species, and their number remains constant throughout embryo development. Unlike PMCs, SMCs are a heterogeneous population of non-skeletogenic cells with several different fates, giving rise to at least four populations, including pigment, blastocoelar, coelomic pouch and circumesophageal muscle cells (Gustafson & Wolpert 1963; Gibson & Burke 1985; Burke & Alvarez 1988; Tamboline & Burke 1992; Venuti *et al*. 1993). Among SMCs, some cells have a potential for skeletogenic differentiation when they happen to be under appropriate

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experimental conditions. The origin of fate-switching SMCs has been elucidated only in *Lytechinus variegatus* species. In fact, it seems that PMC removal from these embryos allows the switching to a skeletogenic fate of some prospective pigment and blastocoelar cells, whose fate had not yet been irreversibly specified (Ettensohn & Ruffins 1993). The potential to change cellular fate is normally suppressed by the PMCs, probably by direct filopodial contacts towards SMCs during the late gastrula stage (Ettensohn 1992; Miller *et al*. 1995). Thus, in the absence of a negative signal transmitted by the PMCs, some SMCs are able to change their fate into a skeletogenic phenotype. This process has been termed SMC transfating. The switching of cell fate appears at a time when late-ingressing SMCs migrate away from the tip of the archenteron, gradually accumulate at PMC-specific target sites and activate a PMC-specific program of gene expression, including de novo expression of all matrix spicule proteins (msp) and surface binding sites for wheat germ agglutinin (WGA) (DeSimone & Spiegel 1986). The result of this regulative event is the development of a correctly patterned skeleton. Therefore, removal of PMCs only delays skeleton development as well as archenteron differentiation, without affecting the timing of invagination (Ettensohn & McClay 1988; Hamada & Kiyomoto 2003).

A noteworthy study has been published very recently in which the author demonstrates that SMCs work as true skeletogenic cells in metamorphosing larvae and adult sea urchins, while PMCs act only until the larval stage (Yajima 2007). These findings update the role previously ascribed to SMCs, which was to differentiate towards all types of mesoderm cells except for skeletogenic ones, and account for the SMCs' potential for skeletogenic differentiation previously described only under experimental conditions.

Recently, we have shown that the inhibition of extracellular matrix (ECM)-ectoderm cells interaction causes a remarkable impairment of skeletogenesis. Briefly, treatment of mesenchyme blastula embryos with monoclonal antibodies (mAb) to *Pl*-nectin, an outer ECM protein, inhibits PMCs to correctly elongate skeletal rods, while ectoderm and endoderm cells are not affected in their differentiation and patterning (Zito *et al*. 1998, 2000, 2003). In such skeleton-defective embryos, besides a reduction in the expression of spicule-specific protein *Pl*-SM30, we found a decrease in the expression of *Pl*-univin, a growth factor of the TGF-β superfamily, which suggests its involvement in ecto-mesoderm inductive events.

In the present study, we take advantage of our skeleton perturbation model to investigate the ability of SMCs to direct skeletogenesis under non-permissive conditions. We examined the expression of specific surface molecules to assess SMC transfating in *P. lividus* PMC-less embryos after treatment with mAb to *Pl*-nectin. In addition, by RT–PCR and *in situ* hybridization, we tested the expression of *Pl*-SM30 in PMC-less embryos untreated and treated with mAb to *Pl*-nectin.

Materials and methods

Embryo culture and micromanipulation

Adult *Paracentrotus lividus* sea urchins were collected along the western coast of Sicily. Eggs were fertilized and embryos cultured at a dilution of 4.000/mL in Millipore Filtered Sea Water (MFSW) containing antibiotics, at 16–18°C. Embryos at different developmental stages were collected by low-speed centrifugation. To prevent ciliary movements, embryos were treated with double strength MFSW for 1 min. After two washes in MFSW, embryos were loaded into microinjection chambers, as described by Kiyomoto *et al*. (2004). PMCs were removed from embryos by flushing the blastocoel with MFSW, as reported previously (Ettensohn & McClay 1988). This micromanipulation was carried out using TransferTips needles (Eppendorf, Hamburg, Germany) with a Cell Tram Oil injector (Eppendorf, Hamburg, Germany) connected to a three-dimensional joystick manipulator (MO-202; Narishige, Tokyo, Japan). After micromanipulation, embryos were collected with the aid of a mouth pipette in small Petri dishes and allowed to develop at 16–18°C until the required stage.

Immunofluorescence

For immunostaining with fluorescein isothiocynate-conjugated wheat germ agglutinin (WGA-FITC), control and micromanipulated embryos were treated with Ca^{2+}-free MFSW for 2 min (Amemiya & Arakawa 1996) and fixed as whole mounts in 4% formaldehyde for 5 min. After rinsing in MFSW, embryos were incubated with WGA-FITC (SIGMA 2 mg/mL, 1:400 in MFSW) for 15 min at room temperature, in the dark, and washed three times in MFSW (modified from Morale *et al*. 1998). For double staining experiments, embryos were fixed in 4% paraformaldehyde in MFSW for 1 h at room temperature. After rinsing twice with phosphate-buffered saline (PBS)-0,1% Tween20 (PBS-T), the embryos were permeabilized with 100% methanol at –20°C. Embryos were washed with PBS-T and incubated with mAb 1D5 (1:10 in PBS-T) overnight at 4°C. After rinsing with PBS-T,

embryos were incubated with TRITC-conjugated, goat antimouse IgG secondary antibody (SIGMA, 1:200 in PBS-T) and WGA-FITC (1:400 in PBS-T) for 1 h at room temperature. Embryos were observed under a Zeiss Axioskop 2 Plus microscope (Zeiss, Arese, Italy), equipped for epifluorescence and recorded by a digital camera system.

