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Expression of *univin*, a TGF-β growth factor, requires ectoderm–ECM interaction and promotes skeletal growth in the sea urchin embryo

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Abstract

Pl-nectin is an ECM protein located on the apical surface of ectoderm cells of *Paracentrotus lividus* sea urchin embryo. Inhibition of ECM–ectoderm cell interaction by the addition of McAb to *Pl*-nectin to the culture causes a dramatic impairment of skeletogenesis, offering a good model for the study of factor(s) involved in skeleton elongation and patterning. We showed that skeleton deficiency was not due to a reduction in the number of PMCs ingressing the blastocoel, but it was correlated with a reduction in the number of *Pl*-SM30-expressing PMCs. Here, we provide evidence on the involvement of growth factor(s) in skeleton morphogenesis. Skeleton-defective embryos showed a strong reduction in the levels of expression of *Pl-univin*, a growth factor of the TGF-β superfamily, which was correlated with an equivalent strong reduction in the levels of *Pl*-SM30. In contrast, expression levels of *Pl*-BMP5-7 remained low and constant in both skeleton-defective and normal embryos. Microinjection of horse serum in the blastocoelic cavity of embryos cultured in the presence of the antibody rescued skeleton development. Finally, we found that misexpression of *univin* is also sufficient to rescue defects in skeleton elongation and SM30 expression caused by McAb to *Pl*-nectin, suggesting a key role for *univin* or closely related factor in sea urchin skeleton morphogenesis.
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Keywords: Sea urchin embryo; Growth factor; Extracellular matrix; Skeletogenesis; Ecto–mesoderm induction

Introduction

Due to its relatively simple mode of development, the sea urchin embryo offers a very useful model for the study of commitment and differentiation of a single cell type and its progeny. This is the case of larger daughters of the micromeres, appearing at 5th cleavage, which later in development give rise to primary mesenchyme cells (PMCs) and have been taken as an example of cell-autonomous specification. PMCs are the only cells in the embryo that synthesize specific matrix proteins essential for the set-up of a quite simple, although species-specific, skeleton. These matrix proteins have been categorized as SM30, SM50, PM27, msp130, etc., on the basis of their molecular weight (George et al., 1991; Kitajima et al., 1996; Katoh-Fukui et al., 1991; Harkey et al., 1995; Leaf et al., 1987; Killian and Wilt, 1996; Wilt, 1999; Illies et al., 2002). Each has been shown to contribute to the elongation of different parts of the skeleton (Guss and Ettensohn, 1997; Kitajima and Urakami, 2000) and to accumulate at different stages of embryo development (Guss and Ettensohn, 1997). Despite the early commitment of PMCs, whose fate does not depend on the physical cell–cell interactions with other blastomeres of the embryo (Okazaki, 1975), growing evidence suggests that inductive signals from the ectoderm regulate both PMCs positioning within the blastocoel and skeletal rod patterning (Ettensohn and Malinda, 1993; Peterson and McClay, 2003; Armstrong and McClay, 1994). These putative ectoderm-derived signals may involve growth factors analogous to

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those present in horse serum (HS) or blastocoelic fluid (BcF), which have been shown to be sufficient at least for in vitro skeleton elongation (Okazaki, 1975; Kiyomoto and Tsukahara, 1991; Page and Benson, 1992). Nevertheless, the identification of putative growth factors involved in skeletogenesis has remained elusive. A few transcription factors have been identified that play a role in skeleton development and may be upstream of ectoderm-derived signals. Misregulation of ectodermal expression of SpMsx, a member of the homeobox-containing genes involved in the BMP pathway and known to be implicated in a variety of epithelial–mesenchymal interactions in vertebrates, has profound effects on the organization of primary and secondary mesenchyme cells within the blastocoel (Tan et al., 1998). Similarly, spatially restricted expression of *Pl*Otp, a *P. lividus* homologue of the orthopedia homeobox-containing gene, has been shown to be involved in short range signaling from the oral ectoderm to the underlying skeletogenic mesenchyme that is required for correct skeletal patterning (Di Bernardo et al., 1999; Di Bernardo et al., 2000).

Our previous studies have demonstrated that, in the sea urchin embryo, the appropriate contact of ectoderm cells with *Pl*-nectin, an outer extracellular matrix (ECM) component, is essential for correct morphogenesis of inner mesoderm structures (Zito et al., 1998, 2000). Briefly, cultures of early or mesenchyme blastulae grown in the presence of monoclonal antibodies (McAb) to *Pl*-nectin contain embryos with defects in the elongation and patterning of spicule rods, although spicule nucleation (triradiate rudiments) was not affected. Since we knew that *Pl*-nectin is apically located on ectoderm and endoderm cells (Matranga et al., 1992), the perturbation experiments suggested that ectoderm cells require contacts with *Pl*-nectin in order to send inductive/permissive signals to PMCs needed for correct skeletogenesis. Furthermore, since we found that in skeleton-deficient embryos ectoderm markers are expressed correctly, dorsoventral axis formation occurred normally and endoderm differentiation and patterning were not affected (Zito et al., 2000), we had an excellent model in which to study specific inductive signals from ectoderm to skeletogenic mesenchyme.

In this paper, we extend our previous studies on sea urchin embryo skeletogenesis and begin to address whether growth factors are involved. Using our perturbation model in which only skeleton elongation and patterning are specifically inhibited by McAb to *Pl*-nectin, we have examined by RT-PCR and in situ hybridization the expression of two growth factors, *Pl-univin* and *Pl*-BMP5-7, in relation to that of the spicule matrix protein *Pl*-SM30. Expression of SM30 and *univin*, as well as spicule elongation, is strongly inhibited by *Pl*-nectin McAb treatment of embryos. Interestingly, misexpression of *univin* can rescue skeletogenesis in these perturbed embryos, suggesting that this or some closely related TGF-β factor is involved with the ectoderm signal to PMCs.

Materials and methods

Embryo culture

Adult *P. lividus* sea urchins were collected along the west Sicily coast. Eggs were fertilized and embryos were 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.

Perturbation assay

Perturbation assays with McAb to *Pl*-nectin (NEVIE11h7) on whole embryos were performed according to Zito et al. (1998). Briefly, purified IgG was added at a concentration of 1, 2, or 3 μg/μl to microtiter plate wells containing 100 embryos/100 μl. Embryos at the swimming blastula (8 h after fertilization) or, alternatively, mesenchyme blastula (16 h after fertilization) were continuously cultured in the presence of the antibody at 16°C.

