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# Regulative specification of ectoderm in skeleton disrupted sea urchin embryos treated with monoclonal antibody to *Pl*-nectin

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*Pl*-nectin is a glycoprotein first discovered in the extracellular matrix (ECM) of *Paracentrotus lividus* sea urchin embryo, apically located on ectoderm and endoderm cells. The molecule has been described as functioning as an adhesive substrate for embryonic cells and its contact to ectoderm cells is essential for correct skeletogenesis. The present study was undertaken to elucidate the biochemical characteristics of *Pl*-nectin and to extend knowledge on its *in vivo* biological function. Here it is shown that the binding of mesenchyme blastula cells to *Pl*-nectin-coated substrates was calcium dependent, and reached its optimum at 10 mM $Ca^{2+}$. Perturbation studies using monoclonal antibody (McAb) to *Pl*-nectin, which prevent ectoderm cell-*Pl*-nectin contact, show that dorsoventral axis formation and ectoderm differentiation were retarded. At later stages, embryos recovered and, even if growth and patterning of the skeleton was greatly affected, the establishment of dorsoventral asymmetry was reached. Similarly, the expression of specific ectoderm and endoderm territorial markers was achieved, although occurring with some delay. Endoderm differentiation and patterning was not obviously affected. These results suggest that both endoderm and ectoderm cells have regulative capacities and differentiation of territories is restored after a lag period. On the contrary, failure of inductive differentiation of the skeleton cannot be rescued, even though the ectoderm has recovered.

**Key words:** calcium-dependent adhesion, development, differentiation, dorsoventral axis formation, extracellular matrix.

## Introduction

Studies on morphogenesis often require the analysis of biochemical and functional properties of the extracellular matrix (ECM) components present in the developing embryo. Knowing how cells adhere to each other and to their ECM is essential to an understanding of some of the most fundamental properties of multicellular systems. Adhesion sorts embryonic cells into germ layers (Dufour *et al.* 1988), guides cell migration and response to extracellular cues (Huttenlocher *et al.* 1995), and underlies both differentiation and the stability of the differentiated state (Adams & Watt 1993). Loss or misregulation of adhesion leads to diseases and cancer (Shapiro 1998). While the overall effects of ECM on cell differentiation are known, the biochemical and molecular bases for these effects have remained unclear.

The ECM of the sea urchin embryo has been shown to play a critical role during morphogenesis (McCarthy & Burger 1987; Wessel & McClay 1987; Ingersoll & Ettensohn 1994; Berg *et al.* 1996; Burke *et al.* 1998). In most cases, the function of ECM molecules has been substantiated by demonstration of loss of normal morphogenesis correlating with a lack of ECM synthesis or with function-blocking antibodies. For example, treatment of embryos with inhibitors of glycoprotein synthesis (Schneider *et al.* 1978; Heifitz & Lennarz 1979), of collagen deposition (Butler *et al.* 1987; Wessel & McClay 1987; Benson *et al.* 1991) or of glycosaminoglycan metabolism (Karp & Solursh 1974; Solursh *et al.* 1986) resulted in the inhibition of gastrulation and spiculogenesis. A differential effect on the expression of tissue-specific molecular markers following the addition of drugs disrupting ECM has been described (Wessel *et al.* 1989; Benson *et al.* 1991). Unfortunately, studies with inhibitors, while they point to possible roles of ECM in development, are often difficult to interpret because there is no direct correlation among the inhibitors used, a specific ECM molecule that might be affected, and the general effects observed. The other approach used to analyze the role of cell–ECM

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interactions during development involves function-blocking antibodies that have been used, for example, in the case of hyaline, where specific monoclonal antibodies (McAb) have been used in perturbation experiments (Adelson & Humphreys 1988). We have used this approach successfully, demonstrating that ectoderm cell–ECM interaction is essential for sea urchin embryo skeletogenesis (Zito *et al.* 1998). The contact with the ECM is established by a protein, called *Pl*-nectin, purified in our laboratory from eggs and embryos using gelatin-Sepharose affinity chromatography (Matranga *et al.* 1992). This protein has been found packed in cytoplasmic granules in unfertilized eggs, while after fertilization it is released into the ECM surrounding the embryo. At later developmental stages, *Pl*-nectin is localized on the apical surface of ectodermal and endodermal cells of the embryo. By *in vitro* adhesion assay, *Pl*-nectin has been shown to be a substrate adhesion molecule for dissociated blastula cells (Matranga *et al.* 1992). Here we extended our previous studies addressing the question if *Pl*-nectin would require calcium ions to mediate cell-substrate adhesion. Furthermore, we asked if the lack of interaction between *Pl*-nectin and ectoderm cells, obtained by the treatment of embryos with McAb to *Pl*-nectin, could affect ectoderm and endoderm cell differentiation.