Count of pigment cells

Count of pigment cells was carried out as described by Kominami (1998) with some modifications. Briefly, control and micromanipulated embryos were incubated with double strength Ca^{2+}-free MFSW for 30 min at room temperature. After rinsing with MFSW, embryos were placed on a glass slide, covered with a coverslip and squashed by removing the MFSW between the coverslip and glass slide with a piece of blotting paper. Spread specimens were observed under a Zeiss Axioskop 2 Plus microscope and the number of pigment cells was counted directly under the microscope.

Perturbation assay

Perturbation assays with mAb to *Pl*-nectin (NEVIE11h7) on whole embryos were carried out according to Zito *et al*. (1998, 2003). Briefly, purified IgGs were added at a concentration of 3 μg/μL to microtiter plate wells containing micromanipulated embryos in a total volume of 100 μL. Embryos were continuously cultured in the presence of the antibody at 16–18°C.

RNA preparation from micromanipulated embryos and relative RT–PCR analysis

PolyA$^+$ RNA was isolated according to the manufacturer's instructions using Dynabeads Oligo (dT)25 (Dynal Biotech) from 12 embryos. One third of the volume of each experimental point was then used in the amplification performed by SuperScript One-Step RT–PCR kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The *Pl*-SM30 primers used were: 5′-TTGGGTTCAGTTGGAGAACC-3′ and 5′-GTTTCGTTGTCTTCGGGGTA-3′ (Zito *et al*. 2003).

In addition, the following primers were used to amplify *Pl*-S24, a transcript encoding a ribosomal protein of the *P. lividus* embryo, as an internal reference control: 5′-CTGATCAGACCATGCTCTAAGGT-3′ and 5′-CCTGATGTCGTCAGTACAACGTA-3′ (Zito *et al*. 2003). The reverse transcription (RT) reaction was carried out for 30 min at 50°C; after denaturation for 2 min at 94°C, polymerase chain reaction (PCR) amplification was carried out for 35 cycles as follows: 94°C, 30 s; 55°C, 45 s; 68°C, 30 s. The total volume of the PCR products was analyzed on a 2% gel. To calculate the relative *Pl*-SM30 and *Pl*-S24 expression, band intensities for each amplification product were quantified by scanning, using a Gel Doc 1000 detection system (BIO-RAD, Hercules, CA, USA) equipped with a Multi-Analyst program, version 1.1. Values obtained were expressed in AU as the percentage volume of band intensities.

Whole-mount in situ *hybridization*

Labeling of probes was carried out as described by Zito *et al*. (2003). Briefly, labeled single-stranded antisense and sense DNA probes were generated by asymmetric PCR in the presence of DIG-dUTP, using a SM30 cDNA of 380 bp as a template. Whole-mount *in situ* hybridization was carried out as described by Croce *et al*. (2003), with some modifications. All prehybridization and hybridization steps were carried out in sterile microtiter-plate wells (96 well plates, Greiner Labortechnik, Longwood, FL, USA), using 20–30 embryos per well. Fixed embryos were re-hydrated and hybridized with 50 ng/mL of DNA probe overnight at 65°C. After washing, embryos were stained, observed under a Zeiss Axioscop 2 Plus microscope and recorded by digital camera. Hybridizations with sense probe were carried out and showed no specific signal above background.

Results

Analysis of SMC transfating in P. lividus *PMC-less embryos*

To study SMC transfating in the Mediterranean species *P. lividus*, we carried out micromanipulation experiments in which all PMCs were removed from mesenchyme blastula embryos, about 16 h after fertilization. The resulting PMC-less embryos were analyzed by microscopic inspection for skeleton development at different time points after micromanipulation. As expected, the removal of PMCs only delayed the morphogenesis of the skeleton that eventually developed correctly both in size and pattern. The first indication of the regulative behavior of SMCs appeared about 10–12 h after PMCs removal (26–28 h postfertilization), when two small triradiate spicule rudiments formed in a ventrolateral position (see below). As development proceeded, spicule rods lengthened and branched in a manner identical to that observed in normal embryos. Twenty-four hours after PMCs removal, embryos were found at the prism (Fig. 1A) or early pluteus (Fig. 1B) stage and eventually they developed as normal