Immunofluorescence

Immunofluorescence experiments were performed on whole-mount embryos according to the method of Coffman and McClay (1990), with some modifications (Zito et al., 2000). Briefly, embryos were fixed for 2 min in ice-cold 0.1% formalin, permeabilized for 2 min in ice-cold MeOH, and then rinsed in MFSW. Embryos were incubated with wheat germ agglutinin–FITC conjugated (WGA-FITC; SIGMA, 1:400 in MFSW) for 30 min on ice in the dark and washed three times in MFSW. Embryos were observed under a Zeiss Axioskop 2 Plus inverted microscope equipped for epifluorescence.

Whole-mount in situ hybridization

Labeling of probes was performed as described by Gianguzza et al. (1995), with some modifications. Briefly, labeled single-stranded antisense and sense DNA probes were generated by asymmetric PCR (Tautz et al., 1992) in the presence of DIG-dUTP, utilizing an SM30 cDNA of 380 b as a template. Whole-mount in situ hybridizations were performed as described by Di Bernardo et al. (1995), with some modifications. All prehybridization and hybridization steps were carried out in microtiter-plate wells, using 30–40 embryos per well. Fixed embryos were rehydrated, digested with 150 μg/ml Proteinase K (Sigma) for 20 min at 37°C, and hybridized with 20 ng/ml of DNA probe overnight at 65°C. After hybridization, embryos were washed, in several steps, in 5× SSC, 0.1% Tween 20, 2× SSC, 0.1% Tween 20, 0.2× SSC, 0.1% Tween 20 at 45°C, and 0.2× SSC, 0.1% Tween 20 at r.t. After staining, embryos were mounted on glass slides and observed under a Zeiss Axioscop 2 Plus inverted microscope. Hybridizations with

sense probe were performed and showed no specific signal above background.

Microinjection experiments

Sea urchin embryos were prepared for microinjection by using a method modified by Cho (1999) for swimming embryos. Briefly, embryos treated with McAb to *Pl*-nectin were aligned in rows on 60-mm Petri dish lids precoated with 1% agarose in MFSW. Microinjection needles from Eppendorf were back-filled with a solution of 32% horse serum (HS). The blastocoel of the embryos received approximately 100 pl of the solution. Microinjection was performed by using a Narishige IM-188 micromanipulator (Narishige CO., LTD) and a Cell Tram Oil injector (Eppendorf). Embryos were then collected with a mouth pipette and allowed to develop in microtiter-plate wells at 16°C.

Isolation and identification of PCR amplification products

To study the relative levels of tissue-specific gene expression in *P. lividus* embryo, homologues of *univin*, BMP5-7, and SM30 were obtained from total RNA by RT-PCR using specific primers designed on the basis of the sequences for *S. purpuratus* (Stenzel et al., 1994; Ponce et al., 1999) and *P. lividus* (Di Bernardo and Spinelli, personal communication), respectively. In addition, we utilized an amplification product obtained with specific primers designed on the basis of the reported sequence for S24, a transcript encoding a ribosomal protein of the *P. lividus* embryo (Sgroi et al., 1996). This amplification product was used as an internal reference control in the following experiments. We obtained PCR products of 259, 117, 384, and 248 bp sizes by amplification with *univin*, BMP5-7, SM30, and S24 primers, respectively, which were cloned and sequenced. The sequences of the primers used were: 5′-ACTGGATCATCGCTCCGATG-3′ and 5′-CATCGGCATCCACAAGCTTC-3′ for *univin;* 5′-TGGCAGGAATGGATCATCGC-3′ and 5′-GAGTGTCTGCACGATGGCGTG-3′ for BMP5-7; 5′-TTGGGTTCAGTTGGAGAACC-3′ and 5′-GTTTCGTTGTCTTCGGGGTA-3′ for SM30; and 5′-CTGATCAGACCATGCTCTAAGGT-3′ and 5′-CCTGATGTCGTCAGTACAACGTA-3′ for S24. The RT-PCR products will be referred to as *Pl-univin* (NCBI Accession No. AJ302364), *Pl*-BMP5-7 (NCBI Accession No. AJ302363), *Pl*-SM30, and *Pl*-S24 in the following.

RNA preparation and relative RT-PCR analysis

Total RNA was isolated by using a single-step method (Chomczynski and Sacchi, 1987) from 100–500 embryos treated with unrelated IgG or IgG to *Pl*-nectin. First-strand cDNA was synthesized by using SuperScript reverse transcriptase (Gibco, BRL) and random hexamers. An amount of cDNA equivalent to 15 embryos was used for each RT-PCR analysis. PCRs contained 0.8 μM concentrations of the appropriate primers, 0.2 mM dNTPs and 1.5 mM $MgCl_2$. PCR amplification conditions were as follows: 94°C, 30 s; 55°C, 45 s; 72°C, 30 s. Preliminary RT-PCR experiments were performed to determine the linear range of amplification for each set of primers (Fig. 3A). We determined that saturation was not reached until after 40 cycles of amplification for each primer pair. Subsequent PCR assays were then carried out for 40 cycles. One-fifth volume of the PCR products was analyzed on a 3% agarose gel. Densitometric analysis was performed by using a Bio-Rad imaging system, and quantitation was performed by using image analysis software (Molecular Analyst).

Preparation of synthetic mRNA for microinjection

For preparation of *univin* mRNA, a template containing *univin* cDNA inserted into pSP64T at the *Bgl*II site was used. Capped, polyadenylated mRNA was synthesized by using the mMessage mMachine kit (Ambion). RNA was purified according to the manufacturer's instructions, resuspended in 30% glycerol filtered through 0.22-μm filters (Millipore). RNA concentrations and quality were analyzed by spectrophotometry and electrophoresis through agarose gels.

Egg injection and treatment with McAb to Pl-nectin

Eggs, dejellied in MFSW pH 4.5, were injected with synthetic mRNA at the concentrations described in Results. Only batches of eggs that gave rise to embryos with normal development after injection of glycerol alone were utilized. Twenty-four hours after microinjection, swimming embryos were collected and part of them were cultured in the presence of McAb to *Pl*-nectin in microtiter-plate wells for the following 24 h, at 16°C. Embryos were examined with a Zeiss Axioscop 2 Plus inverted microscope.