## Materials and Methods

### *Adhesion assay*

To study the role of $Ca^{2+}$ in cell-substrate adhesion, we utilized an assay originally developed by McClay and Fink (1982) and modified by Matranga *et al.* (1992). Briefly, 10 µg/mL of purified *Pl*-nectin, obtained as described by Matranga *et al.* (1992), were bound to microtiter plate wells for 3 h at room temperature. Blanking of the wells was performed by incubation with 10 mg/mL bovine serum albumin (BSA) for 30 min at room temperature. Labeling of embryos was performed by culturing them with 22.2 kBq/mL of [$^3$H]-lysine, 3.2 TBq/mmol specific activity (Amersham, Buckinghamshire, UK), until the mesenchyme blastula stage. Radiolabeled dissociated cells, $1 \times 10^5$ per well, were settled onto *Pl*-nectin-coated wells in seawater, $Ca^{2+}$-free seawater (0 mM $Ca^{2+}$) or increasing concentrations of $Ca^{2+}$ for 1 h at 1 *g*. Then the plates were inverted and spun off for 8 min at 100 *g*. Cell binding was calculated as a percentage relative to the radioactivity associated with $1 \times 10^5$ [$^3$H]-labeled cells counted separately.

### *Perturbation assay*

Morphogenetic effects of the antibodies on whole embryos were tested according to Zito *et al.* (1998). As the same effects were obtained by using Fab fragments or purified immunoglobulin G (IgG), in the experiments described herewith we used purified anti-*Pl*-nectin IgG VIE11h7 or unrelated purified antimouse IgG as control. As already described in Zito *et al.* (1998), purified concentrated IgG was added to microtiter plate wells containing 100 embryos/100 µL, at a concentration of 2 µg/µL. Embryos were allowed to develop at 16°C. The development of single embryos was followed by using chamber slides as previously described (Zito *et al.* 1998) and photographed on a Leitz microscope (Leitz, Wetzlar, Germany), using Ilford FP4125 ISO film, or embryos were fixed with 0.1% formaldehyde and photographed on a Zeiss inverted microscope, using Ilford HP5400 ISO film.

### *Immunofluorescence*

Indirect immunofluorescence was performed on whole-mount embryos using McAb as probes of tissue-specific markers: 5a7 for Ecto V (Coffman & McClay 1990), 5c7 for Endo 1 (Wessel & McClay 1985), UH2–95 for ciliary band (Adelson 1985). The McAb were a kind gift from Prof. D. R. McClay. Whole-mount staining was carried out according to the method of Coffman and McClay (1990) with some modifications. Briefly, embryos were fixed for 2 min in ice-cold 0.1% formalin, permeabilized for 2 min in ice-cold methanol then rinsed in Millipore filtered seawater (MFSW). Embryos were incubated with each McAb tissue culture supernatant (1 : 1 in MFSW) for 30 min in ice, washed three times in MFSW, then incubated for 30 min in fluorescein isothiocyanate (FITC)-conjugated rabbit antimouse IgG (Sigma Chemical Co., St Louis, MO, USA) diluted 1 : 100 in MFSW. Embryos were observed under a Zeiss inverted microscope equipped for epifluorescence.

