Growth factor-mediated mesodermal cell guidance and skeletogenesis during sea urchin gastrulation

Ashrifia Adomako-Ankomah and Charles A. Ettensohn*

SUMMARY
Growth factor signaling pathways provide essential cues to mesoderm cells during gastrulation in many metazoans. Recent studies have implicated the VEGF and FGF pathways in providing guidance and differentiation cues to primary mesenchyme cells (PMCs) during sea urchin gastrulation, although the relative contributions of these pathways and the cell behaviors they regulate are not fully understood. Here, we show that FGF and VEGF ligands are expressed in distinct domains in the embryonic ectoderm of Lytechinus variegatus. We find that PMC guidance is specifically disrupted in Lv-vegf3 morphants and these embryos fail to form skeletal elements. By contrast, PMC migration is unaffected in Lv-fgfa morphants, and well-patterned but shortened skeletal elements form. We use a VEGFR inhibitor, axitinib, to show that VEGF signaling is essential not only for the initial phase of PMC migration (subequatorial ring formation), but also for the second phase (migration towards the animal pole). VEGF signaling is not required, however, for PMC fusion. Inhibition of VEGF signaling after the completion of PMC migration causes significant defects in skeletogenesis, selectively blocking the elongation of skeletal rods that support the larval arms, but not rods that form in the dorsal region of the embryo. Nanostring nCounter analysis of ~100 genes in the PMC gene regulatory network shows a decrease in the expression of many genes with proven or predicted roles in biomineralization in vegf3 morphants. Our studies lead to a better understanding of the roles played by growth factors in sea urchin gastrulation and skeletogenesis.

KEY WORDS: Cell migration, Sea urchin, VEGF, FGF, Gastrulation, Primary mesenchyme cells

INTRODUCTION
Growth factor signaling pathways play essential roles in diverse developmental processes (Hogan, 1999; Wilson and Leptin, 2000; Wu and Hill, 2009; Dorey and Amaya, 2010), including the migration and differentiation of mesoderm during gastrulation. In Drosophila embryos, members of the fibroblast growth factor (FGF) family, pyramus and thisbe, are expressed in the ectoderm and regulate the intercalation, spreading, migration and differentiation of mesoderm cells, which express the FGF receptor heartless (Kadam et al., 2009; McMahon et al., 2010; Winkelbauer and Müller, 2011; Reim et al., 2012). A similar role is played by platelet-derived growth factor (PDGF) during Xenopus gastrulation; in this case, the ligand PDGF-A, expressed in the ectoderm, regulates the orientation and migration of mesoderm cells that express PDGFRα (Ataliotis et al., 1995; Nagel et al., 2004; Damm and Winkelbauer, 2011; Winkelbauer and Müller, 2011). The migration of mesoderm cells away from the primitive streak in the gastrulating chick embryo is regulated by N-cadherin through the PDGF pathway (Yang et al., 2008; Chuai and Weijer, 2009), while FGF4 and FGF8 provide directional cues by acting as attractants and repellants, respectively (Yang et al., 2002; Lunn et al., 2007; Chuai and Weijer, 2009). Additionally, in the mouse embryo, FGF signaling regulates the migration of ingressed mesoderm cells and the formation of paraxial mesoderm (Sun et al., 1999; Ciruna and Rossant, 2001; Boulet and Capecechi, 2012).

The sea urchin embryo, which is optically clear and amenable to both cellular and molecular manipulations, serves as a valuable model with which to study the role of growth factors in gastrulation. Gastrulation in the sea urchin is characterized by the directional migration of mesodermal primary mesenchyme cells (PMCs), which produce the embryonic endoskeleton (Wilt and Ettensohn, 2007; Ettensohn, 2013). PMCs, after undergoing an epithelial-to-mesenchymal transition (EMT), migrate directionally within the blastocoel by means of filopodia and form a characteristic ring pattern (the subequatorial PMC ring), which consists of two ventrolateral clusters (VLCs) of PMCs joined by oral and aboral cell strands. The PMCs fuse as they migrate, forming a single continuous syncytium within which an elaborate calcium carbonate endoskeleton is secreted. Specific cues from the ectoderm of the embryo regulate both the migration and differentiation of the PMCs, thereby determining the structure of the resulting endoskeleton (Ettensohn and McClay, 1986; Ettensohn, 1990; Hardin et al., 1992; Armstrong et al., 1993; Ettensohn and Malinda, 1993; Guss and Ettensohn, 1997). However, relatively few genes expressed in the ectoderm have been shown to play a direct role in regulating PMC behavior (Di Bernardo et al., 1999; Cavalieri et al., 2003; Duloquin et al., 2007; Röttinger et al., 2008; Cavalieri et al., 2011).

