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Localized VEGF signaling from ectoderm to mesenchyme cells controls morphogenesis of the sea urchin embryo skeleton

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During development, cell migration plays an important role in morphogenetic processes. The construction of the skeleton of the sea urchin embryo by a small number of cells, the primary mesenchyme cells (PMCs), offers a remarkable model to study cell migration and its involvement in morphogenesis. During gastrulation, PMCs migrate and become positioned along the ectodermal wall following a stereotypical pattern that determines skeleton morphology. Previous studies have shown that interactions between ectoderm and PMCs regulate several aspects of skeletal morphogenesis, but little is known at the molecular level. Here we show that VEGF signaling between ectoderm and PMCs is crucial in this process. The VEGF receptor (VEGFR) is expressed exclusively in PMCs, whereas VEGF expression is restricted to two small areas of the ectoderm, in front of the positions where the ventrolateral PMC clusters that initiate skeletogenesis will form. Overexpression of VEGF leads to skeletal abnormalities, whereas inhibition of VEGF/VEGFR signaling results in incorrect positioning of the PMCs, downregulation of PMC-specific genes and loss of skeleton. We present evidence that localized VEGF acts as both a guidance cue and a differentiation signal, providing a crucial link between the positioning and differentiation of the migrating PMCs and leading to morphogenesis of the embryonic skeleton.

KEY WORDS: Sea urchin, Embryo, Primary mesenchyme, Cell migration, Guidance, Differentiation, VEGF, Skeleton, Morphogenesis

INTRODUCTION

Cell migration is a fundamental process for the maintenance of adult life as well as for development of the embryo, and its alteration leads to severe pathological states or congenital defects. In the adult, cell migration is involved in wound healing, organ repair and in the immune response. During embryogenesis, migration of cell groups, individual cells or cellular extensions such as axons are essential for coordinated growth. Cell migration is crucial for colonization of gonads by germ cells, gastrulation, construction of complex tubular structures such as the tracheal and vascular systems, development of the nervous and immune systems and the formation of several organs.

Directed cell movement is controlled by external signals that guide migrating cells to their target. Primordial germ cells and leucocytes receive signals through G protein-coupled 7-TM receptors (Raz, 2004; Parent, 2004). Axon guidance relies on specific ligands (netrin, slit, ephrin and semaphorin) and their receptors (Dickson, 2002), some of which are also used by endothelial cells during vascular development (Carmeliet and Tessier-Lavigne, 2005). Recently, gradients of morphogens such as members of the BMP, SHH and WNT families were also shown to act as positional cues in axon guidance (Yoshikawa and Thomas, 2004). Many different cell types respond through tyrosine kinase receptors to signaling molecules controlling migration, such as PDGF in mesoderm cells in *Xenopus* (Nagel et al., 2004), GDNF in enteric neurons in mammals (Natarajan et al., 2002), FGFs in mesoderm cells during gastrulation in mouse and chick (Sun et al., 1999; Yang et al., 2002) and during trachea formation in *Drosophila* (where FGF is also known as BNL – Flybase) (Ribeiro et al., 2002; Sato and Kornberg, 2002), VEGFA in precursors of hematopoietic and neural cells (Hiratsuka et al., 2005; Zhang et al., 2003), PVF1 (VEGF-PDGF) in hemocytes (Cho et al., 2002) and together with EGF-family ligands, Keren and Spitz, in border cells in *Drosophila* (Duchek et al., 2001; Duchek and Rorth, 2001; McDonald et al., 2006).

The primary mesenchyme cells (PMCs) of the sea urchin embryo offer a remarkable model to study the mechanism of cell migration and its involvement in morphogenesis (Fig. 1A). The PMCs synthesize the spicules, which are the mineralized rods that constitute the skeleton of the embryo. At the mesenchyme blastula stage, the PMC precursors (32 cells in most species) leave the blastula wall to become the PMCs, which migrate within the blastocoel and become positioned along the inner ectoderm wall following a stereotypical pattern. At the early gastrula stage they form a characteristic ring with two symmetrical ventrolateral clusters. Spiculogenesis begins by the formation of a tri-radiate spicule rudiment in each ventrolateral cluster. Later, the two rudiments elongate and branch to form the two symmetrical halves of the skeleton. From specification to terminal differentiation, the process takes about two days, and although the number of cells implicated is small, the spatial patterning is stereotypical and the transparency of the embryo allows easy observation of cell behavior.

In the sea urchin embryo, several lines of evidence indicate that ectoderm influences many aspects of skeleton formation, including timing (Ettensohn and McClay, 1986), growth rate (Guss and Ettensohn, 1997), number of spicules and final size (Armstrong et al., 1993). The three-dimensional structure of the skeleton and its bilateral symmetry are foreshadowed and determined by the spatial organization that PMCs can achieve only within the embryo, in intimate contact with the ectoderm wall, suggesting guidance cues from the ectoderm control the well-defined PMC spatial pattern (Gustafson and Wolpert, 1967; Ettensohn, 1990; Malinda and

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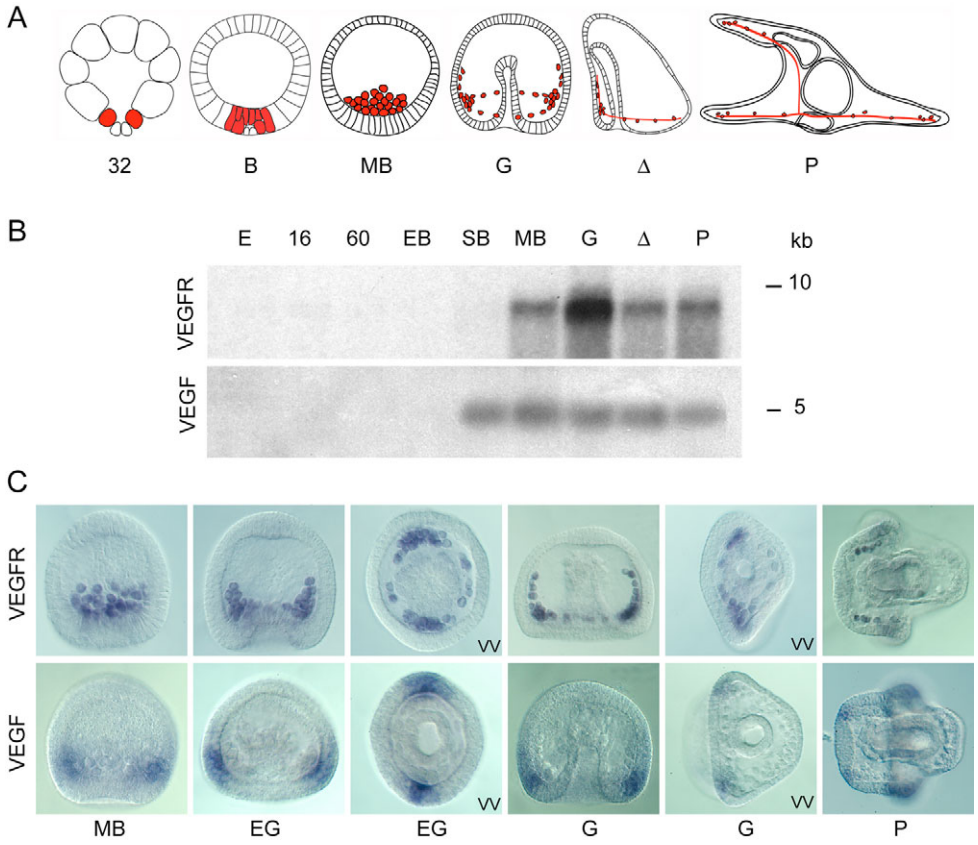