## Results

### Pl-*nectin requires calcium ions for its biological activity*

In the past years it has been demonstrated that interactions between cells and ECM proteins can be achieved by receptors that act in divalent cation-dependent processes (Sigurdson & Lwebuga-Mukasa 1994; Yin *et al.* 1997). Therefore, it was of some interest to investigate the $Ca^{2+}$ involvement in the promotion of cell adhesion to *Pl*-nectin. In the present study, we used an *in vitro* cell-substrate adhesion assay to test the adhesion of dissociated blastula cells to *Pl*-nectin-coated substrates in the presence of $Ca^{2+}$ ions at different concentrations. Figure 1 shows the results of a representative experiment in which the values reported

are expressed as the ratio of percentages of cell bindings to the substrate at different $Ca^{2+}$ concentrations versus control seawater. Each experimental point is the mean of four replicates. The concentration of *Pl*-nectin used to coat microtiter plates was 10 µg/mL, corresponding to $4.8 \times 10^{-7}$ M, assuming the molecular weight of the molecule is 210 kDa. The minimal concentration of $Ca^{2+}$ needed to observe an induction in cell adhesion was 0.5 mM, lower concentrations being completely ineffective. The highest value of cell binding was achieved when $Ca^{2+}$ concentrations were the same as those present in the seawater (10 mM). The experiments demonstrate that dissociated cells bind to *Pl*-nectin in a $Ca^{2+}$-dependent manner. All the adhesion assays were carried out at 16°C, the approximate temperature at which the embryos of *Paracentrotus lividus* develop in the Mediterranean seawater. In other experiments, cells were allowed to adhere to *Pl*-nectin-coated substrates at different temperatures, showing that adhesion is not permitted at temperatures lower than 16°C (data not shown). This can be explained by claiming that the initial adhesion is permitted below 16°C, but its strengthening is not and, therefore, a 100 *g* spin off causes the removal of weakly adherent cells. This explanation is in agreement with cell adhesion studies by Lotz *et al.* (1989). At temperatures as high as 23°C, values of cell binding were similar to those found at 16°C (data not shown).

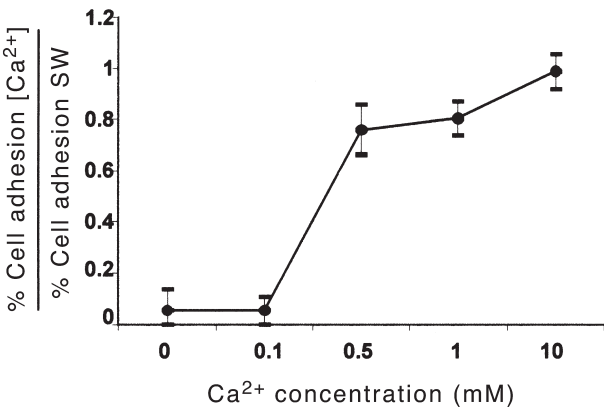


**Fig. 1.** Cell-*Pl*-nectin binding is $Ca^{2+}$ dependent. Each bar represents the mean ± standard error (SE) of four replicates from a representative experiment repeated three times. Values of controls made by plating cells onto bovine serum albumin (BSA), giving typically about 10–20% of unspecific binding, were subtracted. The ratio of percentage of binding from samples at different $Ca^{2+}$ concentrations to control seawater is reported on the ordinate axis. SW, sea water.

### *Perturbation of embryonic development by McAb to* Pl-*nectin*

We have recently shown that *Pl*-nectin-ectoderm cell interactions are necessary for the accomplishment of late skeletogenesis in the embryo of the sea urchin *P. lividus* (Zito *et al.* 1998). We therefore asked whether McAb specific to *Pl*-nectin would affect early events of gastrulation and skeletogenesis, or dorsoventral axis differentiation. We utilized a perturbation assay where McAb to *Pl*-nectin were added to early blastula embryos (8 h from fertilization at 16°C). The development of treated embryos was monitored at different time intervals taking advantage of the use of microchamber slides in which a single embryo could be observed for extended periods of time (Zito *et al.* 1998).

At first we found that treated embryos continued their development after the blastula stage with no apparent problems: primary mesenchyme cells (PMC) ingressed