Recent work has pointed to two pathways – mediated by vascular endothelial growth factor (VEGF) and FGF – in controlling the directional migration of PMCs and the formation of the embryonic skeleton (Duloquin et al., 2007; Röttinger et al., 2008). Duloquin et al. (Duloquin et al., 2007) showed that a VEGF receptor (VEGFR-10-lg) is expressed selectively by migrating PMCs in the sea urchin Paracentrotus lividus, and a VEGF ligand (VEGF3) is expressed in the ectoderm overlying the VLCs. Perturbation of VEGF signaling in this species led to defects in PMC migration and skeletogenesis. Röttinger et al. (Röttinger et al., 2008) identified a similar, complementary pattern of expression of an FGF ligand and receptor (FGFA and FGFR2, respectively) in P. lividus and showed that blocking the FGF pathway also led to defects in skeletogenesis. These studies suggest that FGF and VEGF might have essential...
(and non-redundant) functions in mesoderm cell migration and skeletogenesis in the sea urchin, and raise the question of whether these two pathways regulate the same, or different, cell behaviors during PMC morphogenesis.

In this study, we aimed to further elucidate the roles of VEGF and FGF signaling in gastrulation and skeletogenesis in the sea urchin. We used two-color, fluorescent in situ hybridization to show that, in *Lytechinus variegatus*, *fgfa* and *vegf3* are expressed in distinct domains in the ectoderm. Knockdown of *Lv-vegf3* caused striking defects in the migration and differentiation of PMCs, whereas PMC migration was unperturbed in *Lv-fgfa* morphants, although these formed truncated skeletal elements. Using a second-generation VEGFR inhibitor, axitinib, we found that VEGF signaling is essential for all phases of PMC migration. Importantly, we identified a role for VEGF signaling in biomineralization that was separable from its effects on PMC migration, and showed that VEGF regulates the expression of many biomineralization genes in the PMC gene regulatory network (GRN). This study expands our knowledge of the regulation of mesoderm migration and differentiation by growth factor signaling pathways during gastrulation.

**MATERIALS AND METHODS**

**Embryo culture**

Adult *Lytechinus variegatus* were obtained from the Duke University Marine Laboratory (Beaufort, NC, USA) or Reeftopia (Key West, FL, USA). Adult *Strongylocentrotus purpuratus* were obtained from Pat Leahy (California Institute of Technology, Pasadena, CA, USA). Spawning was induced by intracoelomic injection of 0.5 M KCl and embryos were cultured in artificial seawater (ASW) at 23°C (*L. variegatus*) or 15°C (*S. purpuratus*).

**Cloning of *Lv-vegf3*, *Lv-fgfa*, *Lv-otp* and *Lv-pax2/5/8***

Partial *Lv-fgfa* and *Lv-vegf3* sequences were obtained by PCR using degenerate primers (supplementary material Table S4). Remaining *Lv-vegf3* and 3’ *Lv-fgfa* sequences were obtained by RACE using *L. variegatus* late gastrula cDNA, the GeneRacer Kit with SuperScript III reverse transcriptase, and the Zero Blunt TOPO PCR Cloning Kit for Sequencing (Invitrogen). 5’ *Lv-fgfa* sequence was obtained by screening an *L. variegatus* mid-gastrula cDNA library (a gift from Dr David McClay, Duke University, NC, USA) using partial sequence obtained from degenerate PCR as a probe. GenBank accession numbers for *Lv-fgfa* and *Lv-vegf3* are KF285451 and KF285452, respectively. Comparisons of the sequences of *Lv-vegf3*, *S. purpuratus* and *Paracentrotus lividus* *vegf3* and *fgfa* were carried out using ClustalW (Thompson et al., 1994). A fragment of *Lv-pax2/5/8* and the complete *Lv-otp* coding region were obtained by PCR and cloned into the pCS2+ vector using the EcoRI and XhoI restriction sites.

**Whole-mount in situ hybridization (WMISH)**

Embryos were fixed (Adomako-Ankomah and Ettensohn, 2011) and WMISH and fluorescent WMISH (F-WMISH) were carried out (Lepage et al., 1992; Dulouquin et al., 2007; Sharma and Ettensohn, 2010) as described.

**Microinjection of morpholino antisense oligonucleotides (MOs)**

Microinjections were carried out as described (Cheers and Ettensohn, 2004). Injection solutions contained 20% (vol/vol) glycerol and 0.16% (wt/vol) Texas Red dextran. MOs (supplementary material Table S5) were obtained from Gene Tools. *Lv-fgfa* splice-blocking MO was complementary to the exon 2/intron 2 boundary as described (Röttinger et al., 2008) and was injected at 2 nM and 4 nM. *Lv-fgfa* translation-blocking MO was injected at 4 nM and *Lv-vegf3* and *Sp-vegf3* translation-blocking MOs were injected at 2 nM.