Fig. 1. The primary mesenchyme lineage of the sea urchin embryo and the expression pattern of *VEGFR* and *VEGF* during development. (**A**) Development of the primary mesenchyme lineage (red). The founder cells form at the 32-cell stage. At the mesenchyme blastula stage, the 32 descendants ingress into the blastocoel and become positioned along the ectoderm wall following a stereotypical pattern that includes two symmetrical ventrolateral clusters in which spiculogenesis begins by the formation of a tri-radiate rudiment. (**B**) Northern blot with total RNA isolated from embryos at the indicated stages. (**C**) Spatial distribution of *VEGFR* and *VEGF* transcripts detected by whole-mount in situ hybridization (WMISH). Embryos at the indicated stages were hybridized with sense (not shown) and antisense probes. Lateral views with the animal pole at the top, except for those marked (vv), which are ventral views with the oral side to the left. E, unfertilized egg; 16, 16-cell stage; 32, 32-cell stage; 60, 60-cell stage; EB, early blastula; B, blastula; SB, swimming blastula; MB, mesenchyme blastula; EG, early gastrula; G, gastrula; Δ, prism; P, pluteus.

Ettensohn, 1994). However, little is known at the molecular level about the interaction between ectoderm and the PMCs. Perturbation of the function of several secreted molecules or transcription factors expressed in the ectoderm affects skeleton formation, including nectin (Zito et al., 2000), nodal and BMP2/4 (Duboc et al., 2004), Msx (Tan et al., 1998), coquillette (Tbx2-3) (Croce et al., 2003; Gross et al., 2003), Dri (Amore et al., 2003) and Otp (Di Bernardo et al., 1999; Cavalieri et al., 2003). However, with the exception of Otp (the targets and function of this transcription factor are not known), these molecules appear to be implicated primarily in patterning the oral-aboral (AO) axis, rather than specifically controlling PMC organization, Therefore, the molecular basis of the interaction between ectoderm and PMCs is still not understood, as the signaling molecules and guidance cues implicated in this interaction have not yet been identified.

In this study, we show that VEGF/VEGFR signaling between ectoderm and PMCs plays a key role in the positioning and the differentiation of these migrating cells during gastrulation and, therefore, in the morphogenesis of the sea urchin embryonic skeleton. The guidance function of VEGF/VEGFR appears under different forms in several phyla and may constitute a primordial function conserved during evolution.

MATERIALS AND METHODS

Animals, embryo cultures and treatments

Paracentrotus lividus adults were collected in the bay of Villefranche-sur-Mer, France. Embryos were cultured as described by Lepage and Gache (Lepage and Gache, 1990). Treatment with LiCl was performed according to Ghiglione et al. (Ghiglione et al., 1993) and treatment with $NiCl_2$ as described by Croce et al. (Croce et al., 2003) following Hardin et al. (Hardin et al., 1992).

Cloning *VEGFR* and *VEGF* cDNAs

During a systematic search for receptor tyrosine kinases by RT-PCR using degenerate oligonucleotides, we isolated a fragment encoding part of a protein displaying similarity with VEGFR. This fragment was used to probe a *P. lividus* mesenchyme blastula cDNA library. The longest clone isolated was 5832 bp, with a 300 bp 5′ UTR, an ORF encoding a 1802 amino acid protein, and a 126 bp 3′ UTR.

VEGF cDNA was isolated by RT-PCR using information from published sequences including genomic sequences from the California species *Strongylocentrotus purpuratus*, available from the Baylor College of Medicine (http://www.hgsc.bcm.tmc.edu/blast/blast.cgi?organism=Spurpuratus), followed by screening a *P. lividus* pluteus cDNA library. We isolated a 5790 bp cDNA, with a 1161 bp 5′ UTR, an ORF coding for a 291 amino acid protein, and a 3756 bp 3′ UTR.

EMBL/GenBank accession numbers are AM419057 for *VEGFR* and AM419058 for *VEGF* nucleotide sequences.

Northern blots

Total RNA was extracted by the method of Cathala et al. (Cathala et al., 1983). Northern blots were carried out by standard methods as described by Lepage and Gache (Lepage and Gache, 1990) and Croce et al. (Croce et al., 2003). All probes were ^{32}P-labelled by random priming using the Prime-a-Gene Labelling System (Promega). The *VEGF* probe was a 1.4 kb fragment corresponding to nucleotides 1596-1997 of the cDNA. The 2.2 kb *VEGFR* probe corresponded to nucleotides 2851-5122 of the cDNA.

Whole-mount in situ hybridization

Whole-mount in situ hybridization (WMISH) was performed following Harland (Harland, 1991) and Lepage et al. (Lepage et al., 1992). DIG-labelled probes were revealed using an alkaline phosphatase-conjugated antibody with NBT-BCIP as chromogenic substrates.

The probe for *VEGFR* was a 2920 bp RNA derived from position 2913-5833 of the cDNA. The *VEGF* probe corresponds to the entire cDNA. The probe for *Ske-T* has been described by Croce et al. (Croce et al., 2001). The *MSP130* probe (1.5 kb), which derives from the coding sequence of the *MSP130* cDNA, was a gift from T. Lepage (Université Pierre et Marie Curie and CNRS, Villefranche-sur-Mer, France). The *SM30* (0.9 kb) and *SM50* (1.3 kb) probes were gifts from M. Di Bernardo (Istituto di Biologia dello Sviluppo del CNR, Palermo, Italy).

Microinjection of mRNA and antisense morpholino oligonucleotides

Microinjection into eggs was performed as described by Emily-Fenouil et al. (Emily-Fenouil et al., 1998). The *VEGF* ORF, plus six nucleotides upstream of the initiation codon and the stop codon, was amplified by PCR using Pfu polymerase. The PCR fragment was inserted at the *Bam*HI-*Cla*I sites of pCS2+ (Turner and Weintraub, 1994). The plasmid was linearized with *Not*I. The *pmar1* clone was a gift of P. Oliveri and has been described by Oliveri et al. (Oliveri et al., 2002). Capped mRNAs were synthesized in vitro with the SP6-mMessage-mMachine Kit (Ambion) and injected at 400 and 10 ng/μl for *VEGF* and *pmar1*, respectively. Embryos expressing pmar1 or pmar1 and VEGF were transferred at the blastula stage to Petri dishes coated with 2% agarose in sea water.