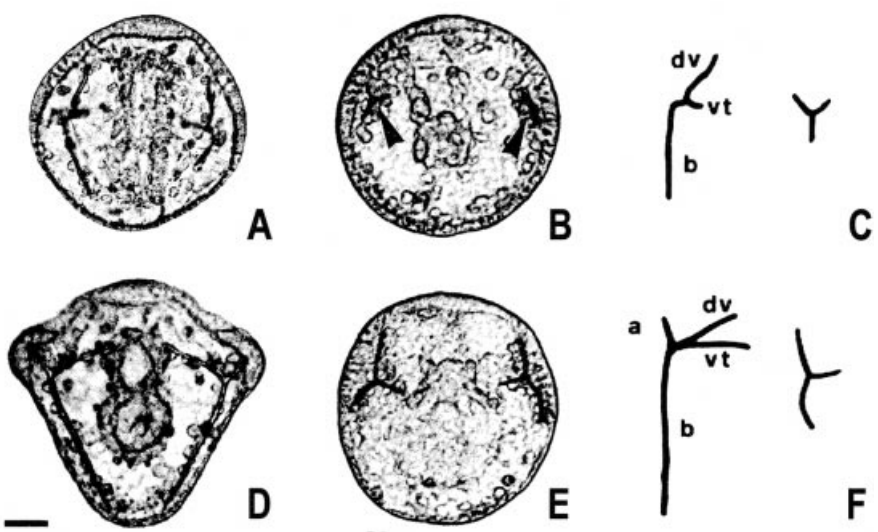


**Fig. 2.** Disruption of skeleton elongation and patterning in embryos treated with monoclonal antibody (McAb) to *Pl*-nectin. Early blastula embryos (8 h postfertilization) were cultured in the presence of unrelated immunoglobulin G (IgG; A,D) or VIE11h7 IgG (B,E) and observed after 30 h (A,B) or 38 h (D,E) from antibody addition. In panel (C) are sketched skeletal rods visible in (A; left) and (B; right); and in panel (F) are those visible in (D; left) and (E; right) in order for the effects on skeletal rod elongation to be readily observed. a, anal rod; b, body rod; dv, dorsoventral connecting rod; vt, ventrolateral transverse rod. Bar, 20 µm.

into the blastocoelic cavity on time and with the usual pattern, and the first step of archenteron invagination began at the expected time. Initiation of skeletogenesis was not affected as treated embryos showed triradiate spicule rudiments, as did the controls (data not shown). The first sign of perturbed development is observed at later stages, after 30 h of treatment, shown by shorter rudiments in antibody-treated embryos than in the controls (Fig. 2A–C). Later, after 38 h of treatment, the body rods that result from the branching of the anonymous rods (see Guss & Ettensohn 1997) were present in control embryos and the anal rods eventually appeared (Fig. 2D,F), while treated embryos showed defects in branching (Fig. 2E,F).