**RT-PCR analysis**

RT-PCR analysis was performed as previously described (Adomako-Ankomah and Ettensohn, 2011). PCR products were cloned into the pCR4-TOPO vector (Invitrogen) for sequencing.

**Axitinib (AG013736) treatments**

A 5 mM stock solution of axitinib (Selleckchem, Houston, TX, USA) was prepared in DMSO and stored at −20°C. Embryos were cultured in ASW containing axitinib at a final concentration of 75 nM (*L. variegatus*) or 50 nM (*S. purpuratus*). Control embryos were cultured in equivalent concentrations of DMSO.

**Immunofluorescence**

Immunofluorescence using monoclonal antibody (mAb) 6a9 was carried out as described (Ettensohn and McClay, 1988). Immunostained embryos were examined using a Zeiss LSM 510 Meta/UV Duoscan inverted spectral confocal microscope.

**Aptosis assay**

The terminal dUTP nick end labeling (TUNEL) assay was carried out using the ApoAlert DNA Fragmentation Assay Kit (Clontech) as described (Voronina and Wessel, 2001). Modifications were introduced to label PMCs with the 6e10 mAb (Hodor et al., 2000), prior to nuclear labeling with 0.2 mg/ml Hoechst stain. Embryos were observed by confocal microscopy as described above.

**Analysis of PMC migration and filopodia extension**

To assess the ability of PMCs in axitinib- and DMSO-treated (control) embryos to target to the vegetal region of the embryo, PMCs were scattered throughout the blastocoele at the mesenchyme blastula stage (prior to the invagination of the archenteron) using a microneedle (Ettensohn and McClay, 1988). Embryos were cultured at 23°C for 5 hours, then fixed in 4% paraformaldehyde (1 hour), washed twice in ASW, and post-fixed in 100% methanol (20 minutes). The distribution of PMCs in the blastocoele was assayed by 6a9 immunostaining.

To further analyze PMC migration, PMCs were removed microsurgically from late mesenchyme blastula stage embryos and placed on fibronectin-coated coverslips as described (Malinda et al., 1995) in a medium of 2% horse serum in ASW. After allowing 30 minutes for attachment, PMCs from DMSO- or axitinib-treated embryos were imaged at 5-minute intervals for 2.5 hours using an Olympus DP71 digital camera. The velocity of migrating PMCs was measured using the Manual Tracking plug-in of ImageJ (NIH). Individual PMCs were tracked either throughout the experiment or until they became untraceable due to clustering.

To quantify filopodial numbers and lengths in axitinib- and DMSO-treated embryos, PMCs were scattered in the blastocoele at the mesenchyme blastula stage, and approximately half of the cells were flushed out of the blastocoele to reduce cell density. This made it possible to visualize unambiguously the filopodia of individual PMCs. Embryos were cultured at 23°C for 1.5 hours post-surgery, and the distribution and length of filopodia present were measured in 6a9-stained embryos using ImageJ.

**PMC fusion assay**

PMC fusion was monitored by dye transfer (Hodor and Ettensohn, 2008). One set of fertilized eggs was injected with a solution of *Lv-vegf3* MO that contained 10% (wt/vol) fluorescein dextran, while another set was injected with a solution of the same MO without the dextran. At the mesenchyme blastula stage, two to six PMCs were transferred from dextran-labeled donor embryos into each unlabeled host embryo. Embryos were observed by confocal microscopy 6 and 24 hours post-surgery to assess the distribution of the dextran within the PMC syncytium.

**Assay of gene expression using the Nanostring nCounter analysis system**

Changes in mRNA levels were assayed using the Nanostring nCounter analysis system (Nanostring Technologies, Seattle, WA, USA) (Geiss et al., 2008). For each experiment, RNA was extracted from 200 control and 200 axitinib-treated or VEGF morphant *S. purpuratus* embryos using the Nucleospin RNA II Kit (Clontech). The code set of probes corresponded to ~80 genes in the PMC GRN (supplementary material Table S2).
RESULTS

VEGF and FGF ligands are expressed in distinct domains in the ectoderm

To compare the roles of VEGF and FGF signaling, we cloned vegf3 and fgfa from L. variegatus, a North American species. ClustalW analysis (Thompson et al., 1994) of the amino acid sequences of VEGF3 and FGFA in L. variegatus, S. purpuratus and P. lividus revealed ~70% and 80% identity, respectively, across species (supplementary material Fig. S1).