Morpholino antisense oligonucleotides (Mos) against splice sites were designed as follows. The sequences of the *P. lividus VEGF* and *VEGFR* cDNAs were aligned against the genomic sequences from *S. purpuratus* (Baylor College of Medicine, see above) to identify intron positions. The 5′-most introns identified were selected. Using primers from adjacent exons, introns were amplified from *P. lividus* genomic DNA. Mos were designed by Gene Tools from sequences of the intron-exon junctions. Mo-VEGF was 5′-CGTGTAACTACTCACATTCATCATAGTC-3′ and covers nucleotide positions 1659-1668 of the cDNA (codons 165-168) and the first 15 bases from the adjacent intron. Mo-VEGFR was 5′-TTAAAGTACAACTT-ACCTGGCGAGC-3′, corresponding to positions 683-691 of the cDNA (codons 127-129) and the following 16 bases from the adjacent intron. Before injection, Mos were dissolved at 1 mM for Mo-VEGF and 0.7 mM for Mo-VEGFR.

For both mRNA and Mo microinjections, more than 100 injected embryos were observed in each experiment and the experiments were repeated at least four times with different egg batches.

Double-injection experiments

In experiments relying on double injection, Mo-VEGF (1 mM) was injected into eggs together with RLDX tracer (rhodamine coupled to dextran 10 kDa, Molecular Probes) to select injected eggs. Later, *VEGF* mRNA (400 ng/μl) was injected into a single blastomere of the eight-cell stage embryo together with FLDX lineage marker (fluorescein coupled to dextran 70 kDa, Molecular Probes). FLDX labelling was observed on living or fixed embryos by fluorescence microscopy. To reveal expression of both the marker gene and the lineage marker we followed the procedure of Thisse et al. (Thisse et al., 2004). Gene expression was first revealed by WMISH as described above. After washing steps, FLDX was revealed using an anti-fluorescein antibody coupled to alkaline phosphatase and Fast Red as substrate (Roche). The substrate NBT-BCIP gives a purple-brown color, whereas Fast Red appears reddish. About 20 double-injected embryos were observed in each experiment, and the experiments were repeated three to four times with different egg batches.

RESULTS

Cloning and characterization of *VEGFR* and *VEGF* cDNAs

Because of their fundamental role during development, we undertook a systematic search for receptor tyrosine kinases expressed in the sea urchin embryo. Among the receptor tyrosine kinase cDNAs that we isolated, we identified a cDNA coding for a member of the VEGFR family. The predicted protein displays the characteristic organization of VEGFR, including an extracellular domain that comprises Ig-like domains and an intracellular kinase domain that displays high similarity with kinase domains of the VEGF family. Furthermore, the phylogenetic analysis carried out by Lapraz et al. (Lapraz et al., 2006) using sequences from the *Strongylocentrotus purpuratus* genome shows that this domain groups with kinase domains from the VEGFR family and not with those from the PDGF family. The protein is, however, atypical in that the extracellular domain is predicted to contain ten Ig-like domains instead of seven and, as far as we know, it is unique to sea urchins.

In addition, we isolated a cDNA coding for a ligand of the VEGF family. The predicted protein consists of a poorly conserved N-terminal domain containing a serine-rich stretch, followed by the conserved typical VEGF/PDGF domain with the cysteine knot and a cysteine-rich C-terminal domain. The corresponding gene was later found in the *S. purpuratus* genome and designated *Sp-VEGF-3* (Lapraz et al., 2006).

Other related genes have been found in the genome of *S. purpuratus* (Sea Urchin Genome Sequencing Consortium, 2006; Lapraz et al., 2006), but their cDNAs have not been cloned. Another VEGFR-family gene codes for a classic VEGFR receptor with seven Ig-like domains. Quantitative PCR measurements (F. Rizzo and M. Arnone, personal communication) on *S. purpuratus* mRNA indicate that *VEGFR-7-Ig* is expressed during embryogenesis, although at a lower level than *VEGFR-10-Ig*. Both VEGFR genes are expressed in coelomocytes of the adult (Sea Urchin Genome Sequencing Consortium, 2006). Two other VEGF-family genes, *Sp-VEGF* and *Sp-VEGF-2* were predicted. In *S. purpuratus*, the expression of *Sp-VEGF2* has been shown to be absent or barely detectable during the first 52 hours of development (J. Rast, personal communication). Tilling data suggest that *Sp-VEGF* is also poorly expressed during embryogenesis (http://www.genboree.org).

Our study focuses on the ten Ig-domain receptor and on the VEGF-3 ligand, referred to here simply as VEGFR and VEGF, respectively.

The *VEGFR* and *VEGF* genes are expressed during the same period in distinct domains

The expression patterns of the *VEGFR* and *VEGF* genes were characterized by northern blot and whole-mount in situ hybridization (WMISH) (Fig. 1B,C). Northern analysis showed that each gene is expressed as a single transcript, around 8 kb for *VEGFR* and 6 kb for *VEGF*. The *VEGFR* and *VEGF* genes are not maternally expressed, but instead have a single phase of zygotic expression that takes place after hatching. *VEGFR* was found to be expressed from mesenchyme blastula to pluteus, with a peak at the gastrula stage. *VEGF* expression began earlier, at the swimming blastula stage, and was maintained at a nearly constant level until the pluteus stage. Thus, the temporal expression profiles of both genes are very similar and largely overlapping. By contrast, the two genes

have distinct domains of expression (Fig. 1C). The *VEGFR* transcripts were detected exclusively in the PMC lineage, and in agreement with the northern data, only after ingression of these cells into the blastocoel and up to the pluteus stage. At the mesenchyme blastula and gastrula stages (i.e. during PMC migration) *VEGFR* was expressed in all PMCs. Later, at the prism stage, expression decreased in cells of the aboral chain. In the pluteus, *VEGFR* transcripts were present in only a few PMCs at the tip of the oral and post-oral arms and at the aboral apex, close to the sites of spicule elongation. The *VEGF* domain of expression did not correspond to a known lineage or territory. Although northern data indicated that they were present, *VEGF* transcripts could not be detected by WMISH at the swimming blastula stage, suggesting that they might be expressed transiently in a diffuse area. From mesenchyme blastula and throughout gastrulation, the *VEGF* transcripts were restricted to two small areas of the ectoderm, close to the ectoderm/endoderm border and to the limit between the oral and aboral ectoderm. This symmetrical ventrolateral localization is coincident with the localization of the PMC clusters that form at the early gastrula stage along the ectoderm wall. At the pluteus stage, the *VEGF* transcripts were detected only in a few ectodermal cells at the tip of the arm buds, close to the *VEGFR*-expressing cells.

The complementary expression of these potential partners between two interacting tissues suggests that VEGF signaling might play a role in the organization of the PMCs. At a later stage, it might also participate in the control of the growth of the spicule rods.