RNA preparation from microinjected embryos and relative RT-PCR analysis

PolyA^+ RNA was isolated according to the manufacturer's instructions using Dynabeads Oligo $(dT)_{25}$ (Dynal Biotech) from 15–20 embryos. Then, a single-step RT-PCR was carried out using SuperScript One-Step RT-PCR kit (Invitrogen) according to the manufacturer's instructions. The RT reaction was performed for 30 min at 45°C; after denaturation for 5 min at 94°C, PCR amplification was performed for 35 cycles as follows: 94°C, 30 s; 55°C, 45 s; 72°C, 30 s. Total volume of the PCR products was analyzed on a 3% agarose gel stained with ethidium bromide.

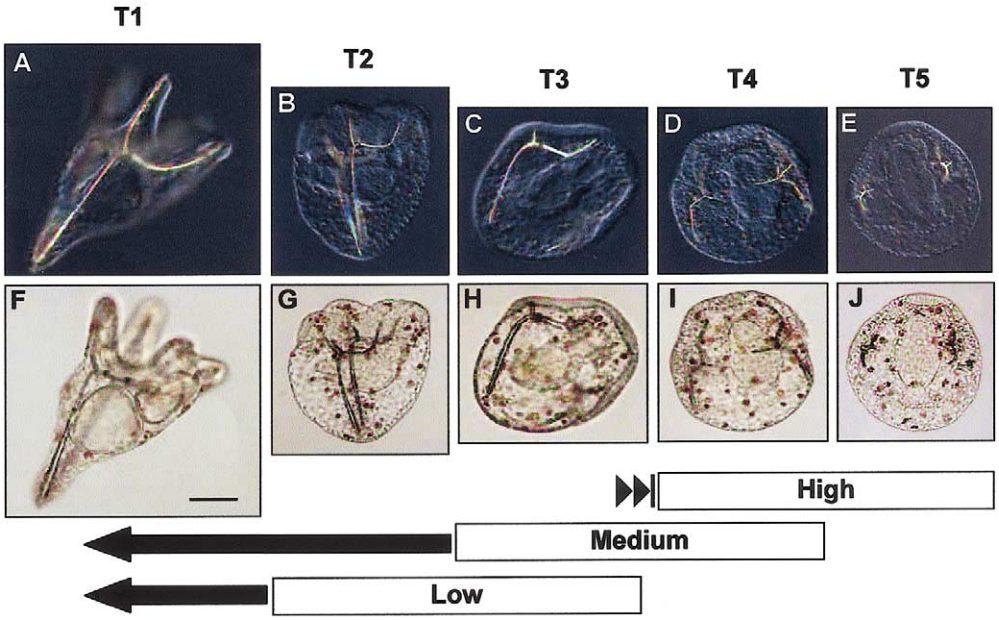

Fig. 1. Skeleton defects obtained after culturing embryos in the presence of different concentrations of McAb to *Pl*-nectin. (A, F) Type 1 embryo with normal skeleton; (B, G) Type 2 and (C, H) type 3 embryos with partial skeleton defects; (D, I) Type 4 and (E, J) type 5 embryos with strong skeleton defects. High concentrations of McAb to *Pl*-nectin (High) inhibit skeleton development (arrowhead); medium (Medium) and low (Low) concentrations of McAb to *Pl*-nectin allow skeleton development (arrows). Control embryos were cultured in the presence of unrelated IgGs. Scale bar, 50 μm.

Results

PMC ingression and specific gene expression in normal and perturbed embryos

We have previously shown that culture of early blastula embryos in the presence of McAb to *Pl*-nectin greatly affects both skeleton growth and patterning, but has no effects on ectoderm and endoderm differentiation (Zito et al., 1998, 2000). Embryos were classified on an arbitrary scale where the severity of skeleton deficiencies was evaluated from types 1 to 4, type 1 (T1) being normal embryos, type 2 (T2) and type 3 (T3) embryos with partial skeleton defects, and type 4 (T4) embryos with the most dramatic skeleton defects (Zito et al., 1998). In addition, we have recently identified a fifth morphology, defined as type 5 (T5) embryos, showing the presence of the triradiate spicule rudiments as the sign of the initiation of spiculogenesis, but with no further growth. Control embryos were cultured in the presence of unrelated IgGs and did not show any apparent defects when compared with embryos raised in normal sea water.

In order to study the effects of skeleton impairment at the levels of mRNA and protein expression, it was important to obtain embryos with different degrees of skeleton defects on a very reproducible basis. This was achieved by culturing embryos continuously for 24 h with different concentrations of McAb to *Pl*-nectin, and it is summarized in Fig. 1, where different skeleton defective morphologies are shown. Using low concentrations of McAb, corresponding to 1 μg/μl, we observed about 90% of the embryos with weak (Fig. 1B and G, T2) or intermediate (Fig. 1C and H, T3) skeleton defects. In this case, embryos eventually recover skeleton development 48 h after the treatment and approach the morphology of control embryos (Fig 1. A and F, T1). Using medium McAb concentrations, corresponding to 2 μg/μl, 90% of embryos showed intermediate (Fig. 1C and H, T3) or strong (Fig. 1D and I, T4) skeleton defects. However, even in this case, embryos eventually developed a quite normal skeleton 48 h after the treatment. A complete inhibition of skeleton development was obtained with high McAb concentrations, corresponding to 3 μg/μl. In this case, about 90% of the embryos exhibited T4 (Fig. 1D and I) and T5 (Fig. 1E and J) morphologies and, what is more striking, they were blocked in skeleton elongation and never developed a normal skeleton, even 48 h after the treatment. Concentrations of McAb to *Pl*-nectin higher than 3 μg/μl were obviously toxic, since they caused a general impairment of development, and embryos eventually died within 24 h of culture.