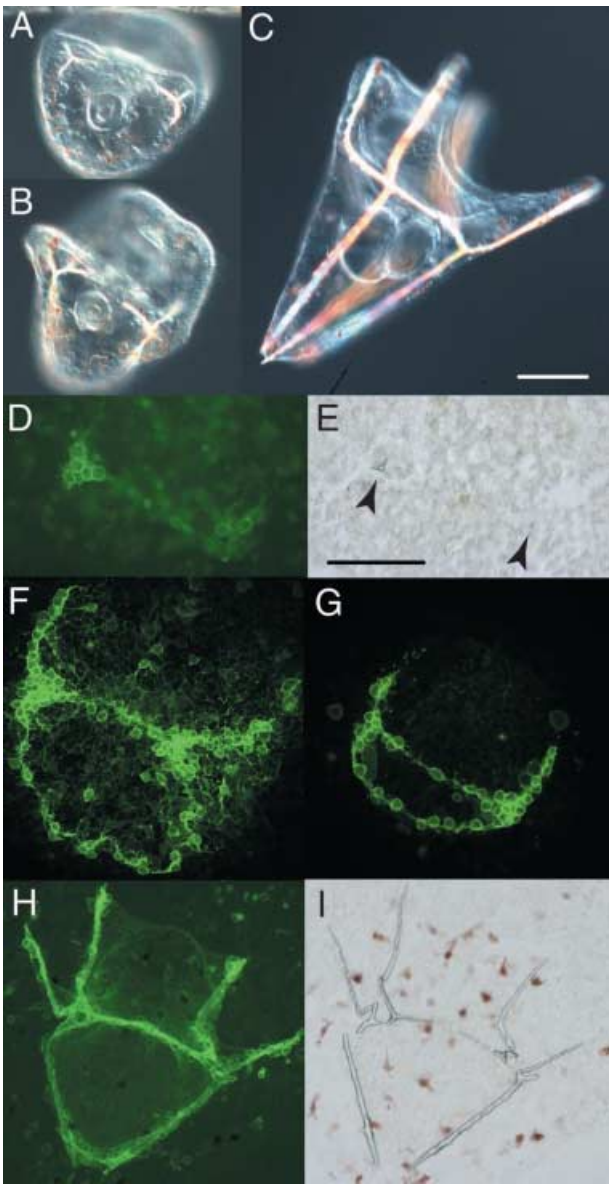

Fig. 1. Secondary mesenchyme cell (SMC) transfating and skeleton development in *Paracentrotus lividus* embryos. Primary mesenchyme cell (PMC)-less embryos observed 24 h (A, B) and 48 h (C) after PMCs removal. Fluorescein isothiocyanate-conjugated wheat germ agglutinin (WGA-FITC) staining of squash preparations of embryos labeled 10 h (D), 24 h (F) and 48 h (H) after PMCs removal. Bright fields (E) and (I) correspond to (D) and (H), respectively. Arrowheads in (E) indicate spicule rudiments. Normal embryo 24 h after fertilization (G). (D) and (E) are at higher magnification. Bars, 50 μm.

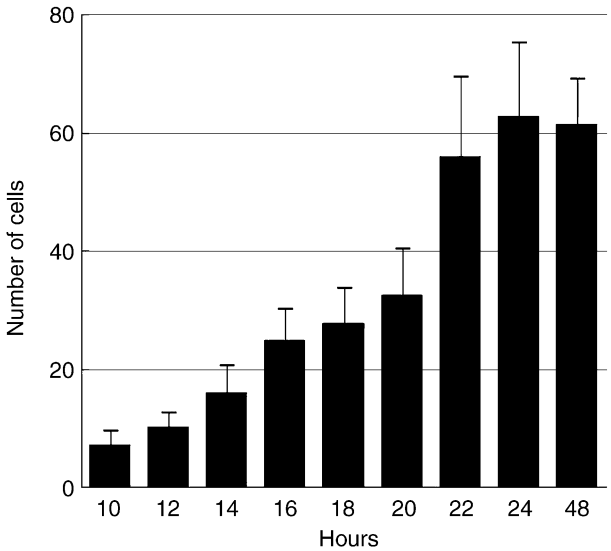

Fig. 2. Number of wheat germ agglutinin (WGA)-positive cells during development after primary mesenchyme cell (PMC) removal. PMC-less embryos were stained with fluorescein isothiocyanate-conjugated wheat germ agglutinin (WGA-FITC) at different time points after micromanipulation. Each bar represents the mean of 6–50 embryos scored in two to seven independent experiments ± SD.

plutei within the next 24 h (48 h after PMCs removal, 64 h postfertilization; Fig. 1C). To gain further information on SMC transfating in this species, PMC-less embryos were stained with WGA-FITC and observed. We found that WGA-positive cells were always grouped in two ventrolateral clusters after 10 h (Fig. 1D,E). Then, an increasing number of WGA-positive cells arranged themselves in a subequatorial ring (Fig. 1F) and eventually dispersed regularly along the skeleton rods within 48 h (Fig. 1H,I). Since we observed an obvious increase in the number of putative transfated SMCs with respect to PMCs present in control embryos (compare Fig. 1F with Fig. 1G), a number of experiments were carried out in order to count WGA-positive cells at different time points after micromanipulation. Figure 2 shows results of two to seven experiments in which 6–50 embryos have been scored for each time point. The analysis of micromanipulated embryos revealed an almost constant increase in the number of WGA-positive cells between 10 and 20 h after PMCs removal, from 7 ± 2 to 32 ± 8 cells, the latter number being very close to that of PMCs counted in normal pluteus embryos of the *P. lividus* species (Zito *et al.* 2003). Surprisingly, the number of WGA-positive cells roughly doubled, reaching an average of 56 ± 13 between 20 and 22 h after PMCs removal. Later, their total amount slightly increased and reached about 62 cells at 24 and 48 h. The differences in the median values among each group are statistically significant by Kruskal–Wallis One Way Analysis ($P < 0.001$). As a second marker for SMC transfating and to further characterize WGA-positive cells, we