We performed two-color F-WMISH to compare directly the expression domains of Lv-fgfa and Lv-vegf3 at various developmental stages (Fig. 1). Lv-fgfa showed a highly dynamic pattern of expression whereas Lv-vegf3 expression was steadily maintained in ectodermal territories associated with PMC accumulation and skeletal growth. Both genes were first detectable at the hatched blastula stage (Fig. 1A–A′), when Lv-fgfa was expressed in a ring of ectoderm that spanned the equator of the embryo and in the presumptive PMCs at the vegetal pole. Lv-vegf3 was also expressed in a ring in the ectoderm, but in a territory more vegetal to, and not overlapping with, the domain of Lv-fgfa expression. This separation of expression domains persisted at the mesenchyme blastula stage, although at this stage the expression of both genes was generally restricted to the ectoderm overlying the VLCs (Fig. 1B–B′). As the archenteron began to invaginate, the expression domains of Lv-fgfa and Lv-vegf3 in the ectoderm overlapped slightly (Fig. 1C–C′). This overlap persisted as the archenteron elongated (Fig. 1D–D′), but was no longer evident at the late gastrula stage (Fig. 1E–E′), when Lv-vegf3 continued to be expressed in the ectoderm overlying the VLCs (Fig. 1E′) while Lv-fgfa was downregulated in these domains and was expressed mostly in two apical domains of ectoderm and in PMCs (Fig. 1E). Lv-fgfa expression at the prism stage was largely restricted to the PMCs, whereas Lv-vegf3 expression persisted in the ectoderm adjacent to these cells (Fig. 1F–F′). At the pluteus stage, Lv-vegf3 was expressed in the ectoderm at specific regions overlying the elongating postoral and anterolateral rods, while Lv-fgfa was expressed in PMCs associated with these rods and in the gut (Fig. 1G–G′). Additionally, we observed that the cognate receptors Lv-vegfr-10-lg and Lv-fgfr-2 were expressed selectively by PMCs in patterns identical to those reported in P. lividus (Duloquin et al., 2007; Röttinger et al., 2008) (data not shown).

VEGF signaling plays a more prominent role than FGF signaling in PMC migration and skeletogenesis in L. variegatus

To compare the functions of FGF and VEGF in L. variegatus, we knocked down the expression of each ligand. Skeletogenesis was blocked in embryos injected with 2 mM Lv-vegf3 translation-blocking MO (Fig. 2A–B′), a phenotype very similar to that observed in P. lividus (Duloquin et al., 2007). Likewise,
skeletogenesis was eliminated in *S. purpuratus* embryos injected with 2 mM *Sp-vegf3* translation-blocking MO (supplementary material Fig. S2B,B′). The *Lv-vegf3* and *Sp-vegf3* MOs differed at 8/25 nucleotide positions, demonstrating the specificity of the morphant phenotype. However, unlike results obtained in *P. lividus* (Röttinger et al., 2008), skeletogenesis was not eliminated in embryos injected with 2 mM splice-blocking *Lv-fgfa* MO, and these morphants formed extensive, although truncated, skeletons (Fig. 2C,C′). Embryos injected with as much as 4 mM *Lv-fgfa* MO also formed skeletal elements (Fig. 2D′), although these embryos showed toxicity effects due to the high MO concentration (Fig. 2D).

Following the same splice-blocking strategy used by Röttinger et al. (Röttinger et al., 2008), we designed the *Lv-fgfa* MO to overlap the exon 2-intron 2 boundary, leading to the exclusion of exon 2, which contains the highly conserved FGF domain (Fig. 2E). We demonstrated the effectiveness of the MO by RT-PCR analysis of 200 control embryos and 200 embryos injected with 1, 2 or 4 mM *Lv-fgfa* MO and observed the expected shift in the size of the *Lv-fgfa* transcript corresponding to an exclusion of exon 2 in embryos injected with all concentrations of *Lv-fgfa* MO (Fig. 2F, bright bands). At the MO concentrations used in our subsequent analysis (2 mM), we did not detect normally spliced *Lv-fgfa* mRNA by RT-PCR. The structure of the mis-spliced form of the mRNA lacking exon 2 was confirmed by sequencing. Embryos injected with an *Lv-fgfa* translation-blocking MO at a range of concentrations likewise formed bilaterally symmetrical, truncated skeletal elements (supplementary material Fig. S3B,D).

We examined the distribution of PMCs in *Lv-vegf3* and *Lv-fgfa* morphants by 6a9 immunostaining. At the late gastrula stage, PMCs in control embryos were arranged in a subequatorial ring with VLCs (Fig. 3A, arrow). PMCs in *Lv-vegf3* morphants did not form VLCs (Fig. 3B,B′), although most of the cells remained localized in the vegetal hemisphere. By contrast, PMCs in *Lv-fgfa* morphants formed a well-patterned ring comparable to that of control embryos (Fig. 3C,C′). At the pluteus stage, PMCs in control embryos were aligned along a well-developed skeleton (Fig. 3D,D′), whereas PMCs in *Lv-vegf3* morphants were scattered primarily within the vegetal region of the embryo (Fig. 3E,E′). Strikingly, in *Lv-vegf3* morphants, PMCs were highly fragmented at the pluteus stage (Fig. 3E, inset), but not at earlier stages (Fig. 3B, inset). We showed by a TUNEL assay that this fragmentation was not due to apoptosis (supplementary material Fig. S4). In *Lv-fgfa* morphants at the pluteus stage, PMCs were arranged in a pattern similar to that observed in control embryos, although defects in the morphology of the skeleton were apparent (Fig. 3F,F′).