One explanation for the perturbation effects observed is that a reduced number of PMCs ingresses into the blastocoel. Alternatively, the skeleton defects observed could have been the result of a reduction in the number of PMCs expressing spicule-specific proteins necessary for the construction of the skeleton. To compare the number of PMCs in normal embryos and those treated with McAb to *Pl*-nectin, we stained them with WGA-FITC. We found that, although control embryos had a well-defined skeleton, typical of an early pluteus stage (Fig. 2A), and perturbed embryos had a gastrula-like shape with short triradiate spicule rudiments (Fig. 2B), the total number of ingressed PMCs in T1 (Fig. 2C) and T4 (Fig. 2D) embryos did not

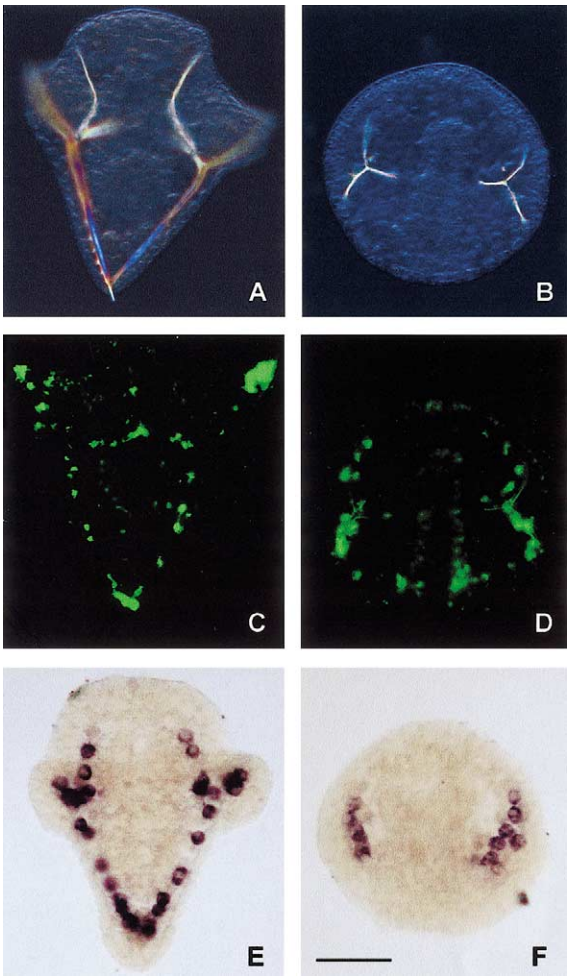

Fig. 2. Number of PMCs and specific gene expression in perturbed embryos. (A, B) brightfield micrographs of type 1 and type 4 embryos, respectively. (C, D) Direct immunofluorescence with WGA-FITC of type 1 and type 4 embryos, respectively. (E, F) In situ hybridization analysis with the *Pl*-SM30 antisense probe of type 1 and type 4 embryos, respectively. Scale bar, 50 μm.

change. To determine whether in control and perturbed embryos all PMCs were actively expressing skeleton-specific genes, we analyzed the expression of the SM30 gene, which encodes a spicule matrix protein, that has already been shown to be affected by external cues (Urry et al., 2000). Hence, we performed in situ hybridization experiments on whole-mount embryos coming from the same perturbation experiment, by using a *Pl*-SM30 anti-sense probe on control and perturbed embryos. As shown in Fig. 2, 32 out of 36 PMCs present in T1 (control) embryos express *Pl*-SM30 (Fig. 2E), whereas in T4 embryos, many fewer of the ingressed PMCs were positive (Fig. 2F). Hybridizations with sense probe showed no specific signal above background (not shown). The embryos shown in Fig. 2E and F are representative examples of 4 experiments that were scored and used for a statistical analysis of in situ hybridization. In conclusion, we found that *Pl*-SM30 transcript was detected in 14 ± 7 PMCs in T4 (perturbed) embryos, while 32 ± 7 PMCs were positive in T1 (control) embryos, indicating about a 43% reduction in the number of PMCs actively expressing the *Pl*-SM30 gene.

Microinjection of horse serum rescues skeleton-defective embryos

In vitro cultures of PMCs in the presence of HS or BcF have shown the elongation of spicules and therefore indicated the need for some kind of signals coming from the environmental medium (Okazaki, 1975; Harkey and Whitely, 1980; Kiyomoto and Tsukahara, 1991). We hypothesized that skeleton deficiencies found in embryos cultured in the presence of McAb were caused by the insufficiency or lack of putative growth factor(s). If this hypothesis is correct, then microinjection of HS into the blastocoelic cavity of perturbed embryos would lead to the partial or total recovery of skeleton deficiencies caused by McAb to *Pl*-nectin. To test this assumption, we performed preliminary experiments in which very early blastula embryos (6 h after fertilization) were first injected with different concentrations of HS, namely 8, 32, and 64%, and then cultured in the presence of McAb to *Pl*-nectin for 42–48 h. We found that any of the HS concentrations used for microinjection were able to rescue skeleton deficient embryos, as indicated by the remarkable increase in the number of T2 and T3 embryos, reaching about 90% of the total (not shown). To further demonstrate that HS is sufficient to rescue perturbed embryos, we designed another experimental procedure as follows: first, we cultured embryos in the presence of McAb to *Pl*-nectin for different lengths of time (3, 6, and 22 h), and then we microinjected them with 100 pl 32% HS. This concentration of HS was adopted for two reasons: first, to be sure to have a moderate excess of growth factor available in the blastocoel, and second, because considering the volume of injected blastocoel equal to 523 pl, this concentration would give a 6% HS theoretical final concentration in the blastocoel. This value is very close to the 4% HS concentration used for in vitro experiments of spicule growth using isolated PMCs (Okazaki, 1975; Harkey and Whitely, 1980). As control embryos, we took those that were cultured for the same period of time in the presence of McAb to *Pl*-nectin, but never received the microinjection of HS. Results are summarized in Table 1. In a first series of experiments, we asked if HS could rescue embryos cultured for a short time in the presence of McAb to *Pl*-nectin. Mesenchyme blastula embryos (16–17 h postfertilization) were cultured for either 3 or 6 h (trials 1 and 2, respectively) in the presence of McAb to *Pl*-nectin before HS microinjection. Controls, with no HS microinjection, were skeleton-defective and showed between 90 and 80% of T4 and T5 morphologies, respectively. In contrast, microinjection of HS rescued skeleton development with percentages of T2–T3 embryos between 60 and 68% in one case (3 h) and between 87.5 and 100% for the other (6 h). In a second series of experiments, we asked if HS microinjection could rescue skeleton develop-