Organization of the PMCs and of the skeleton correlate with the expression of *VEGF* in embryos mispatterned along the AV and OA axes

VEGF is expressed in very restricted areas precisely positioned along the animal-vegetal (AV) and oral-aboral (OA) axes. Alterations of patterning along these axes are known to produce abnormal skeleton. Patterning of the AV axis is controlled by the maternal components of the canonical WNT pathway (Logan et al., 1999; Emily-Fenouil et al., 1998; Huang et al., 2000; Vonica et al., 2000) and can be perturbed by LiCl. Treatment with LiCl vegetalizes the embryo, i.e. it alters cell fate specification along the AV axis by shifting the ectoderm/endoderm border towards the animal pole, resulting in overdevelopment of endoderm at the expense of ectoderm and a morphological radialization of the embryo. The perturbation of the skeleton depends on the degree of vegetalization (e.g. Emily-Fenouil et al., 1998). Organization along the OA axis is controlled by the nodal and BMP2/4 pathways (Duboc et al., 2004). The OA polarity is strongly affected by treatment with $NiCl_2$, the target of which is not yet identified. Treated embryos are oralized and produce multiple tri-radiate spicule rudiments along the circumference (Hardin et al., 1992). The phenotype of embryos treated with $NiCl_2$ is similar to those overexpressing nodal (Duboc et al., 2004).

The expression of the *VEGFR* and *VEGF* genes in perturbed embryos is shown in Fig. 2. In all cases, the PMC precursors were apparently not affected and the PMCs ingressed on schedule and expressed *VEGFR* at a normal level. However, the expression of *VEGF* and the behavior of the PMCs were modified. In embryos treated with LiCl, the enlarged endoderm was exogastrulated and the ectoderm was reduced to a small area around the animal pole. *VEGF* was expressed in this area at the tip of the embryo, and the PMCs, which expressed *VEGFR*, formed a single cluster close to the expression domain of *VEGF*. In some cases, a single abnormal spicule formed (Fig. 2A) (Emily-Fenouil et al., 1998).

In embryos treated with $NiCl_2$, the PMCs did not form the two ventrolateral clusters and did not line up to form the oral and aboral chains. Two different phenotypes were observed depending on egg batches: PMCs were either arranged around the circumference of the embryo and formed a variable number of spicule rudiments as already described (Hardin et al., 1992); or the PMCs formed a single vegetal aggregate that was later carried to the center of the blastocoel by the invaginating archenteron and eventually made a spicule. In both cases, the bilateral symmetry of the skeleton was lost. In all cases, WMISH showed that the expression domain of *VEGF* was not limited to the two ventrolateral patches, but formed a ring around the embryo (Fig. 2B).

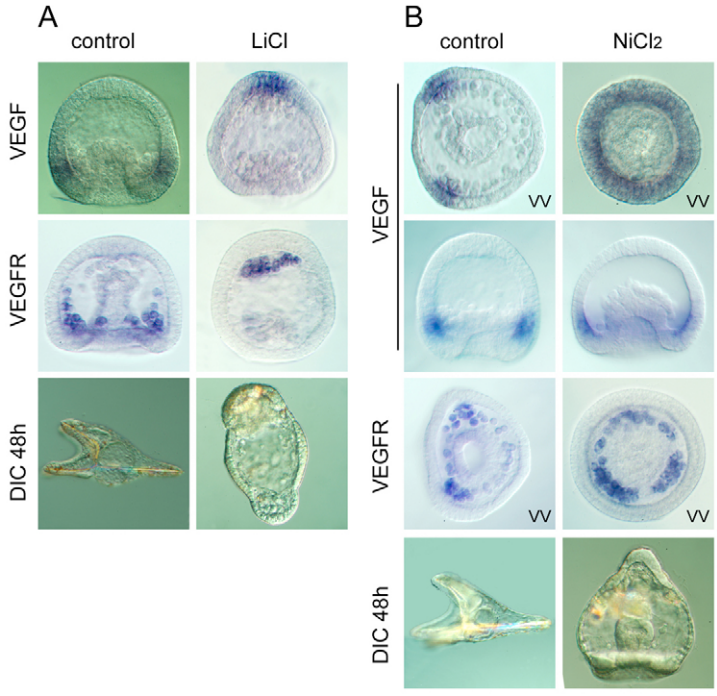

Fig. 2. Expression pattern of *VEGFR* and *VEGF* during perturbed development. (**A**) Perturbation along the AV axis. Sea urchin embryos were treated with 30 mM LiCl. Detection of *VEGF* and *VEGFR* transcripts by WMISH (as in Fig. 1) on gastrula-stage embryos and DIC images of 48-hour-old embryos. Lateral views with the animal pole at the top. (**B**) Perturbation along the OA axis. Embryos were treated with 0.5 mM $NiCl_2$. WMISH of early gastrulae-stage embryos and DIC images of 48-hour-old embryos. Lateral views with the animal pole at the top, or ventral views with the oral side to the left when OA polarity is present (vv).

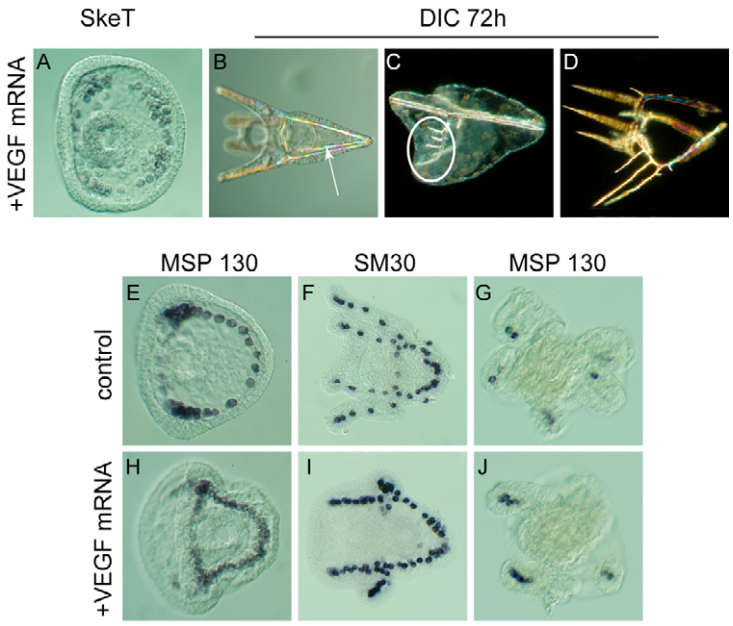

Fig. 3. Overexpression of *VEGF* perturbs skeletogenesis. Sea urchin embryos overexpressing *VEGF* following microinjection of *VEGF* mRNA into the egg. (**A**) WMISH with a *Ske-T* probe at the gastrula stage. Vegetal view. (**B-D**) DIC images of pluteus-stage embryos. Arrow, supernumerary elements; circle, abnormal branching. (**E-J**) WMISH with probes for (E,H) *MSP130* at prism stage, (F,I) *SM30* and (G,J) *MSP130* at pluteus stage.