We tested whether signaling through the VEGF and FGF pathways regulated the expression of *Lv-vegf3* or *Lv-fgfa*. *Lv-vegf3* expression was strongly upregulated in *Lv-vegf3* morphants (Fig. 4A,B). We also observed a striking downregulation of *Lv-fgfa* in PMCs, from the mesenchyme blastula stage throughout later development (Fig. 4C,D). *Lv-fgfa* was expressed in an expanded territory in the ectoderm at the late gastrula stage in *Lv-vegf3* morphants (Fig. 4D), although this expression domain narrowed to a pattern comparable to that of controls by the prism stage (data not shown). Upon *Lv-fgfa* knockdown, *Lv-vegf3* was slightly upregulated, an effect that was more pronounced at mesenchyme blastula (Fig. 4E) than at late gastrula (Fig. 4F) stages. Furthermore, as previously described in *P. lividus* (Röttinger et al., 2008), *Lv-fgfa* expression was strongly upregulated in the PMCs and ectoderm of *Lv-fgfa* morphants (Fig. 4G,H). The increase in *Lv-vegf3* and *Lv-fgfa* mRNA levels in the corresponding morphants is likely to reflect negative feedback of each pathway on transcription, although we cannot exclude the possibility that mRNA stability is affected (directly or indirectly) by the MOs.

We also examined two other genes expressed in the ectoderm that have been shown to play a role in regulating skeletogenesis: *otp* (Di Bernardo et al., 1999; Cavalieri et al., 2003) and *pax2/5/8* (Cavalieri et al., 2011). *Lv-pax2/5/8* expression was slightly downregulated in *Lv-vegf3* morphants (supplementary material Fig. S5A,A′) and strongly downregulated in *Lv-fgfa* morphants (supplementary material Fig. S5C,C′). *Lv-otp* was unaffected by the knockdown of either *Lv-vegf3* or *Lv-fgfa* (supplementary material Fig. S5B,B′,D,D′). As *Lv-fgfa* knockdown produced only modest defects in skeletogenesis and had no detectable effect on PMC migration, we focused our attention on further elucidating the role of VEGF signaling in PMC morphogenesis.

**VEGF signaling controls PMC pathfinding throughout gastrulation**

VEGF MO injection blocks VEGF signaling throughout embryogenesis. To determine the temporal requirements for VEGF signaling, we used a second-generation VEGFR inhibitor, axitinib, which specifically blocks VEGF signaling at low (nM) concentrations (Hu-Lowe et al., 2008; Bhargava and Robinson, 2011). *L. variegatus* embryos cultured in 75 nM axitinib from early cleavage developed no skeletal elements (Fig. 5B,B′), a phenotype identical to that of VEGF morphants (Fig. 2B). As in *Lv-vegf3* morphants, PMCs in arixinib-
treated embryos did not form VLCs (Fig. 5D,D’). At the pluteus stage, PMCs in axitinib-treated embryos exhibited the same fragmented phenotype observed in Lv-vegf3 morphants (Fig. 5F). Similarly, PMC migration and skeletogenesis were inhibited in S. purpuratus embryos cultured in 50 nM axitinib from early cleavage (supplementary material Fig. S2C,C’,F,F’). These findings confirmed that axitinib phenocopies VEGF knockdown.

PMC migration can be considered to occur in two phases. During the initial phase (early gastrulation), PMCs disperse from the site of ingression and arrange themselves in the subequatorial ring. During the second phase (late gastrulation), a chain of PMCs migrates from each VLC towards the animal pole. These cells produce the dorsoventral, anterolateral, and recurrent rods of the larval skeleton. To test whether VEGF signaling is required for the second phase of PMC migration, we cultured embryos in axitinib from the early gastrula stage, when PMCs were in the process of forming the subequatorial ring (Fig. 6A,A’). We fixed control and axitinib-treated embryos at the late gastrula stage and assessed the distribution of PMCs by immunostaining. PMCs in DMSO-treated control embryos migrated normally and formed two chains of cells that extended from the VLCs towards the animal pole (Fig. 6C,C’). No strands of PMCs migrated towards the animal pole in axitinib-treated embryos, however, despite the fact that VLCs were visible in 80% of embryos (Fig. 6B,B’). Based on these observations, we conclude that VEGF signaling is required for both the early and late phases of PMC migration.