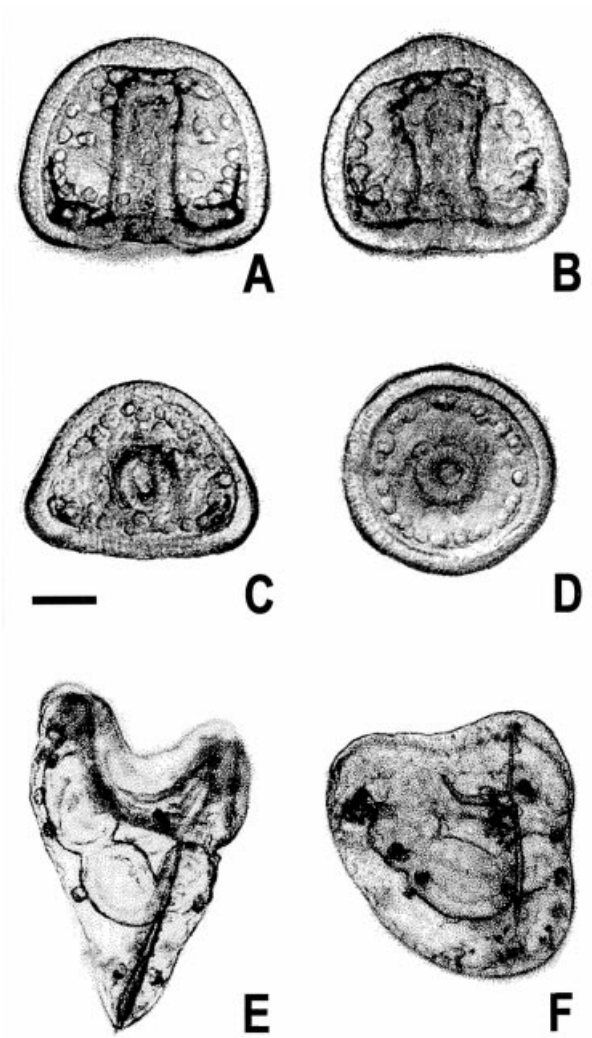


**Fig. 3.** Ectoderm differentiation and dorsoventral axis formation are delayed in embryos treated with monoclonal antibody (McAb) to *Pl*-nectin. Early blastula embryos (8 h postfertilization) were cultured in the presence of unrelated immunoglobulin G (IgG; A,C,E) or McAb VIE11h7 IgG (B,D,F) and observed after 24 h (A–D) or 42 h (E,F) from antibody addition. Embryos in (C,D) are viewed from the vegetal pole. Bar, 20 μm.

We also observed a delay in the polarization of the ectoderm along the oral–aboral axis. In fact, the two ventrolateral thickenings of the ectoderm, which normally appear at the late gastrula stage (Fig. 3A,C), were not present at this stage in treated embryos (Fig. 3B,D). The establishment of dorsoventral polarity is clearly observed in embryos after 42 h of treatment, when severe skeleton deficiencies are observed (Fig. 3E,F).

It is known that ectodermal differentiation involves the formation of distinctly different oral and aboral derivatives (Davidson 1989; Wikramanayake & Klein 1997). In treated embryos the specification of ectoderm territories occurred normally although with some delay: the squamous epithelium of aboral ectoderm as well as the characteristic animal cap thickening of the oral ectoderm was observed (compare Fig. 2A with Fig. 2E). A transient delay was observed in digestive apparatus development, which was exclusively restricted to the first step of archenteron elongation (data not shown). On the contrary, compartmentalization of the endoderm in the three parts of the intestine is found in embryos observed after 42 h of treatment (Fig. 3F). No obvious differences from the control embryos were found so far as specification and patterning are concerned.

### *Expression of tissue-specific markers in embryos treated with McAb to* Pl*-nectin*

As treatment of embryos with McAb against *Pl*-nectin resulted in developmental delays and in some morphogenetic abnormalities, we asked whether tissue differentiation was also affected. Embryos were treated with unrelated McAb (Fig. 4A–D) or with McAb to *Pl*-nectin (Fig. 4E–H), fixed after 42 h of development and stained with McAb to ectodermal (Fig. 4B,C,F,G) and endodermal territorial markers (Fig. 4D,H). Figure 4A,E show bright fields of embryos at the same stage and with the same orientation as those shown in Fig. 4(B–D,F–H), respectively. We used McAb Ecto V that detects an antigen that becomes restricted to the oral ectoderm and foregut (Coffman & McClay 1990). As shown, perturbed embryos expressed Ecto V antigen in a restricted region, which seems to correspond to the oral ectoderm (Fig. 4F). Control embryos showed an intense staining of the stomodeum (Fig. 4B). The other ectoderm-specific marker used was UH2-95, an antibody that specifically stains the ciliary band (Adelson 1985), a structure arising late in development at the border separating oral and aboral ectoderm (Cameron *et al.* 1993). In perturbed embryos McAb UH2-95 stains a structure that is likely to be the ciliary band (Fig. 4G), although its morphology is abnormal with respect to control embryos (Fig. 4C). Similarly, for endoderm territorial markers, the pattern of expression of Endo 1,