Thus, when the *VEGF* domain was shifted along the AV axis and reduced to a single spot at the animal pole of the embryo, all the PMCs aggregated into a single cluster adjacent to the *VEGF* source. In radialized embryos, the absence of symmetrical bilateral clusters of PMCs and the skeletogenic defects observed correlate with the radialization of the VEGF expression domain. Therefore, PMC positioning and cluster formation appear to be closely linked to the localization, extent and shape of the *VEGF* expression domain.

Misexpression of *VEGF* provokes defects of skeletogenesis

To gain insight into the function of VEGF signaling, we expressed *VEGF* in the whole embryo by microinjecting a *VEGF* mRNA into the egg. The injected embryos developed almost normally, except for abnormalities restricted to the skeletogenic lineage (Fig. 3). At the early gastrula stage, the PMC ring did not display its regular organization. The two ventrolateral clusters were present, but PMCs formed a variable number of additional aggregates that looked irregular and loosely packed (Fig. 3A). At the pluteus stage, the normal elements of the skeleton were present and gave the embryo its normal global shape. However, some spicule rods were duplicated (Fig. 3B,C) and some rods had aberrant multiple ramifications (Fig. 3D). Thus, the uniform expression of exogenous VEGF superimposed on endogenous VEGF expression allows formation of a basic skeleton but produces supernumerary elements and irregular branching.

Monitoring expression of marker genes (see below) revealed that the number of PMCs was approximately twice that in control embryos (Fig. 3E,J). In the early pluteus, a probe for *SM30* transcripts marked about 70 PMCs, as opposed to 29 in the control. At the late pluteus stage, *MSP130* transcripts were detected in two spots at the tip of the arms of normal embryos and in four spots in embryos overexpressing *VEGF*. This observation reveals a potential influence of VEGF on cell division that might have a physiological role in species in which PMCs undergo their last round of division shortly after ingression. However, it is unlikely that the artifactual increase in cell number observed here leads by itself to additional aggregation of the PMCs and to the skeleton abnormalities observed, as it has been shown by Ettensohn (Ettensohn, 1990) that embryos with three times the normal complement of PMCs form a normal skeleton. Therefore, perturbation of skeletogenesis by overexpression of *VEGF* suggests that VEGF plays a direct role in spiculogenesis by the PMCs.

Loss-of-function of *VEGF* or *VEGFR* perturbs PMC positioning, downregulates differentiation genes and blocks skeleton formation

Loss-of-function for the *VEGF* or *VEGFR* gene was achieved by microinjection of morpholino antisense oligonucleotides (Mos). The phenotypes obtained in more than 90% of the embryos were specific to PMC development and furthermore were identical for both gene knockdowns (Fig. 4). This suggests that the effects are specific and that VEGF and VEGFR are interacting partners.

In embryos that were left to develop, the morphological defects were first detected at the early gastrula stage. The normal complement of PMCs formed and ingressed, but did not lead to the classic PMC ring. Neither the ventrolateral clusters nor the oral or aboral chain formed. At the pluteus stage, instead of the elongated shape typical of the *P. lividus* pluteus, the embryos were of a squatty appearance with poorly extended arms. The AV and OA polarities were present, the digestive tripartite gut had developed and the mouth opened, and pigmented cells were visible within the ectoderm. However, from early gastrula to prism, no spicule rudiment was ever observed and at the pluteus stage bright spicule rods could not be seen, indicating that the skeleton was totally absent.

To further follow the behavior of the PMCs, we monitored expression of *Ske-T* (also known as *T-brain*) and *MSP130*, two PMC-specific genes that begin to be expressed before PMC ingression and thus before VEGF signaling could operate. *Ske-T* encodes a T-box transcription factor that is expected to play a regulatory role at an intermediate level between specification and the terminal differentiation genes (Croce et al., 2001; Fuchikami et al., 2002; Oliveri et al., 2002) (C.G., unpublished). *MSP130* codes for a cell surface protein implicated in the biomineralization process, probably by regulating calcium transport (Carson et al., 1985; Leaf et al., 1987). As shown in Fig. 4, both genes were expressed by the

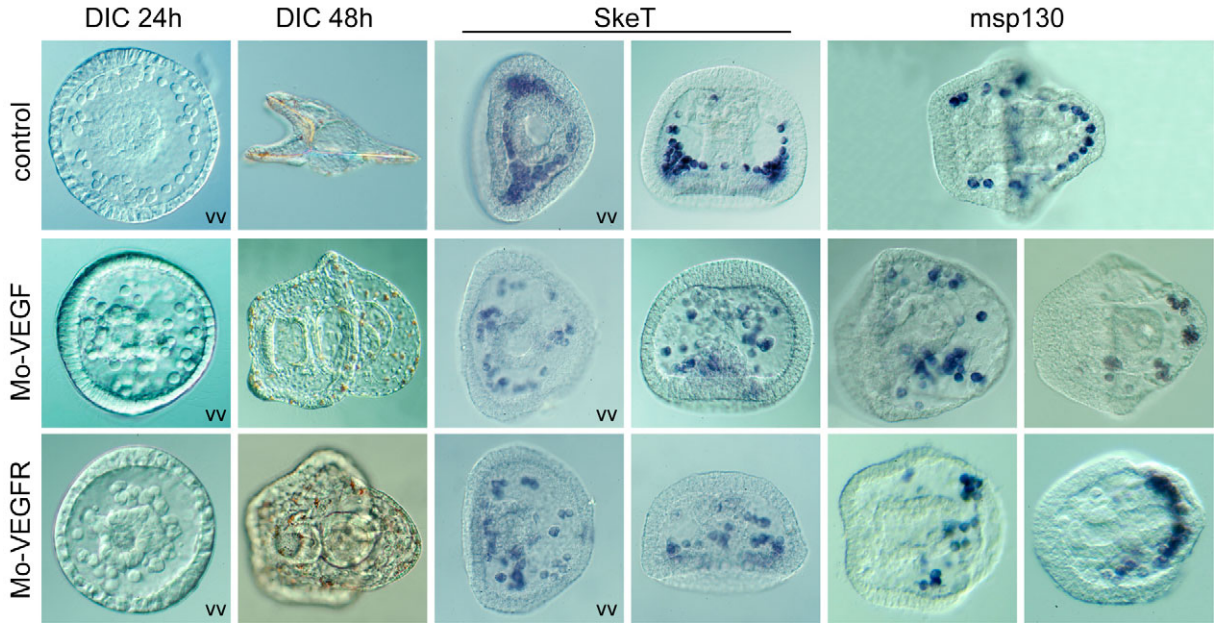

Fig. 4. *VEGF* or *VEGFR* loss-of-function blocks PMC spatial patterning and skeleton formation in sea urchin. Embryonic treatment (control or injected with Mo-VEGF or Mo-VEGFR) is indicated on the left of each row. In each column is shown morphology at 24 and 48 hours, expression of *Ske-T* at the gastrula stage and expression of *MSP130* at the pluteus stage. vv, vegetal view.