Upon Lv-vegf3 knockdown or VEGFR inhibition by axitinib treatment, PMCs did not disperse randomly through the blastocoel but remained mostly in the vegetal hemisphere. To examine whether this behavior might be the result of VEGF-independent guidance cues in the blastocoel, we displaced PMCs throughout the blastocoel in control (Fig. 7A,A’) and axitinib-treated embryos at the mesenchyme blastula stage and examined the distribution of PMCs 5 hours later. In 28/30 cases, PMCs in control embryos returned to the vegetal region.
and formed a subequatorial ring, usually with two chains of cells extending towards the animal pole (Fig. 7C,C'). By contrast, in 30/30 axitinib-treated embryos, many PMCs remained in the animal half of the embryo (Fig. 7B,B'). Comparable results were obtained when PMCs in Lv-vegf3 morphants were scattered in the same manner (data not shown). Aggregates of PMCs remained scattered throughout the blastocoel of axitinib-treated embryos even 10 hours post-surgery (Fig. 7D,D'), whereas extensive, well-patterned skeletal elements were visible in control embryos (Fig. 7E,E'). We therefore found no evidence of a VEGF-independent mechanism that targets PMCs to the vegetal hemisphere.

**VEGF signaling is not required for PMC motility per se or for PMC fusion, but regulates the number and length of filopodia**

In axitinib-treated embryos and Lv-vegf3 morphants, PMCs dispersed from the site of ingression and migrated along the basal surface of the ectoderm, indicating that cell motility per se was not blocked. We also used an *in vitro* assay to examine PMC motility (Fig. 8). Control PMCs migrated actively on fibronectin-coated coverslips and formed aggregates, as did PMCs that were isolated from axitinib-treated embryos and cultured continuously in the presence of the inhibitor (Fig. 8A). Axitinib treatment resulted in a very small, statistically insignificant decrease in average PMC velocity ($P=0.1298$) (Fig. 8B). These results support the view that VEGF signaling has a specific role in PMC guidance but is not required for PMC motility.

Because PMC pathfinding is likely to be mediated by filopodia, we analyzed the effect of VEGF signaling on the number and length of filopodia. PMCs in control embryos showed a stereotypical elongated morphology with filopodia that extended mostly from the two ends of each cell (Fig. 9A). PMCs in axitinib-treated embryos, however, were more rounded and had fewer, shorter filopodia (Fig. 9B). PMCs in control embryos ($n=57$) had ~5 filopodia per cell, of an average length of 11.5 µm. By contrast, PMCs in axitinib-treated embryos ($n=63$) had ~2 filopodia per cell, of an average length of 4.8 µm (Fig. 9C,D). These differences between control and axitinib-treated embryos were highly significant ($P=7.68\times10^{-8}$ and $P=5.15\times10^{-29}$ for filopodia number and length, respectively). These findings strongly suggest that VEGF signaling regulates aspects of filopodial dynamics, i.e. the formation of these cell protrusions and/or their stability.

**Filopodia also mediate PMC-PMC fusion** (Hodor and Ettensohn, 2008). We used a dye-transfer assay to test whether PMC fusion was blocked in Lv-vegf3 morphants. In 20/21 morphant embryos, dextran spread from a small number of transplanted, labeled PMCs to all host PMCs by the late gastrula stage (Fig. 10A,A'). dextran label was still visible in the fragmented PMCs of Lv-vegf3 morphant embryos (Fig. 10B,B').

**VEGF signaling is required continuously for skeletogenesis**

Why do PMCs fail to produce skeletal elements when VEGF signaling is blocked? One possibility is that VEGF regulates biomineralization directly, by regulating the expression of biomineralization genes. Alternatively, VEGF might regulate...
biomineralization indirectly, by affecting the ability of PMCs to migrate directionally and accumulate in the VLCs, where local ectodermal cues other than VEGF might induce biomineral secretion. To distinguish between these possibilities, we treated embryo cultures with axitinib at various stages of development (Fig. 11). The frequencies of morphant phenotypes that we observed upon drug addition at each stage of development are shown in Table 1. Embryos treated with axitinib beginning at the hatched blastula (Fig. 11B,B') or early gastrula (Fig. 11D,D') stages developed no skeletal elements when control embryos reached the pluteus stage (Fig. 11A,A'). Embryos treated with axitinib from the mid-gastrula (Fig. 10E,E'), late gastrula (Fig. 11F,F') or prism (Fig. 11G,G') stages developed truncated skeletal elements.

Surprisingly, we noted that in embryos cultured in axitinib from the mid- or late gastrula stages, specific skeletal rods were affected to varying degrees. The body rods and recurrent rods extended mesenchyme blastula (Fig. 11C,C') or early gastrula (Fig. 11D,D') stages developed no skeletal elements when control embryos reached the pluteus stage (Fig. 11A,A'). Embryos treated with axitinib from the mid-gastrula (Fig. 10E,E'), late gastrula (Fig. 11F,F') or prism (Fig. 11G,G') stages developed truncated skeletal elements.