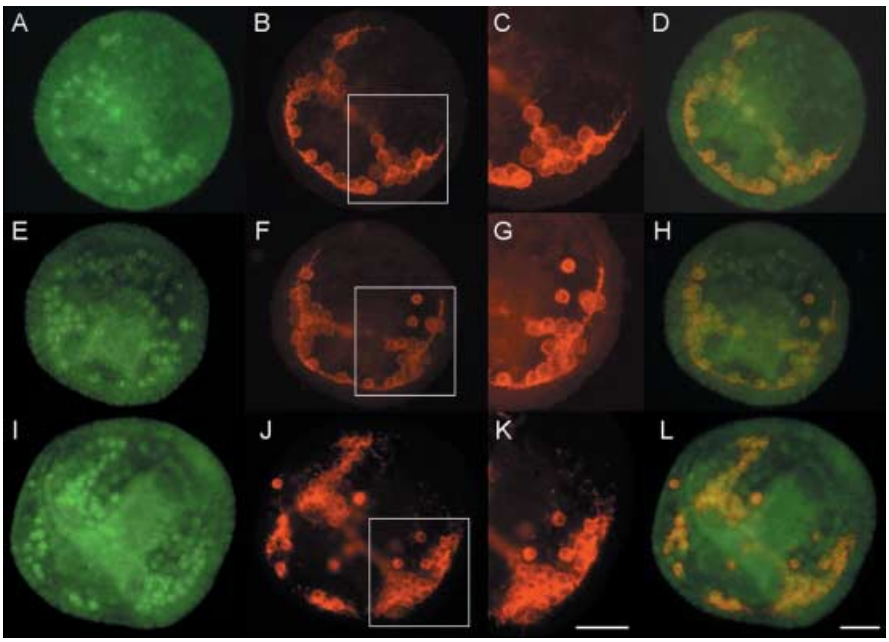

Fig. 3. Localization of msp130 glycoprotein in late gastrula (A-D) and primary mesenchyme cell (PMC)-less embryos observed 18 h (E–H) and 24 h (I–L) after PMCs removal. (A, E, I) Fluorescein isothiocyanate-conjugated wheat germ agglutinin (WGA-FITC) staining; (B, F, J) staining with monoclonal antibodies (mAb) 1D5; (C, G, K) higher magnification of parts of B, F, J, respectively; (D, H, L) merged images of A–B, E–F and I–J, respectively. Bars, 20 μm.

used mAb 1D5, an antibody that recognizes the msp130 glycoprotein, a cell surface protein normally expressed only in PMCs (Anstrom *et al.* 1987; Farach *et al.* 1987; Leaf *et al.* 1987). We carried out double-staining on whole-mount late gastrulae and PMC-less embryos harvested 18 and 24 h after PMCs removal, using WGA-FITC and mAb 1D5. We found that the number of msp130-positive cells in 18 h-PMC-less embryos was close to that of control gastrulae (compare Fig. 3F,G with 3B,C), matching WGA expression at this stage (compare Fig. 3A,D with 3E,H). On the contrary, we counted a higher number of msp130-positive cells in 24 h-PMC-less embryos than in control gastrulae (compare Fig. 3J,K with 3B,C), which exactly matched the increased number of WGA-positive cells (Fig. 3I,L). *In situ* hybridization experiments on whole-mount embryos using a msp130 antisense probe produced a similar result, namely, an increased number of msp130-positive cells in 24 h-PMC-less embryos with respect to control gastrulae (not shown).

A further issue to be clarified was the origin of transfated SMCs, which is almost controversial among different sea urchin species. In fact, in *L. variegatus* it was shown that they originate from fate-switching of pigment cells, since a reduction in the number of these cells was observed in PMC-less embryos (Ettensohn & Ruffin 1993); on the contrary, in *Hemicentrotus pulcherrimus* the number of pigment cells did not change under the same conditions (M. Kiyomoto, unpubl. data, 2003). It was then of some interest to count the number of pigment cells

Table 1. Number of pigment cells counted in normal and micromanipulated embryos

		Hours after micromanipulation (hours postfertilization)					
Trial no.	PMCs	22 (38)	24 (40)	30 (46)	46 (62)	47 (63)	50 (66)
1	+			33 ± 3		41 ± 6	40 ± 5
	–			31 ± 5		38 ± 5	40 ± 2
2	+	31 ± 1	33 ± 2		30 ± 4		
	–	29 ± 2	29 ± 4		33 ± 6		

PMCs, primary mesenchyme cells.

at different time points after PMCs removal. Table 1 summarizes results from two independent experiments where an average of eight embryos per each point was scored. At first, we found that the number of pigment cells in normal embryos comprised between 30 and 40, from 38 to 66 h postfertilization. Interestingly, their number remained close to that of normal embryos in PMC-less embryos observed at the same time, corresponding to an interval ranging from 22 to 50 h after PMCs removal. The differences in the average numbers of control and micromanipulated embryos are not statistically significant by unpaired *t*-test (between $P > 0.06$ and $P > 0.7$).

Treatment of PMC-less embryos with mAb to PI-*nectin*

Recently, we have demonstrated that the inhibition of outer ECM–ectoderm cells interactions, by culturing mesenchyme blastula embryos in the presence of