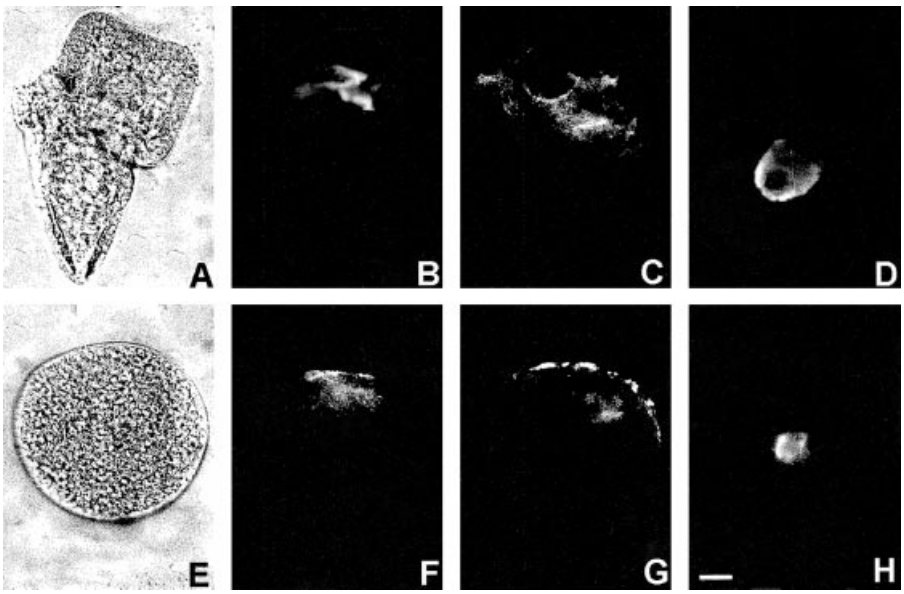


**Fig. 4.** The expression of specific ectoderm and endoderm markers is maintained in embryos treated with monoclonal antibody (McAb) to *Pl*-nectin. Bright field (A,E) and immunofluorescence (B–D,F–H) views of control embryos treated with unrelated immunoglobulin G (IgG; A–D) or VIE11h7 IgG (E–H) for 42 h. Embryos were decorated with ectodermal and endodermal specific antigens as follows: Ecto V (B,F); UH2-95 ciliary band antigen (C,G); Endo 1 (D,H). Bar, 20 μm.

a midgut and hindgut marker (Adelson 1985) was indistinguishable in perturbed embryos from controls (Fig. 4D,H). Thus, while abnormal morphogenesis affecting mesodermal structures occurs in antibody-treated embryos (see also Zito *et al.* 1998), ectoderm and endoderm territories differentiate and express their specific markers in an appropriate pattern.

## Discussion

Results presented in the current paper show that $Ca^{2+}$ ions are required in order for *Pl*-nectin-mediated cell-substrate adhesion to be achieved. An explanation for this result implies the direct binding of calcium ions to the protein, which promotes its biological function in mediating cell adhesion. Another possible explanation is that cell-*Pl*-nectin interactions might be mediated by a cell membrane receptor, such as those belonging to the integrin receptor family, which require calcium ions for their binding and function. It has been suggested that cations might act as allosteric activators of integrin function (Yin *et al.* 1997). For example, it has been shown that, depending on the cell lines used, adhesion on different ECM components has different divalent cation requirements (Sigurdson & Lwebuga-Mukasa 1994; Yin *et al.* 1997). Recently, the presence in the sea urchin embryo of integrins, structurally similar to those characterized in other eukaryotic systems, has been demonstrated (Marsden & Burke 1997, 1998; Hertzler & McClay 1999). However, whether this cation-dependent interaction might be attributed to the presence of an integrin-like receptor or not remains to be determined. On the other hand, ongoing studies attempting isolation of a putative receptor for *Th*-nectin, a homologous protein previously isolated from the Japanese species *Temnopleurus hardwiiki* (Yokota *et al.* 1994), suggest the presence of a non-integrin-like type of receptor (Y. Yokota, pers. comm., 2000). Further studies are required in order to characterize the cell-binding domain in the *Pl*-nectin molecule and to identify the receptor(s) involved in cell-*Pl*-nectin interaction.

We extended our previous studies addressing the question as to whether the lack of interaction between *Pl*-nectin and ectoderm cells, obtained by the treatment of embryos with McAb to *Pl*-nectin, could affect ectoderm and endoderm cell differentiation. We found that invagination of endoderm cells at the vegetal plate occurred normally and without delay in the presence of the anti-*Pl*-nectin antibody. This finding indicates that apparently epithelial cells do not use *Pl*-nectin as a substrate to initiate invagination and favor the possibility that other ECM molecules present in the apical lamina are mediating this event; for example, hyaline (Fink & McClay 1985), apical lamina proteins (Burke *et al.* 1998) and apextrin (Haag *et al.* 1999). On the other hand, the striking regulative capacities of endoderm territories of the sea urchin embryo are well known (McClay & Logan 1996) and Wessel and Wikramanayake (1999) have recently reviewed it in a comprehensive work.