Fig. 7. VEGF signaling is required for the targeting of PMCs to the vegetal hemisphere. At the mesenchyme blastula stage, a micropipette was used to scatter PMCs, redistributing most of the cells into the animal hemisphere. Fluorescence (A-E), and DIC-merged (A'-E') images of a control embryo 0 hours post-surgery (A,A'), and axitinib-treated (B,B',D,D') and DMSO-treated control (C,C',E,E') embryos 5 hours (B,B',C,C') and 10 hours (D,D',E,E') after surgery. At 5 hours, almost all PMCs have migrated to the vegetal hemisphere and have formed a subequatorial ring in control embryos, whereas in axitinib-treated embryos most PMCs remain in the animal hemisphere. At 10 hours, a well-patterned skeleton is visible in control embryos, whereas PMCs in axitinib-treated embryos are aggregated within the blastocoel.

Fig. 8. VEGF signaling is not required for PMC translocation in vitro. (A) DIC images of PMCs treated with DMSO (control) or axitinib show that PMC motility and cell clustering on fibronectin-coated coverslips are not perturbed when VEGF signaling is blocked. (B) The average velocities of migrating control (n=223) and axitinib-treated (n=200) PMCs are not statistically different (P=0.1298). Error bars indicate s.e.m.
S. purpuratus
Blocking VEGF signaling in design of a Nanostring probe set (supplementary material Table S2). Used because the availability of a sequenced genome facilitated the S. purpuratus expression of genes in the PMC GRN. (Figs 11, 12), we tested the role of this pathway in regulating the Because VEGF signaling regulates biomineralization directly in the PMC GRN VEGF signaling regulates the expression of genes (supplementary material Fig. S7B). We also examined the reversibility of the effects of VEGF inhibition on skeletogenesis. Embryos were cultured in axitinib from early cleavage and the inhibitor was washed out at various stages of development (supplementary material Fig. S7). Most embryos recovered fully from the effects of blocking VEGF signaling if axitinib was removed prior to the end of gastrulation (supplementary material Fig. S7F-H). The postoral and anterolateral rods grow via the activity of a plug of PMCs at their tips; these PMC clusters were truncated (Fig. 12). The postoral and anterolateral rods fail to elongate normally (supplementary material Fig. S6).

VEGF signaling regulates the expression of genes in the PMC GRN
Because VEGF signaling regulates biomineralization directly (Figs 11, 12), we tested the role of this pathway in regulating the expression of genes in the PMC GRN. S. purpuratus embryos were used because the availability of a sequenced genome facilitated the design of a Nanostring probe set (supplementary material Table S2). Blocking VEGF signaling in S. purpuratus either with an Sp-vegf3 translation-blocking MO or by culturing embryos in 50 nM axitinib from early cleavage perturbed PMC migration and differentiation, as in L. variegatus (supplementary material Fig. S2). Differences normally in the dorsal region of the embryo (Fig. 11E, F', arrows, Fig. 12) whereas the postoral and anterolateral rods were severely truncated (Fig. 12). The postoral and anterolateral rods grow via the activity of a plug of PMCs at their tips; these PMC clusters were apparent in axitinib-treated embryos, although the rods failed to elongate normally (supplementary material Fig. S6).

DISCUSSION
Growth factors play a crucial role in regulating mesoderm morphogenesis during gastrulation, but the specific growth factors involved and the cell behaviors they control vary across species. For example, in the chick embryo, mesoderm migration is regulated by a combination of signals (PDGF, FGF and VEGF) (Chuai and Weijer, 2009), whereas our results indicate that in the sea urchin [which lacks PDGF and PDGF receptor genes (Lapraz et al., 2006)] PMC migration is controlled predominantly by one growth factor (VEGF3). In several organisms, it has been shown that a single growth factor influences multiple cell behaviors, although the details vary. During Xenopus gastrulation, PDGF regulates the orientation, radial intercalation and directional migration of mesodermal cells (Nagel et al., 2004; Damm and Winklbauer, 2011). PDGF signaling also regulates cell polarization and the formation of cellular processes by mesendodermal cells in zebrafish, but apparently not their directional migration (Montero et al., 2003). In Drosophila, FGF regulates several mesoderm cell behaviors,

Fig. 9. VEGF signaling regulates the number and length of PMC filopodia. (A, B) Fluorescence images of control (A) and axitinib-treated (B) embryos 1.5 hours after PMCs were scattered within the blastocoel. PMCs in control embryos extend more filopodia than PMCs in axitinib-treated embryos and these filopodia are longer in controls. (C, D) The average number of filopodia per cell (C) and the average length of filopodia (D) extended by PMCs in control embryo (n=57) and axitinib-treated embryos (n=63). Error bars indicate s.e.m.