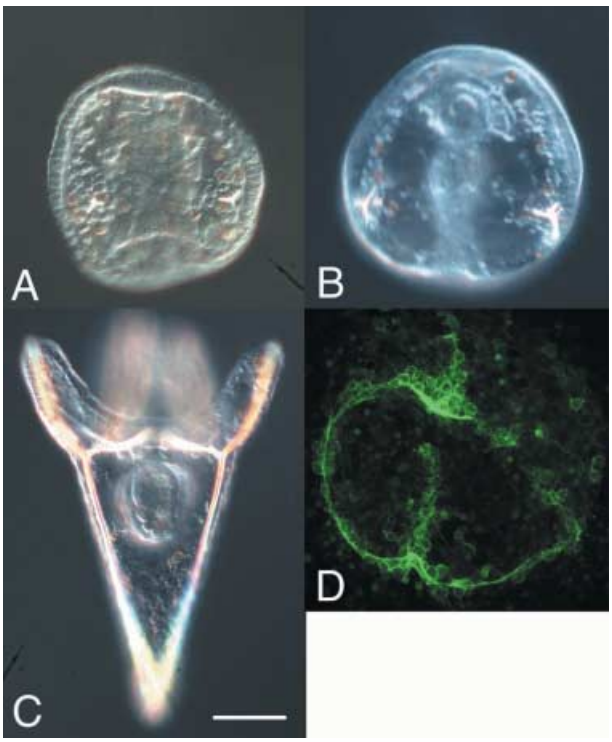

Fig. 4. Secondary mesenchyme cell (SMC) transfating in embryos treated with mAb to *Pl*-nectin. Primary mesenchyme cell (PMC)-less embryos cultured in the presence of monoclonal antibodies (mAb) to *Pl*-nectin for 24 (A) and 48 (B) hours. Normal embryo (C), cultured for the same period of time as in (A). Fluorescein isothiocyanate-conjugated wheat germ agglutinin (WGA-FITC) staining of PMC-less embryo (D) treated as in (A). Bars, 50 μm.

mAbs to *Pl*-nectin, caused a complete and permanent inhibition of skeleton development (Zito *et al*. 2003). The extent of malformations was directly correlated with reduced mRNA levels of *Pl*-SM30, a specific spicule matrix protein whose synthesis is regulated in space and time during skeletogenesis (George *et al*. 1991; Guss & Ettensohn 1997; Kitajima & Urakami 2000; Urry *et al*. 2000; Zito *et al*. 2003). To test whether mAbs to *Pl*-nectin could affect embryos in which transfated SMCs were directing skeletogenesis, we designed micromanipulation experiments in which PMC-less embryos were cultured in the presence of mAbs to *Pl*-nectin within 2 h after PMCs removal. Henceforth these embryos will be referred to as P^-/A^+, where P stands for PMCs and A for antibodies. PMC-less embryos with no antibodies treatment will be referred to as P^-/A^-. Normal embryos cultured in the absence or presence of mAbs to *Pl*-nectin for the same period of time will be referred to as P^+/A^- and P^+/A^+, respectively. Embryos were analyzed at the morphological level 24 and 48 h after PMCs removal and mAb addition. We found that, after 24 h of culture in the presence of mAb, P^-/A^+ embryos showed a remarkable impairment of skeletogenesis (Fig. 4A), similar to that observed in normal embryos cultured in the presence of mAb to *Pl*-nectin (Zito *et al*. 2003). Such skeleton-defective embryos typically showed two small triradiate spicule rudiments and no arms, while normal polarization of the ectoderm along oral-aboral axis as well as endoderm differentiation and patterning were not affected. Embryos never elongated their spicule rudiments, even after prolonged periods of culture in the presence of mAb to *Pl*-nectin (Fig. 4B). On the contrary, we have just shown that PMC-less embryos cultured in the absence of mAbs to *Pl*-nectin (P^-/A^- embryos) developed to the pluteus stage by 48 h after PMCs removal (Fig. 1C). As expected, P^+/A^- control embryos, cultured in MFSW for the same periods of time, were at the pluteus stage (Fig. 4C). It is noteworthy that, as previously observed for P^-/A^- embryos, also P^-/A^+ embryos (18 scored) showed an almost doubled number of WGA-positive cells (69 ± 11), arranged in a subequatorial ring (Fig. 4D).

Expression of a skeleton specific gene in micromanipulated and perturbed embryos

In addition to the morphological criteria used for the assessment of SMC transfating, a molecular analysis of the expression of *Pl*-SM30 transcripts was carried out by RT–PCR on normal and PMC-less embryos cultured in the absence or presence of mAb to *Pl*-nectin and harvested 24 h after PMC removal (Fig. 5). Five independent experiments were carried out and results expressed in arbitrary units as the percentage of band intensities volumes. As an internal control, we used S24, a gene encoding for a ribosomal protein, which is expressed at similar levels in all stages used (see also Zito *et al*. 2003). In agreement with our previous results (Zito *et al*. 2003), we found a reduction in *Pl*-SM30 expression, corresponding to a threefold (P^+/A^+) and a sixfold (P^-/A^+) decrease, in those embryos cultured in the presence of mAb to *Pl*-nectin, with respect to P^+/A^- control embryos. A 2.2-fold decrease in *Pl*-SM30 expression was observed in PMC-less embryos (P^-/A^-) with respect to controls. Differences in *Pl*-SM30 expression levels, calculated for each experimental group of embryos with respect to controls, are highly statistically significant as assessed by a *t*-test ($P < 0.0001$).

The low levels of *Pl*-SM30 expression in each group of P^- embryos, namely, both untreated and treated with mAb to *Pl*-nectin, paralleled the morphological observations and suggested that probably a small number of transfated SMCs were actively expressing

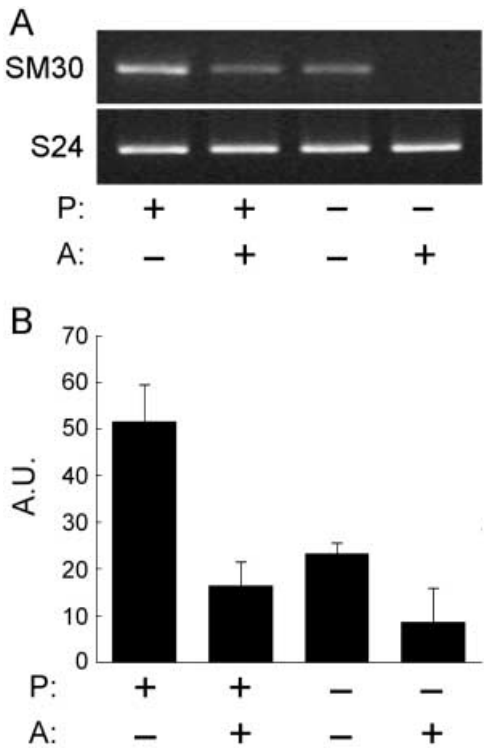

Fig. 5. Relative reverse transcription-polymerase chain reaction (RT–PCR) analysis of the *Pl*-SM30 transcript levels in normal (P^+) and primary mesenchyme cell (PMC)-less (P^-) embryos cultured in the absence (A^-) or presence (A^+) of mAb to *Pl*-nectin, 24 h after PMCs removal. (A) A representative RT–PCR of SM30 and S24, a transcript encoding a ribosomal protein, used as internal control; (B) histogram shows the quantitative analysis of five independent experiments ± SD. Values obtained from measurement of band intensities for each amplification product are expressed in arbitrary units.