PMCs in control embryos and in embryos injected with Mos against *VEGF* or *VEGFR*. However, whereas in control embryos the PMCs were positioned following the stereotypical pattern, in about 90% of the embryos in which VEGF/VEGFR signaling has been impaired, the PMCs were disorganized. In most embryo batches (80%), they were randomly dispersed within the blastocoel, whereas in some embryo batches (20%) they formed several small aggregates randomly positioned within the vegetal part of the embryo and were often found later near the aboral apex.

To further characterize this phenotype, we probed expression of PMC-specific late differentiation genes. Several proteins are directly involved in the synthesis of the spicules, among which SM30 and SM50 are components of the organic matrix (Benson et al., 1987; George et al., 1991; Wilt, 2002). The *SM30* and *SM50* genes are turned on after PMC ingression and although they are expressed with slightly different spatial patterns (Guss and Ettensohn, 1997), their transcripts can be detected in most PMCs (Fig. 5). In embryos in which expression of *VEGF* or *VEGFR* was blocked, both *SM30* and *SM50* were strongly downregulated. *SM30* transcripts were never detected, whereas *SM50* transcripts were sometimes detectable in a few cells (Fig. 5).

Because *VEGFR* has a special function among PMC-specific genes, we next asked whether continuous expression of *VEGFR* itself might depend upon VEGF signaling. In normal embryos, *VEGFR* was expressed in all PMCs from ingression to the gastrula stage (Fig. 1C and Fig. 5). In embryos in which VEGF signaling had been impaired by injection of Mo-VEGF, *VEGFR* transcripts were detected in PMCs at the mesenchyme blastula stage but were undetectable at the gastrula stage (Fig. 5). This indicates that after an autonomous early phase, *VEGFR* expression is maintained by VEGF signaling. The late expression domains of *VEGF* and *VEGFR* are very restricted, closely adjacent and localized at all skeletal extension sites (Fig. 1C), suggesting that this coupling between VEGF and expression of *VEGFR* might participate in the control of skeletal growth.

These results show that VEGF/VEGFR signaling is required for the proper positioning of the PMCs, the formation of the ventrolateral clusters, the maintenance of *VEGFR* expression, the activation or maintenance of terminal differentiation genes functioning in spiculogenesis, and the production of spicules.

Ectopic *VEGF* expression can direct spicule formation in embryos in which endogenous *VEGF* function is blocked

We next addressed whether an exogenous source of *VEGF* expression in embryos in which the endogenous pathway has been blocked was capable of directing spicule formation. As the Mo-VEGF is targeted against a splice junction, it should not inhibit synthetic *VEGF* mRNA. In order to carry out this experiment, we choose embryonic batches in which injection of Mo-VEGF alone led to a strong dispersion of the PMCs. In these batches, co-injection of Mo-VEGF and *VEGF* mRNA led to a recovery of spicule formation in about 50% of the embryos.

In the first series of experiments, we co-injected Mo-VEGF and *VEGF* mRNA into unfertilized eggs (Fig. 6A). The resulting pluteus-stage embryos displayed AV and OA polarities with a tripartite gut, but their overall shape was abnormal. This shape resulted from the presence of spicule elements with abnormal shapes and variable and incorrect positioning. Thus, spicule rods, sometimes with branching, could be rescued by broadly supplied exogenous VEGF, but skeletal morphology could not.

In the preceding experiments, VEGF was uniformly expressed in the embryo. In order to create a more localized source of VEGF, Mo-VEGF was first microinjected into the egg and then at the 8-cell stage *VEGF* mRNA was microinjected into a single blastomere together with a fluorescent lineage marker (Fig. 6E). The injected embryos were allowed to develop until non-injected embryos reached the pluteus stage. Injection into one blastomere at the 8-cell stage was random in terms of whether it was in an animal or vegetal

blastomere. As VEGF is normally expressed in ectoderm, embryos displaying lineage marker in the ectoderm were selected. Control embryos injected with Mo-VEGF developed without forming any spicules as described above. Differential interference contrast (DIC) and fluorescence microscopy showed that double-injected embryos formed a single spicule rod of variable shape, close to the lineage tracer and thus to the *VEGF*-expressing cells (Fig. 6F-H). Moreover, *SM30* was expressed in the PMCs that were found in aggregates adjacent to the ectoderm patches expressing *VEGF* (Fig. 6I-K).

Thus, in embryos devoid of endogenous VEGF signaling, ectopic localized expression of VEGF can rescue, at close distance, PMC aggregation, *SM30* activation and spicule formation.

VEGF triggers formation of spicule primordia within a population of PMCs

It is known that isolated micromeres cultured in sea water do not produce spicules but can do so if the medium is supplemented with serum or blastocoelar fluid (Okazaki, 1975; Kiyomoto and Tsukahara, 1991). To address whether exposure to VEGF could induce PMCs to synthesize spicules, we produced a population of PMCs by taking advantage of the powerful ability of the transcriptional regulator pmar1 to specify PMC fate (Oliveri et al., 2002). When *pmar1* mRNA is injected into an egg, most cells of the embryo are converted to a PMC fate, forming a ball of loosely connected mesenchyme cells that express some PMC markers but do not form spicules. To obtain such PMC balls and to expose

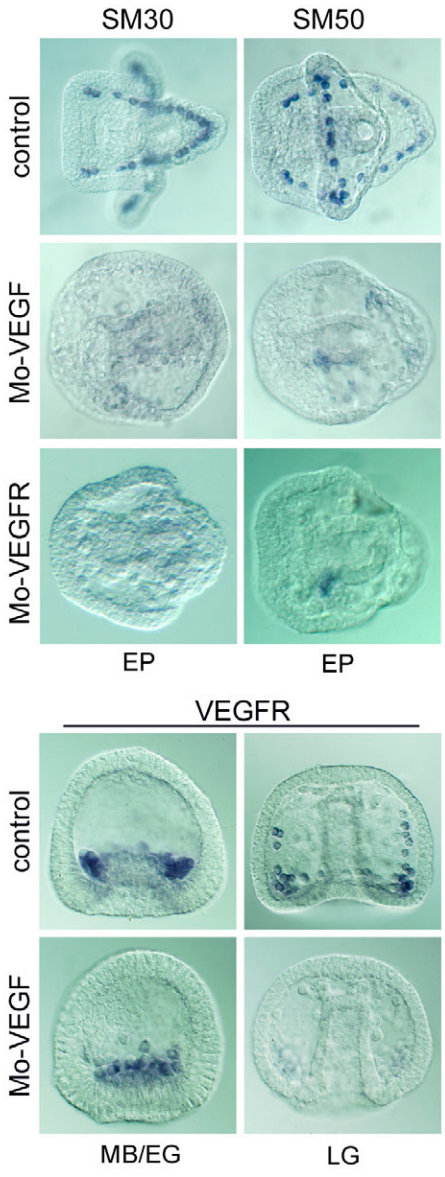

Fig. 5. VEGF/VEGFR signaling controls expression of PMC-specific genes. *SM30*, *SM50* and *VEGFR* transcripts detected by WMISH at the indicated stages in control sea urchin embryos and those injected with Mo-VEGF or Mo-VEGFR. MB, mesenchyme blastula; EG, early gastrula; LG, late gastrula; EP, early pluteus.