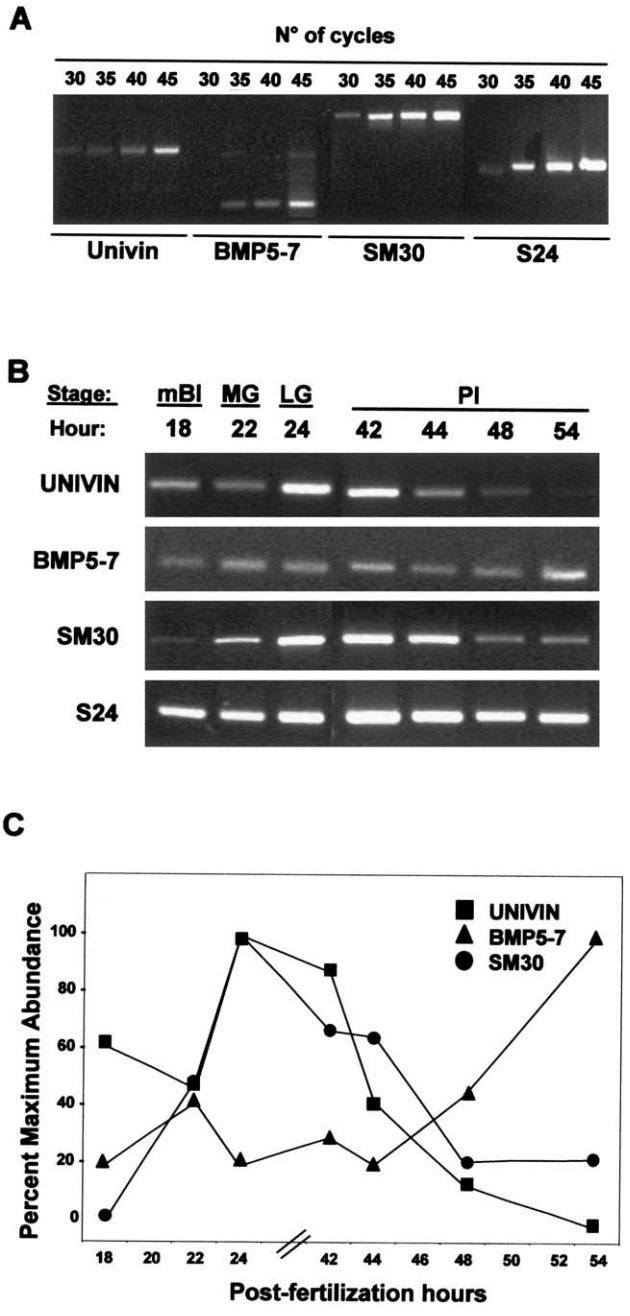

Fig. 3. Relative RT-PCR analysis of the levels of *Pl-univin*, *Pl*-BMP5-7, and *Pl*-SM30 transcripts during development. (A) Validation of the assay: RT-PCRs were carried out for 30, 35, 40, and 45 cycles for each set of primers as indicated. Amplification products were loaded on the same agarose gel and bands visualized by ethidium bromide. (B) Electrophoretic analysis of RT-PCR products obtained for each set of primers with cDNA from different stages. (C) Histogram showing the quantitation of RT-PCR products from the gel shown in (B). Values, obtained from measurement of band intensities for each amplification product, were normalized to the *Pl*-S24 values. Maximal values were normalized to developmental stages with the highest levels of expression of a particular mRNA. Stages: mB1, mesenchyme blastula; MG, middle gastrula; LG, late gastrula; Pl, pluteus larva. Hour: hours from fertilization.

ment even after longer periods of culture in the presence of the antibody. Early blastula embryos (6 h postfertilization) were cultured for 22 h in the presence of high concentrations of antibody to obtain T4 embryos before HS microinjection. In the bottom panel of Table 1 (Trial 3) are results of a representative experiment showing that all the embryos that received HS microinjection, observed 24 h later, regained skeleton development (100% T2–T3); conversely, we found that uninjected control embryos had 100% of inhibition (T4–T5). In particular, we found that in uninjected control embryos, all PMCs ingressed the blastocoel, ventrolateral clusters were formed and started to make triradiate spicules that didn't continue to elongate, supporting a specific role for HS in spicule elongation.

Expression of univin, BMP5-7, and SM30 in normal and skeleton-defective embryos

Since we found that microinjection of HS, which is a rich source of growth factors, resulted in the recovery of spicule growth in perturbed embryos, and since skeletogenesis in other systems is known to depend heavily on members of the BMP family (Wozney et al., 1988), we decided to analyze the expression of two genes encoding such factors as well as that of a gene coding for a spicule matrix protein (SM30), which is active during the spicule elongation phase of skeletogenesis (Guss and Ettensohn, 1997; Kitajima and Urakami, 2000). The two candidates selected were *univin* and BMP5-7, whose gene sequences from the *S. purpuratus* sea urchin embryo have been reported (Stenzel et al., 1994; Ponce et al., 1999). In our experiments, expression of *univin* and BMP5-7 was monitored during normal and perturbed spiculogenesis, with the aim of finding a correlation between their gene expression levels and skeleton abnormalities. At first, we carried out a stage-dependent analysis of the two BMPs members and SM30 on control embryos from mesenchyme blastula to pluteus stages. Amplification product band intensities were measured by a densitometric image scanning analyzer as shown in Fig. 3B and C. Values were normalized to the *Pl*-S24 values. We found a stage-dependent change in the transcript levels of *Pl-univin* and *Pl*-SM30. Specifically, *Pl-univin* and *Pl*-SM30 transcript levels increased from the beginning of gastrulation through the late pluteus stage, coinciding with the time of spicule elongation. The concentration of both *Pl-univin* and *Pl*-SM30 transcripts decreased at 44 h of development when skeletogenesis was almost complete. In contrast, *Pl*-BMP5-7 transcript levels were almost constant and low from the mesenchyme blastula to the early pluteus stage, increasing only after 48 h of development.