the skeleton-specific SM30 gene. Therefore, we carried out *in situ* hybridization experiments on whole-mount embryos, using a *Pl*-SM30 antisense probe on normal or PMC-less embryos, cultured in the absence or presence of mAb to *Pl*-nectin and harvested 24 and 48 h after PMCs removal. As expected, SM30-positive cells were found along the skeleton rods of P^+/A^- embryos (Fig. 6A,E), whereas a reduced number was found in all the other categories (Fig. 6B–D,F,H), except for PMC-less embryos (P^-/A^-, Fig. 6G), which showed an almost normal number of SM30-positive cells 48 h after PMCs removal. Figure 7 shows results of quantitative analysis from two to four independent experiments in which an average of 15 embryos were scored for each experimental point. In control embryos, 40 h post fertilization, we detected 25 ± 7 SM30-positive cells (Fig. 7A). At the same time, corresponding to 24 h after PMCs removal, we found only a few SM30-positive cells in all embryos treated with mAb to *Pl*-nectin (P^+/A^+ and P^-/A^+), whereas 7 ± 5 positive cells were scored in PMC-less embryos (P^-/A^-). Forty-eight hours after PMCs removal, the number of SM30-expressing cells in P^-/A^- embryos increased up to 27 ± 7, which is close to the number counted in P^+/A^- control embryos (30 ± 4) (Fig. 7B). On the contrary, all the skeleton-defective embryos (P^+/A^+ and P^-/A^+) still showed a reduced number of SM30-positive cells, namely 5 ± 5 and 7 ± 5, respectively. Hybridizations with a sense probe showed no specific signal above background (not shown). The differences in the mean number of SM30-positive cells, calculated for each experimental group of embryos with respect to controls, are highly statistically significant as assessed by a *t*-test ($P < 0.0001$, except for P^-/A^- at 48 h after PMCs removal, which is $P < 0.05$).

Discussion

In our previous papers, we demonstrated that interactions between ectoderm and *Pl*-nectin, an

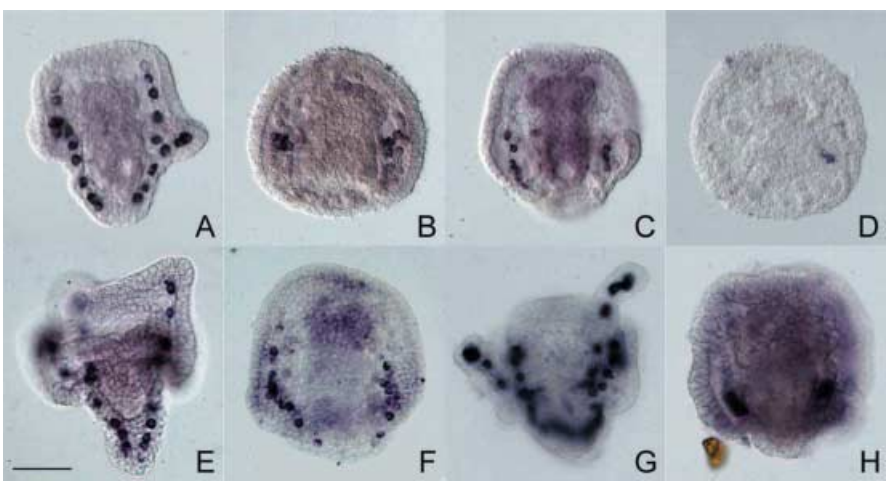

Fig. 6. Localization of *Pl*-SM30 mRNA in micromanipulated and monoclonal antibodies (mAb) to *Pl*-nectin treated embryos, 24 (A–D) and 48 (E–H) hours after primary mesenchyme cell (PMC) removal. (A, E) Untreated embryos (P^+/A^-); (B, F) normal embryos cultured in the presence of mAb to *Pl*-nectin (P^+/A^+); (C, G) PMC-less embryos cultured in the absence of mAb to *Pl*-nectin (P^-/A^-); (D, H) PMC-less embryos cultured in the presence of mAb to *Pl*-nectin (P^-/A^+). Bar, 50 μm.