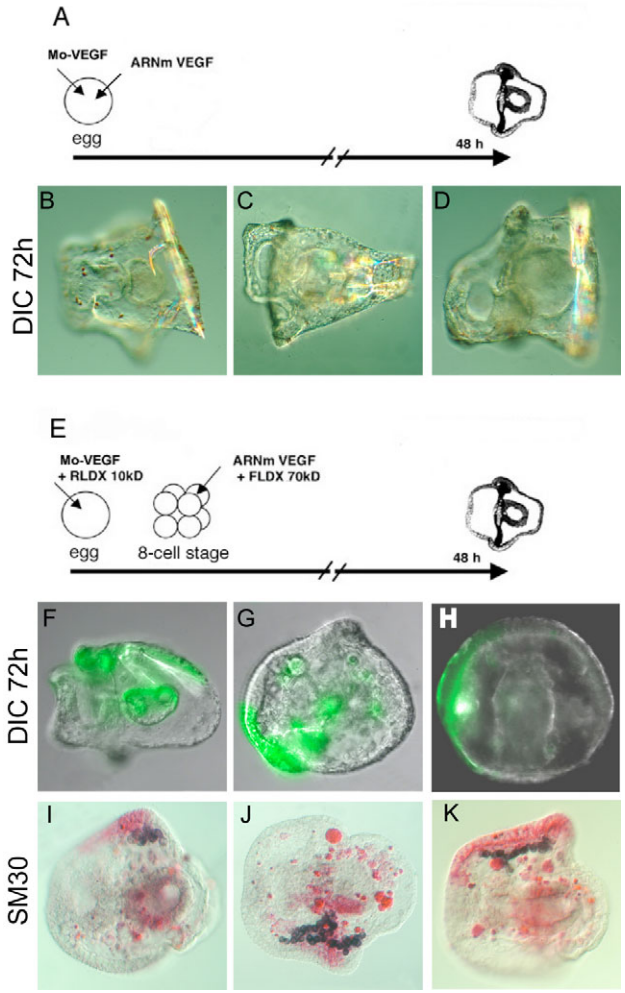

Fig. 6. Rescue of spicule formation by expression of *VEGF* mRNA in sea urchin embryos injected with Mo-VEGF. (**A**) Co-injection of Mo-VEGF and *VEGF* mRNA into unfertilized eggs. (**B-D**) Phenotypes of 72-hour-old embryos treated as in A. (**E**) Localized ectopic expression of *VEGF* obtained by microinjection of Mo-VEGF into the egg, and *VEGF* mRNA together with a fluorescent lineage marker into one blastomere at the 8-cell stage. (**F-K**) 72-hour-old embryos treated as in E were split into two batches for (F-H) DIC and fluorescence microscopy, with the DIC/fluorescence signal superimposed to show spicule formation, or (I-K) immunolocalization of lineage marker (light red) and WMISH with a *SM30* probe (brown); dispersed spherical red 'dots' are microinjection artifacts.

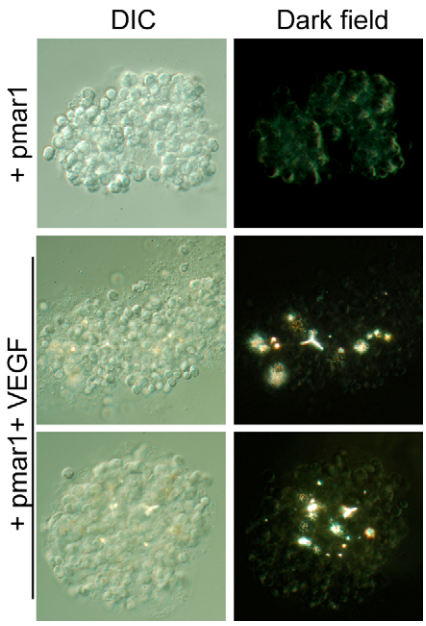

Fig. 7. VEGF induces spicule formation by PMCs. DIC and dark field images of 72-hour-old sea urchin embryos injected at the egg stage with *pmar1* mRNA or co-injected with *pmar1* and *VEGF* mRNAs.

them to VEGF, we coexpressed *pmar1* and *VEGF*. Embryos derived from eggs injected with *pmar1* mRNA or with *pmar1* and *VEGF* mRNAs are shown in Fig. 7. Embryos expressing only *pmar1* developed as previously described (Oliveri et al., 2002) and did not form any spicule or spicule primordium. However, embryos expressing both *pmar1* and *VEGF* displayed the same morphology, but many refractive dots typical of spicule material and some spicule primordia were visible. Therefore, VEGF is sufficient to trigger formation of spicule primordia in a population of aggregated cells reoriented towards a PMC fate. This indicates that VEGF is a differentiation signal sufficient to initiate skeletogenesis by PMCs.

DISCUSSION

The unique fate and the easily observable behavior of the primary mesenchyme cells during embryogenesis of the sea urchin have long attracted attention. The striking stereotypical positioning adopted by these cells during their migration is the basis for the morphogenesis of the embryonic skeleton. It has been anticipated that the whole process results from interplay between the intrinsic properties of the PMCs and the influence from the ectoderm, but almost nothing was known of the interaction between these two tissues at the molecular level. Our results show that VEGF/VEGFR signaling mediates a crucial interaction between ectoderm and PMCs, playing a key role in the morphogenesis of the embryonic skeleton. The evidence for this is as follows. (1) *VEGF* and *VEGFR* are both expressed in largely overlapping periods spanning the period of PMC spatial patterning and skeleton formation. (2) The receptor is expressed only in the PMCs. The ligand is expressed in two very restricted areas of the ventrolateral ectoderm overlying the PMC clusters. (3) Perturbations of embryonic axes lead to modifications of the *VEGF* expression territory that correlate well with the associated alterations in PMC positioning. (4) Misexpression of *VEGF* leads to skeletal abnormalities. (5) When VEGF/VEGFR signaling is impaired, PMC positioning is perturbed, and differentiation does not occur (some skeletogenic genes are downregulated and spicules are not formed). (6) In embryos in which endogenous *VEGF* expression has been blocked, ectopic localized expression of *VEGF* drives local formation of PMC aggregates, activation of skeletogenic genes and formation of spicule rods. (7) Embryos in which most cells have been transformed into mesenchyme cells do not produce spicule primordia unless they express *VEGF*.

This VEGF/VEGFR signaling system provides a molecular basis for the interaction between ectoderm and the PMCs that has been outlined in several studies (for reviews, see Gustafson and Wolpert, 1967; Decker and Lennarz, 1988; Ettensohn et al., 1997; Wilt, 2002). The complementary expression of the ligand in the ectoderm and of the receptor in the PMCs demonstrates a unidirectional interaction between these two tissues. The symmetrically localized expression of VEGF constitutes a spatial cue that is required for the formation of the two ventrolateral clusters and the formation of the spicule rudiments.