During normal development, the cells of the aboral ectoderm flatten from their original cuboidal shape to form a squamous epithelium. This phenomenon does not occur in the oral ectoderm where the cells remain

thickened. These morphogenetic features have been taken as cues to identify the correct differentiation of the ectoderm (Cameron *et al.* 1993; Berg *et al.* 1996). In embryos perturbed by anti-*Pl*-nectin antibody, ectoderm differentiation is at first delayed. However, its specification is regulative enough to restore normality after a lag period of about 8 h, but skeletogenesis cannot recover, probably because it is on a timed sequence which, if disrupted, results in the failure to rescue a normal skeleton. We also observed a delay in dorsoventral axis formation as the two ventrolateral thickenings in the ectoderm, which normally appear at the gastrula stage embryo, failed to appear in 24 h-treated embryos. After 38 h of treatment the obvious effect is that embryos have no arms (see also Zito *et al.* 1998). However, tissue-specific markers appear in the ectoderm correctly: spatio-temporal expression of the oral ectoderm-specific antigen Ecto V antigen was maintained. As we also found the expression of ciliated band antigen, the present results suggest that both oral and aboral ectodermal cells differentiate and are capable of promoting the required interactions to form the ciliary band (Cameron *et al.* 1993). The expression of Endo 1, a molecular marker known to identify midgut and hindgut, confirms that the disruption of the contact between presumptive endoderm cells and *Pl*-nectin substrates do not affect the differentiation of endodermal territories.

It has been documented that local induction of ectoderm cells to underlying PMC is needed for skeletogenesis to occur (Ettensohn & Malinda 1993). Therefore it can be inferred that the proper *Pl*-nectin-mediated ECM–ectoderm cell interaction constitutes a permissive signal that ultimately acts on the PMC differentiative program, through a yet to be identified transduction pathway. Thus, inhibition of the ectodermal cell-*Pl*-nectin interaction blocks the ectoderm–mesoderm signaling required for correct skeletogenesis to occur. Earlier we showed that the antibody has no effect when PMC are directly treated with it (Zito *et al.* 1998). Therefore the effect is on ectoderm cells, which then are incapable of correctly patterning the PMC. That incorrectness may only be a timing delay: the mesoderm cells are operating on a control sequence of events and require patterning information at a certain stage. That patterning information is not present at the right time because ectoderm cells are in a lag period caused by loss of contact with the matrix. Later they recover and differentiate appropriately, but by then the PMC are beyond the ability to use that information for skeletogenesis and probably not anymore responsive to cues sent by ectoderm cells. If so, the prediction would be that antibody treatment at late stages might interfere with adhesion. However, if the ectoderm is already providing patterning information, the PMC will make normal spicules even in the presence of the antibody. This model, while taking care of all the experimental observations, doesn't say what is happening at the molecular level concerning ectoderm induction to PMC. In a hypothetical model, the interaction between *Pl*-nectin and ectodermal cells is mediated by a transmembrane receptor that possibly activates an unknown pathway that causes the release in the blastocoel of diffusible factors (i.e. growth factors). Whatever is the cause, the effect is that the presence of putative growth factors promotes spicule growth and patterning by the expression of specific PMC genes. Even though these specific receptors and growth factors have not been identified yet, there is already evidence in the literature for the presence of both integrin-like receptors (Marsden & Burke 1997; Hertzler & McClay 1999) and growth factors (Ramachandran *et al.* 1993; Stenzel *et al.* 1994; Yamasu *et al.* 1995; Hwang *et al.* 1999; Ponce *et al.* 1999). Moreover, functional demonstration of the involvement of growth factors in the morphogenesis of the sea urchin embryo has been reported in the case of rescued gastrulation and spiculogenesis in those embryos whose ECM had been disrupted (Govindarajan *et al.* 1995; Ramachandran *et al.* 1995, 1997). If the synthesis of specific growth factors is involved in mediating ecto-mesoderm induction in the sea urchin embryo, perturbed embryos can be rescued by microinjection of heterologous factors (F. Zito *et al.*, unpubl. data, 2000). In addition, it could be possible to show that in skeleton-deficient embryos the levels of endogenous growth factors are different from those found in control embryos. Now that molecular tools are available, it should be possible to study these inductive cell interactions in our model system where *Pl*-nectin-mediated ECM–ectoderm cell interaction has been prevented.

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