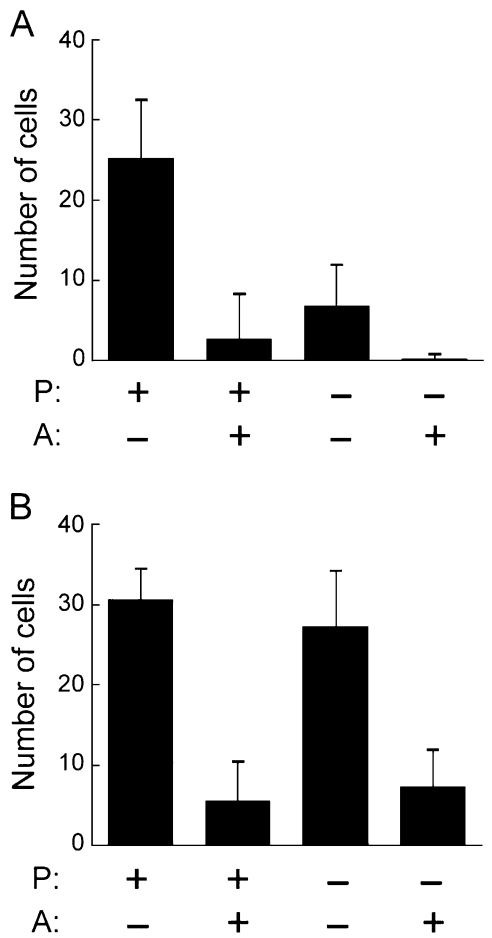

Fig. 7. Number of SM30-positive cells in micromanipulated and monoclonal antibodies (mAb) to *Pl*-nectin treated embryos. Each bar represents the mean ± SD of cells scored 24 h (A) and 48 h (B) after primary mesenchyme cell (PMC) removal.

outer ECM protein, are required for correct skeleton development. In particular, dramatic skeleton defects were observed in embryos treated with mAb to *Pl*-nectin as a consequence of reduced or absent inductive signal(s) in the blastocoel (Zito *et al*. 2003; Kiyomoto *et al*. 2004). One such signal has been identified in the TGF-β growth factor *Pl*-univin, whose involvement in ecto-mesoderm induction events has been elucidated (Zito *et al*. 2003).

In this report, we focused on transfated SMCs in embryos exposed to mAb to *Pl*-nectin after PMCs removal, to investigate the competence of fate-switching cells to respond to environmental cues needed to synthesize spicules. At first, we described the time schedule of SMC transfating in *P. lividus* embryos, taking into account some main features of this event: changes in the arrangement of cells inside the blastocoel, expression of PMC-specific cell surface molecules and spiculogenesis.

We then demonstrated that, despite cells being arranged in PMC-specific target sites, normal skeleton development occurred with some delay, and SMC transfating resulted in a significant increase in the number of WGA/msp130-positive cells with respect to the number of SM30-expressing cells, which instead was close to that of PMCs in normal embryos. Moreover, interfering with interactions between ectoderm cells and *Pl*-nectin in PMC-less embryos resulted in perturbation of skeleton development, paralleled by reduced levels of *Pl*-SM30 expression. These observations support the conclusion that, although phenotypic transfating of previously non-skeletogenic cells does not need ectoderm inductive signals, such signals are necessary in order for transfated SMCs to synthesize a normal skeleton.

Cell number and origin of transfated cells in P. lividus *PMC-less embryos*

The first sign of phenotypic SMC transfating in *P. lividus* appeared about 10 h after PMCs removal, when we found few strongly stained WGA-positive cells inside the blastocoel. Moreover, we observed that WGA-positive cells grouped in two ventrolateral clusters as soon as they first appeared and shortly arranged in a regular subequatorial ring. At the same time, two small triradiate spicule rudiments began to be observed in ventrolateral positions. As expected, the removal of PMCs in *P. lividus* embryos only delayed skeleton synthesis by several hours, since it did not interfere with the regular elongation and patterning of skeletal rods. Slightly different situations have been described concerning the early steps of the transfating process among sea urchin species. In *H. pulcherrimus*, the first skeletogenic cells, detected by staining with a PMC-specific mAb, which recognizes glycoproteins of the same group of msp130 (Shimizu *et al*. 1988; Shimizu-Nishikawa *et al*. 1990), appear at the tip of the archenteron when this reaches the animal pole, before they scatter into the blastocoel (Hamada & Kiyomoto 2003). Differently, in *L. variegatus* the expression of both WGA-binding sites and msp130 glycoprotein appear on transfated SMCs when they are already scattered inside the blastocoel but before they arrange themselves in the characteristic ventrolateral clusters (Ettensohn & McClay 1988; Ettensohn 1992). Thus, in *H. pulcherrimus* and *L. variegatus* the expression of PMC-specific surface molecules precedes the organization of transfated cells in the typical ring pattern and consequently in the onset of skeletogenesis. On the contrary, in *P. lividus* embryos the expression of

PMC-specific molecules and cells clustering occur almost at the same time.

The appearance at later stages of an abnormal number of WGA/msp130-positive cells associated with the skeleton was completely unexpected. In fact, we counted about 60 WGA/msp130-positive cells from 22 h on after PMCs removal, both in embryos untreated and treated with mAb to *Pl*-nectin, while the number of PMCs in normal *P. lividus* species was about 36 ± 2 at the pluteus stage (Zito *et al*. 2003). The reason for the doubling of WGA/msp130-positive cells is still unclear. To the best of our knowledge, similar examples have not been described yet. To explain our findings, we can hypothesize that some of them transfate partially and show at least two of the PMC features characteristic of early skeletogenesis (WGA-binding molecules and msp130 glycoproteins), never switching completely to a skeletogenic phenotype. Thus, truly transfated SMCs should be considered only those expressing the three skeletogenic markers (WGA-binding proteins, msp130 proteins and SM30 gene), in agreement with information accumulated in different species. Alternatively, it could be possible that a number of SMCs switch to skeletogenic fate within 20 h of PMCs removal (about 32 ± 8, see Fig. 2), and, by 22 h, they divide for an unknown reason. The observation that the doubling of cells occurred in a very short interval (about 2 h) would be in agreement with such a cell division hypothesis. On the contrary, data from published reports are in disagreement with this hypothesis. In fact, in *L. variegatus* embryos, after a final round of division carried out shortly after ingression, PMCs do not undergo DNA synthesis and therefore do not divide during the entire period of skeletogenesis (Stephens *et al*. 1986; Ettensohn & McClay 1988; Nislow & Morrill 1988; Tanaka & Dan 1990; Ettensohn & Malinda 1993). Furthermore, no case of cell division has been observed in *L. variegatus* micromanipulated embryos. In fact, it has been shown that the replacement of missing PMCs by SMCs is quantitative, which means that the embryo regulates the number of SMCs that switch fate accordingly to the number of PMCs removed. Moreover, no case of converting cells undergoing division was recorded during this process (Ettensohn & McClay 1988). Experiments are in progress to elucidate this point.