The parallel expression of *Pl-univin* and *Pl*-SM30 suggested a possible involvement of *Pl-univin* and not *Pl*-BMP5-7 during skeletogenesis. In order to investigate this hypothesis, we monitored by RT-PCR assay the expression of *Pl-univin*, *Pl*-BMP5-7, and *Pl*-SM30 transcripts in em-

Table 1
Effect of HS microinjection on embryos pretreated with McAb to *Pl*-nectin

Trial	IgG addition (h post-fertilization)	HS injection (h after IgG addition)	Inhibition	Recovery
			% T4–T5 (n° embryo/total)	% T2–T3 (n° embryo/total)
1a	16	—	90 (27/30)	10 (3/30)
1b	16	3	32 (7/22)	68 (15/22)
1c	16	6	40 (9/23)	60 (14/23)
2a	17	—	81 (42/52)	19 (10/52)
2b	17	6	0	100 (7/7)
2c	17	6	12.5 (1/8)	87.5 (7/8)
3a	6	—	100 (6/6)	0
3b	6	22	0	100 (4/4)

(−), no HS injection.

bryos with strong skeleton defects, cultured in the presence of the antibody for 24 h, and harvested 42 h after fertilization. Values obtained from measurement of band intensities for each amplification product were normalized to the *Pl*-S24 values, which were assumed to be unaffected by treatment with McAb to *Pl*-nectin. Fig. 4 shows the ethidium bromide stained gel of a representative RT-PCR experiment and a quantitative analysis of five different experiments. *Pl-univin* expression is strongly reduced in perturbed embryos with strong skeleton defects with respect to control embryos. Similarly, *Pl*-SM30 expression is reduced in perturbed embryos (T4 and T5 morphologies). It should be noted that about the same average decrease is found when measuring gene expression by RT-PCR (2-fold) and the number of SM30-positive cells by in situ hybridization (43%) (see Fig. 2). On the contrary, the levels of expression of *Pl*-BMP5-7 remained low and constant regardless of the skeleton fate and did not differ between control and perturbed embryos.

Microinjection of univin mRNA prevents embryos from skeleton defects and rescues perturbed embryos

Differential expression of *univin* in control and perturbed embryos suggests that it is involved in skeletal growth. Interestingly, it was already shown by in situ experiments that *univin* mRNA is accumulated in progressively restricted areas outlining a circumequatorial band for stages between cleavage and gastrula, and at the growing arms at the pluteus stage (Stenzel et al., 1994), i.e., at times and places consistent with a role in promoting skeletogenesis. To show direct evidence of *univin* involvement in skeletogenesis, we asked whether injection of mRNA encoding full-length *S. purpuratus univin* could reverse the skeleton defects caused by treatment with the McAb to *Pl*-nectin. Synthetic, capped, polyadenylated mRNA was injected into sea urchin eggs and, about 20–27 h after fertilization, embryos were cultured in high antibody concentrations for about 24 h before microscopic investigation. We found that, about 20 h after fertilization, before the addition of the antibody, *univin* mRNA-injected embryos and glycerol-injected controls were indistinguishable as they both reached the mesenchyme blastula stage (not shown). When development was monitored 24 h after culture in the presence of the antibody, a noteworthy difference among cultures was observed: while glycerol-injected control embryos were at the pluteus stage (Fig. 5A), glycerol-injected embryos cultured in the presence of McAb to *Pl*-nectin showed the typical T4 morphology, with an undeveloped skeleton and no arms (Fig. 5B). On the contrary, embryos injected with *univin* mRNA, although cultured for 24 h in the presence of McAb to *Pl*-nectin, were partially rescued in their skeleton development as assessed by the typical T2–T3 morphology (Fig. 5C and D). In Table 2 are collected data coming from different experiments in which embryos, microinjected with

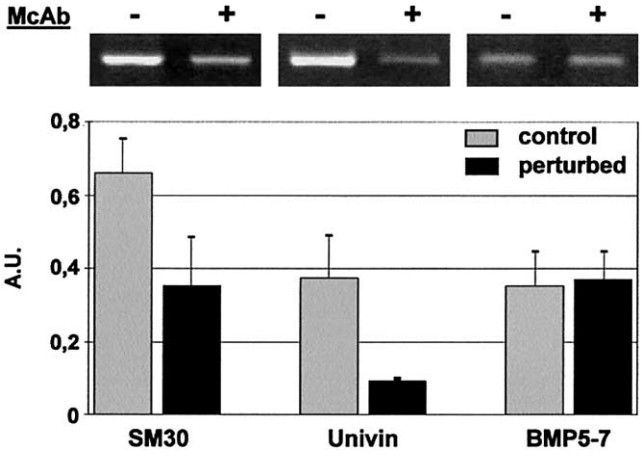

Fig. 4. Relative RT-PCR analysis of the levels of *Pl*-SM30, *Pl-univin*, and *Pl*-BMP5-7 transcripts in control (McAb −) and perturbed embryos (McAb +). A representative ethidium bromide stained gel is shown in the upper panel. Histogram shows the quantitative analysis of five different experiments, for which the SD has been calculated. Values obtained from measurement of band intensities for each amplification product were normalized to the *Pl*-S24 values and are expressed in arbitrary units.

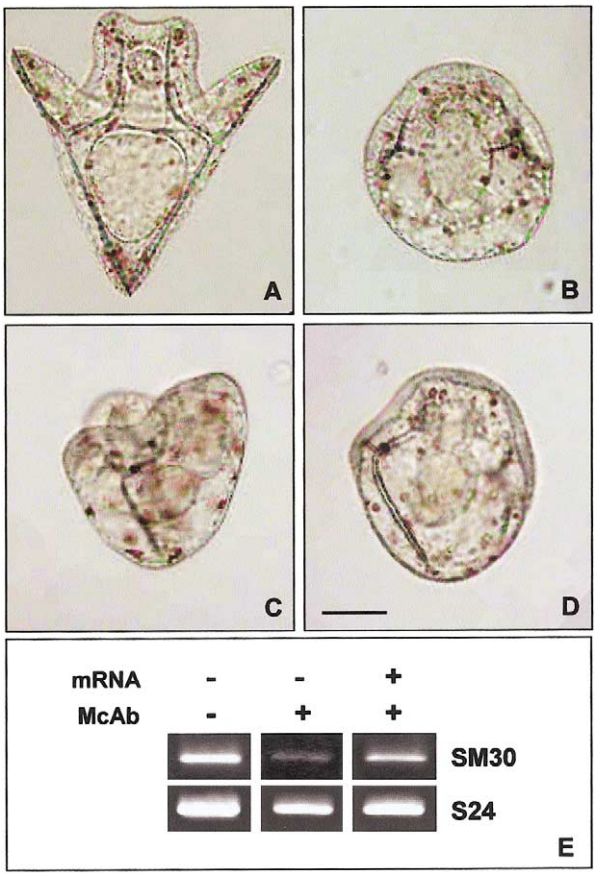

Fig. 5. Rescue of skeleton development after injection of *univin* mRNA. Glycerol (control; A, B) or synthetic *univin* mRNA (C, D) was injected into eggs of *P. lividus*, which were fertilized and, after 20 h of development, were cultured for other 24 h in the absence (A) or in the presence (B, C, D) of McAb to *Pl*-nectin. Scale bar, 50 μm. (E) Relative RT-PCR analysis of *Pl*-SM30 and *Pl*-S24 levels of control, perturbed, and rescued embryos. Shown is a representative ethidium bromide gel of three different experiments.