Furthermore, our findings fit with previous important observations. First, restriction of VEGFR to the PMCs explains why only PMCs are competent to respond to the guidance signal (Ettensohn and McClay, 1986). Second, PMCs from mesenchyme blastula injected into the blastocoel of younger blastula form a pattern only when the host reaches the mesenchyme blastula stage (Ettensohn and McClay, 1986), which corresponds to the onset of *VEGF* expression. Conversely, the permanent expression of *VEGF* and *VEGFR* during gastrulation explains why late PMCs, having already completed their migration, are still capable of responding to ectoderm directional cues when transplanted to a new host (Ettensohn, 1990). Third, by using embryos of different sizes and manipulating the OA axis, Armstrong et al. (Armstrong et al., 1993) have shown that normal embryos always form two rudiments, whatever their size, whereas radialized embryos display supernumerary spicule rudiments in proportion with their size. This follows from the transformation of the *VEGF* expression domain from two symmetrical patches into a continuous ring the size of which is proportional to the size of the embryo. Fourth, it has been predicted that signaling from the ectoderm should regulate expression of PMC-specific products (Guss and Ettensohn, 1997). Indeed, when VEGF/VEGFR signaling is impaired, the two PMC-specific genes *SM50* and *SM30* are repressed, although a third one, *MSP130*, is not significantly affected. The expression patterns of these three genes are different (Guss and Ettensohn, 1997). *MSP130* begins to be expressed before PMC ingression, whereas *SM50* and *SM30* are turned on at early and late mesenchyme blastula, respectively. During the initial phase of PMC patterning, *MSP130* and *SM50* are expressed uniformly in all PMCs, whereas *SM30* is expressed at a higher level in the ventrolateral clusters. Furthermore, in PMC cultures, all cells express *SM50*, whereas *SM30* is expressed only by cells that participate in spicule synthesis, and the expression of *SM30* is extremely sensitive to the presence of serum (see Guss and Ettensohn, 1997). In addition, misexpression of pmar1 upregulates *MSP130* strongly and *SM50* moderately, but does not significantly affect *SM30* expression (Oliveri et al., 2002). Thus, it appears that *MSP130* is not controlled by VEGF as it is probably part of the autonomous program of the PMCs. *SM50* might be turned on autonomously, but VEGF signaling is required to maintain a high level of expression. *SM30*, which is expressed in the PMC clusters and sensitive to the ectoderm signal, requires VEGF signaling. Finally, our results support the view that the endogenous signal from ectoderm and the signal provided by serum or blastocoelar fluid might be similar (Ettensohn et al., 1997), but this remains to be demonstrated

through serum fractionation. In summary, VEGF/VEGFR signaling appears to be a major element of the interaction between ectoderm and PMCs.

Identification of the function of VEGF opens the way to a better understanding of where this signaling event fits in the regulatory interactions that built the embryo, and of its influence upon the migratory behavior of the PMCs.

The sea urchin embryo is particularly well suited to unravel gene regulatory networks such as the network that controls mesendoderm formation (Davidson et al., 2002). VEGF/VEGFR signaling is a bridge between the ectoderm network, which is largely unknown, and the PMC network (Oliveri et al., 2002). Although PMCs have a largely autonomous program of differentiation, the PMC network cannot go to completion without an input from the ectoderm. VEGF is a spatial cue that directs the formation of the ventrolateral clusters, controls expression of *VEGFR* and of spiculogenic genes and probably regulates skeletal growth. VEGF, therefore, establishes a coupling between morphogenesis and differentiation. It will be important for future studies to integrate these signaling factors within their respective networks. In contrast to *VEGFR* expression, *VEGF* expression is not lineage-restricted and thus results from fine patterning of the ectoderm. The spatial pattern of *VEGF* is reminiscent of the pattern of the homeobox gene *Otp* (Di Bernardo et al., 1999), but it is unlikely that *Otp* controls *VEGF* as *Otp* seems to be activated later than *VEGF* and in a territory much smaller than the *VEGF* domain. The VEGF expression domain overlaps that of another signaling molecule: in an independent study on the function of FGF during gastrulation, E. Röttinger and T. Lepage (personal communication) have shown that FGF signaling plays a role in PMC patterning. The spatial expression of the two ligands is linked to the main embryonic axes, but otherwise VEGF and FGF signals are independent and functionally nonredundant (our unpublished results and T. Lepage, personal communication) and are thus both required for correct morphogenesis of the primary mesenchyme.

How migrating mesenchyme cells detect the signal transmitted from the ectoderm and modulate their behavior is another essential question. Pioneering studies (Gustafson and Wolpert, 1961) and a more recent work (Malinda et al., 1995) have shown that PMCs contact the blastocoel wall through long filopodia and move through contraction of these filopodia. Gustafson and Wolpert (Gustafson and Wolpert, 1961) observed that PMCs randomly explore the blastula wall with dynamic filopodia, and suggested that they became trapped as the ectoderm displays areas with differential adhesive properties. No such patterning of the ectoderm has been discovered so far. Instead, our findings point to a role for VEGF, as in other systems. For example, during angiogenesis of the mouse retina, endothelial tip cells expressing VEGFR2 (KDR – Mouse Genome Informatics) extend long filopodia that detect steep gradients of VEGF and guide their migration (Gerhardt et al., 2003). In the sea urchin embryo, VEGF signaling might stabilize PMC filopodia that randomly explore the ectoderm wall and contact the *VEGF* expression domains or closely approach a local VEGF gradient. Thus, PMCs would reach their target through short-range contact guidance.

VEGF, first identified as a regulator of vascular permeability, was later shown to have a very important role in vascularization and angiogenesis. Recently, VEGF was shown to be a guidance cue for endothelial, hematopoietic and neural precursors in vertebrates (Gerhardt et al., 2003; Hiratsuka et al., 2005; Zhang et al., 2003). VEGF (PVF1) is also a guidance cue for border cells and blood cells in *Drosophila*. Border cells are follicular cells that migrate towards the oocyte during oogenesis. Border cells express the receptor (PVR, similar to both VEGFR and PDGFR), whereas the oocyte expresses VEGF (Duchek et al., 2001). Hemocytes, which are produced in the head and migrate throughout the body, express VEGFR (PVR), whereas VEGF is expressed along their migration pathways (Cho et al., 2002; Wood et al., 2006). As *Drosophila* has no blood vessels, Cho et al. (Cho et al., 2002) suggested that the VEGF pathway might have originally functioned in blood cells and was later recruited for vascular development. In the sea urchin embryo, which lacks a vascular system and blood cells, VEGF might carry out an ancestral function. We showed that the guidance function of VEGF, already described in protostomes and in vertebrate deuterostomes, is also present in sea urchin, a nonchordate deuterostome, and thus may indeed constitute a primordial function conserved during evolution.

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