A further issue to be elucidated in *P. lividus* embryos is the origin of transfated SMCs, which is also controversial among different sea urchin species. In fact, in *L. variegatus* embryos, pigment and blastocoelar cells have been identified as the populations of SMCs that carry out skeletogenic fate-switching in PMC-less embryos, since their number decreases considerably (Ettensohn & Ruffin 1993). On the contrary, transfated SMCs seem to have a different, although unknown, origin in *H. pulcherrimus*, since the number of pigment cells does not change after PMCs removal (M Kiyomoto, unpubl. data, 2003). However, it should be highlighted that the number of pigment cells is rather variable among sea urchin species, although timing of their specification may be common. In fact, *L. variegatus* embryos have about 100 pigment cells (Ettensohn & Ruffin 1993), *S. purpuratus* about 28 ± 5 (Gibson & Burke 1985), *H. pulcherrimus* between 40 and 60 (Kominami 1998), while in *P. lividus* embryos we counted between 30 and 40 cells at late stages. In agreement with what was observed in *H. pulcherrimus*, we did not record any reduction in the number of pigment cells in PMC-less embryos, which instead remains close to that of normal embryos. In any case, the small number of pigment cells counted in *P. lividus* could not account for the 60–70 WGA-positive cells counted in our PMC-less embryos. Unfortunately, we were not able to detect the number of other SMC populations in normal and in PMC-less embryos, thus we cannot exclude any of them as potential source of transfated cells.

Cell–ECM interaction is required for SM30 gene expression in PMC-less embryos

The interest in studying the competence of transfated SMCs to respond to ectoderm signals, necessary to synthesize spicules, came from our findings reporting the effects on skeleton development by inhibition of ECM–ectoderm cell interactions. Indeed, we showed that treatment with mAb to *Pl*-nectin produced embryos with remarkable skeleton defects, but with a normally compartmentalized gut and polarized ectoderm (Zito *et al*. 2003). Furthermore, the skeleton deficiencies were correlated with a reduction in the number of SM30-expressing PMCs. Here, we found that also skeleton development and *Pl*-SM30 expression by transfated SMCs were affected when the interactions between ectoderm and *Pl*-nectin were prevented. The almost normal number of SM30-expressing cells counted at late stages in PMC-less embryos, never treated with mAb to *Pl*-nectin, was in agreement with the morphological observations showing the presence of a normal developing skeleton. It has already been emphasized that, despite the presence of doubled WGA/msp130-positive cells, the number of SM30-expressing cells counted by *in situ* hybridization remained almost equal to that of PMCs expressing SM30 in normal embryos. A great

deal of evidence indicates that skeleton elongation and patterning are regulative and not autonomously specified events, meaning that they greatly depend on the microenvironment that surrounds PMCs. In agreement, PMCs from skeleton-defective embryos obtained after different treatments, for example with mAb to *Pl*-nectin or with $NiCl_2$, are able to synthesize skeleton if introduced into the blastocoelic cavity of normal PMC-less embryos (Armstrong *et al*. 1993; Kiyomoto *et al*. 2004). Furthermore, these results are in agreement with the hypothesis that ectoderm cells supply the signal(s) necessary both for skeleton development in normal embryos or for resuming the skeletogenic program in experimentally perturbed ones. Our experiments using mAb to *Pl*-nectin led us to link, for the first time, ecto-mesodermal signaling with the interactions between ectoderm and the apical ECM. In particular, we proposed a model in which the correct association of ectoderm cells with *Pl*-nectin allows these cells to produce the signal(s) required for spicule growth. We identified *Pl*-univin, a growth factor of the TGF-β family, as a signaling molecule in ecto-mesoderm induction events (Zito *et al*. 2003). The results showed here, together with data from published reports, suggest as a whole that SMCs could be responsive to the action of *Pl*-univin or related factors, but this happens only when they switch their fate to the skeletogenic one. Thus, transfated SMCs synthesize a normal skeleton in PMC-less embryos, when the ectoderm inductive signal is present. On the other hand, they produce short defective spicules or a completely radialized skeleton in mAb to *Pl*-nectin-treated and in $NiCl_2$-treated embryos, respectively, when the ectoderm inductive signal is missing (Ettensohn & McClay 1988; Hardin *et al*. 1992; Zito *et al*. 2003).

Taken together, these findings lead us to some conclusions. First, ectoderm inductive signals are not involved in the phenotypic transfating events of previously non-skeletogenic cells. Second, an unknown regulative mechanism, other than the suppressive signal operated by PMCs, seems to be involved in the transfating of the right number of SMCs in the *P. lividus* embryo. Third, pigment cells are not the prospective SMCs able to fate-switch in the *P. lividus* species. Fourth, transfated SMCs respond to the signal(s) originating from *Pl*-nectin-interacting ectoderm, which induces skeleton elongation and patterning.

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