different concentrations of *univin* mRNA, were monitored for rescue of skeleton development. Significance was determined according to the Student's t test ($P < 0.05$), by pooling the numbers of the two groups of perturbed uninjected and injected embryos, regardless of *univin* mRNA dose. The number of rescued embryos increased when higher concentrations of *univin* mRNA were injected, suggesting a dose-response effect. No aberrations in skeleton development were observed when embryos were microinjected with *univin* mRNA and allowed to develop without McAb addition (not shown). In addition to the morphological criteria used for the assessment of developmental recovery of skeleton elongation, a molecular analysis of the expression of *Pl*-SM30 transcripts was performed by RT-PCR on control, perturbed, and rescued embryos. Fig. 5E shows the results of a representative experiment, in which *Pl*-S24 expression is also shown. As predicted, *Pl*-SM30 mRNA levels are significantly increased in skeleton recovering embryos, indicating that *univin* mRNA injection rescues the expression of *Pl*-SM30.

Discussion

In this report, we show that perturbation of skeleton development in sea urchin embryos by interfering with interactions of ectoderm cells with *Pl*-nectin in the ECM results in reduced levels of *Pl-univin* expression, parallel to reduced levels of *Pl*-SM30 expression. Moreover, we demonstrate that misexpression of *univin* rescues these embryo defects, promoting skeletal growth. These observations support the conclusion that the sea urchin *Pl-univin* gene is involved in the ectoderm–mesoderm induction that regulates skeletogenesis.

In our previous papers, we showed that skeleton defects observed after treatment with McAb to *Pl*-nectin are not caused by a direct action of McAb on PMCs. Instead, they probably depend on a loss of the interaction between ectoderm cells and *Pl*-nectin, indirectly causing a loss of the signal from ectodermal cells to the PMCs (Zito et al., 1998, 2000). The primary observations that support this conclusion are that skeletal growth arrest occurs when the McAb is added outside the embryo and not when it is injected into the blastocoel where the PMCs reside (Zito et al., 1998). This hypothesis is consistent with the finding that, although PMCs are rigidly specified early in development to differentiate as skeletogenic cells (Okazaki, 1975), skeleton morphogenesis is governed by ectoderm-to-PMC interactions (Armstrong et al., 1993; Guss and Ettensohn, 1997). Diffusible growth factors have been suggested to mediate the ectoderm–mesoderm induction. Until now, three members of the TGFβ family have been identified in the sea urchin embryo: *univin* (Stenzel et al., 1994), BMP5-7 (Ponce et al., 1999), and *Lv*BMP2-4 (Angerer et al., 2000). Among these, *univin* is a good candidate to act as diffusible growth factor since, according to its known reported sequence, it shows features typical of a secreted protein (Stenzel at al., 1994). Furthermore, *univin* gene expression has been shown to become progressively restricted to areas outlining a circumequatorial band for stages between cleavage and gastrula, and in the growing arms at the pluteus stage in *S. purpuratus* (Stenzel at al., 1994), i.e., at times and places

Table 2
Rescue of skeletogenesis by injection of *univin* mRNA

mRNA injected (μg/μl)	% Rescued (n° embryo/total)	% Perturbed (n° embryo/total)
–	0 (0/8)	100 (8/8)
0.8	5 (1/19)	95 (18/19)
–	0 (0/8)	100 (8/8)
0.8	21 (5/23)	78 (18/23)
–	0 (0/7)	100 (7/7)
0.8	66 (4/6)	33 (2/6)
–	0 (0/20)	100 (20/20)
1.2	50 (15/30)	50 (15/30)
–	48 (27/56)	52 (29/56)
1.2	74 (29/39)	26 (10/39)

(–), 30% glycerol injected

consistent with a role in promoting skeletogenesis. BMP5-7 was also taken into consideration in the present study because of its homology to some BMPs, which, in other systems, have been shown to have an osteoinductive activity (review in Hogan, 1996). We considered BMP2/4 a less likely candidate for promoting skeletal growth based on its temporal and spatial patterns of expression and its demonstrated role in aboral ectoderm differentiation (Angerer et al., 2000).

Defects in skeleton elongation and patterning do not depend on the number of ingressed PMCs

First, we asked whether skeleton defects observed in treated embryos were caused by a reduction in the total number of PMCs. We found no significant changes in the number of PMCs ingressed into the blastocoelic cavity and also in their localization and arrangement in embryos with strong skeletal defects. We have previously shown that the first sign of perturbed development of the skeleton was observed at later stages, when in control embryos triradiate spicule rudiments elongated and branched, giving rise to the various skeletal rods. In the most severe phenotypes, we could observe only a slight elongation of the triradiate spicules, correctly positioned in the ventrolateral side of the blastocoel. In moderately perturbed embryos, these spicules eventually branched to give rise to short body rods and anal rods, with roughly undeveloped anterolateral rods (Zito et al., 2000). These results indicate that the defects in skeleton elongation and patterning do not depend on the number of ingressed PMCs, but instead result from failure of PMCs to deposit skeletal material. Altered skeletal growth has also been observed in embryos treated with $NiCl_2$, which have the normal number of PMCs, but produce as many as a dozen small triradiate skeletal spicules (Hardin et al., 1992). There are other evidences that skeletal growth is regulated and not autonomously specified in the PMCs. For example, embryos with supernumerary PMCs, as many as two to three times the normal number, produce normal skeletons with no changes in their mass or pattern even with the extra PMCs (Ettensohn, 1990). Moreover, in half- and quarter-sized dwarf larvae, PMCs produced skeletons with a normal pattern, although reduced in size (Takahashi and Okazaki, 1979). Taken all together, these findings indicate that elongation and patterning of the skeleton are regulative events and signaling pathways are required.

Microenvironmental cues are essential for skeleton development

The defects in skeleton elongation and patterning can be reversed by microinjection of HS, the same medium that promotes skeletal growth in in vitro cultures of micromeres or PMCs (Okazaki, 1975; Page and Benson, 1992). Although the fundamental factor(s) has not yet been identified, it is likely to be a growth factor(s) since HS contains many such proteins. In addition, we found that, although the PMCs from McAb-treated embryos do not produce elongated spicules, they retain competence to do so. Recently, we have demonstrated that PMCs transplanted from skeleton-defective embryos are still able to position themselves at ventrolateral clusters and to synthesize skeleton if introduced into the blastocoelic cavity of normal PMCs-deprived embryos (Kiyomoto et al., submitted). The competence of PMCs to respond to environmental signals lasts for considerable periods of time if compared with their normal time schedule of skeleton development. These results support the hypothesis that the signal is reduced or absent in the blastocoel of embryos treated with McAb to *Pl*-nectin. When it is supplied, the PMCs resume the skeletogenic program. Evidence that it is cells other than PMCs that supply the signal comes from several studies. PMCs from full-size donor embryos form a skeleton with an appropriately reduced size when transplanted into a dwarf host (Armstrong and McClay, 1994). In agreement, PMCs from $NiCl_2$-treated embryos, in which they have a radialized distribution, form normal bilateral clusters when transplanted into normal PMC-deprived hosts. On the contrary, normal PMCs transplanted into radialized PMC-deprived hosts produce radialized skeleton (Armstrong at el., 1993). In addition, Ettensohn and Malinda (1993) have shown that, if ectoderm from the tip of pluteus arms is microsurgically ablated, spicule growth is considerably reduced. In conclusion, skeleton elongation greatly depends on the microenvironment in which PMCs live, when the right cues are provided.

Cell–ECM interaction is required for SM30 and univin gene expression

The interest in monitoring the expression of SM30, a specific spicule matrix protein, in skeleton-defective embryos came from findings reporting that the expression of this gene is responsive to external cues (Urry et al., 2000) and occurs during spicule elongation (George et al., 1991). We found that embryos, in which skeleton growth was arrested by interfering with ectoderm–ECM interactions, have a reduced number of SM30-expressing PMCs that are localized along the triradiate spicule rudiments. Accordingly, we found a comparable reduction in the levels of SM30 mRNA. In agreement, in vitro experiments demonstrated that withholding serum from micromere cultures severely depresses SM30 accumulation, but it does not completely eliminate it (Urry et al., 2000). Furthermore, short-range signals from different ectodermal territories appear to regulate the expression of PMC-specific gene products like SM30 that are rate-limiting in skeletal biosynthesis, thereby locally influencing skeletal rod growth (Guss and Ettensohn, 1997).

A major question is what is the molecular nature of the signals from ectoderm that regulate skeletal patterning. We interpreted our results as an effect of decreased concentrations of unknown inductive factor(s) in the blastocoel.

Therefore, we investigated the levels of expression of *univin* and BMP5-7 growth factors. An indication of *univin* involvement in skeletogenesis came from normal embryo development, where its expression increases, paralleling *Pl*-SM30, at the time of spicule elongation, in contrast to *Pl*-BMP5-7. Furthermore, we found that the expression of the TGF-β growth factor gene, *univin*, shows similar sensitivity as SM30 to *Pl*-nectin McAb, whereas a related growth factor, *Pl*-BMP5-7, did not, raising the possibility that *univin* could be the ectoderm-to-PMC signal. The fact that misexpression of *univin* in McAb treated embryos can rescue defects in skeleton elongation and SM30 expression provide strong support for this notion. These data indicate that a TGF-β signaling pathway is required for skeletal growth, and a number of experiments discussed above indicate that the ectoderm produces the signal. Whether the signaling ligand is *univin* or some closely related factor is not yet clear, and is being tested with loss-of-function experiments currently in progress. This model predicts that the PMCs should express a TGF-β receptor. Recently, one that is similar to vertebrate Type I receptor, Alk2, which can mediate both activin and BMP signals, has been found to be expressed in ingressed PMCs of *S. purpuratus* embryos (L.A., unpublished observations). Whether this receptor mediates the skeleton-promoting signal is not yet known.

Unexpectedly, although *univin* misexpression can rescue skeletal defects produced by *Pl*-nectin McAb treatment, it does not cause any detectable aberrations in development in the normal embryo. The reason for this is not clear. A limiting condition could be the proteolytic processing of *univin*. This is the case for Vg1, which also does not elicit phenotypes on injection of mRNA encoding unprocessed protein, since it yields relatively large amounts of precursor but rather little mature protein (Thomsen and Melton, 1993; Dohrmann et al., 1996). Furthermore, it has been suggested that processing of Vg1 could be highly restricted spatially (Kessler and Melton, 1995; Dohrmann et al., 1996). *Univin* is closely related to Vg1 and, from its nucleotide sequence, it seems to be proteolytically processed (Stenzel et al., 1994). Therefore, if situations similar to the above mentioned were true for *univin*, then the lack of a misexpression phenotype but the presence of a PMC rescue phenotype could be explained. Alternatively, the *univin* receptor could be PMC-specific and spicule growth could be unaffected by high ligand concentration.

Other signaling pathways are required either directly or indirectly for skeletogenesis. For example, Ramachandran et al. (1995) observed that PDGF-BB and TGF-α rescued skeletogenesis, as well as gastrulation in ECM-disrupted embryos. They also showed that antibodies against human PDGF-B, TGF-α, and their respective receptors inhibited gut and spicule differentiation and morphogenesis (Ramachandran et al., 1995; Govindarajan et al., 1995). Because perturbations of these signals have effects on multiple cell types, including the ectoderm, it is possible that the effect on skeletogenesis is indirect. In contrast, the TGF-β pathway discovered here is different in that it is specific for spicule growth; our skeleton-defective embryos have normal gut and ectoderm differentiation (Zito et al., 2000). Our data support a model in which some ectodermal cells secrete processed *univin* or a related factor into the blastocoel where it signals PMCs to synthesize SM30 and other spicule matrix proteins required for spicule growth. The ability of these ectodermal cells to produce the signal depends on their association with *Pl*-nectin in the apical ECM.

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