Fig. 10. VEGF signaling does not regulate PMC fusion. Fluorescence (A, B), and DIC-merged (A', B') images of Lv-vegf3 morphant hosts 6 hours (A, A') and 24 hours (B, B') after a few FITC-dextran labeled PMCs were transferred from Lv-vegf3 morphant donors. PMCs are fusion competent, as shown by the spread of dextran throughout the syncytium 6 hours post-surgery (A) and in scattered fragmented PMCs 24 hour post-surgery (B).
including EMT, intercalation, spreading, and attachment to the epithelium prior to migration (Wilson and Leptin, 2000; Winklbauer and Müller, 2011). In sea urchins, VEGF3 regulates the formation of cellular processes and directional migration. In contrast to mesodermal cells in *Drosophila* and *Xenopus*, however, the ingression and spreading of PMCs is independent of growth factor-mediated signaling. In addition, VEGF signaling is not required for PMC translocation or fusion, but only for guidance. We hypothesize that the effect of VEGF3 on the number and length of PMC filopodia is associated in some way with the defect in pathfinding, although the relationship is unclear.

In vertebrate endothelial cells, VEGF regulates filopodial extension and acts as a chemoattractant to direct cell migration (Gerhardt et al., 2003; Gerhardt, 2008; Shamloo et al., 2008). These findings, and the observation that PMCs accumulate at ectopic sites of VEGF expression (Duloquin et al., 2007), indicate that VEGF3 functions as a chemoattractant in the sea urchin. Our finding that *Lv-vegf3* expression is initially confined to a ring of ectoderm at the vegetal pole of the blastula and subsequently resolves to the ectoderm overlying the VLCs might account for the observation that vegetal directional cues are established even before PMC ingression (Ettensohn and McClay, 1986) and for the gradual emergence of the two PMC clusters during gastrulation (Malinda et al., 1995; Gustafson and Wolpert, 1999). Our findings further suggest that VEGF3 signaling is required for the second phase of PMC migration, during which chains of PMCs extend from the VLCs towards the animal pole. At present, owing to embryo to embryo variability in the timing of VEGF3 expression in the animal hemisphere during late gastrulation, the role of VEGF3 in PMC migration remains uncertain.

---

**Table 1. Distribution of phenotypes in axitinib wash-in experiments**

<table>
<thead>
<tr>
<th>Stage at which axitinib was added</th>
<th>DMSO control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenotype</td>
<td>n %</td>
</tr>
<tr>
<td>No skeleton</td>
<td>202 100</td>
</tr>
<tr>
<td>Tiny skeletal deposits (specks)</td>
<td>0 0</td>
</tr>
<tr>
<td>Branched skeletal rudiments</td>
<td>0 0</td>
</tr>
<tr>
<td>Extended rods, with prominent body and dorsoventral connecting rods</td>
<td>0 0</td>
</tr>
<tr>
<td>Short pluteus skeleton</td>
<td>0 0</td>
</tr>
<tr>
<td>Wild-type skeleton</td>
<td>0 0</td>
</tr>
<tr>
<td>Total</td>
<td>202 100</td>
</tr>
</tbody>
</table>

Bold type indicates the prevalent phenotype observed.

---

**Fig. 11. VEGF signaling regulates biomineralization independently of its role in PMC guidance.** DIC (A-G) and polarized light (A’-G’) images of a control embryo (A,A’), and embryos treated with axitinib at the hatched blastula (B,B’), mesenchyme blastula (C,C’), early gastrula (D,D’), mid-gastrula (E,E’), late gastrula (F,F’), or prism (G,G’) stages. Inhibition of VEGF signaling prior to the mid-gastrula stage completely blocks biomineralization (B’-D’), whereas VEGFR inhibition at or after mid-gastrula leads to the formation of truncated skeletal elements (E’-G’). Arrows (A’,E’,G’) indicate body rods. Diagrams indicate the stage at which axitinib was added to embryo cultures (PMCs are represented in red and skeletal elements in blue).
Although VEGF signaling is required for biomineral formation (Duloquin et al., 2007), it has been unclear whether this is a secondary effect of disrupting the normal pattern of PMC migration, which might prevent PMCs from receiving a different ectodermal signal. In this regard, the ectoderm overlying sites of skeletal rod growth is a local source of signaling factors other than VEGF3, including FGFA and WNT5. Using axitinib to inhibit VEGF signaling late in development (Fig. 12). We report that the expression of vegfr-10-Ig and fgfa expression in the ectoderm is likely to be indirect. We report that the expression of fgfa in PMCs (but not in the ectoderm) is dependent on VEGF3, an effect that might be mediated directly by VEGFR-10-Ig. This provides evidence of cross-talk between these two pathways, which function synergistically in other systems (Tomene et al., 2010). Cavaleri et al. (Cavaleri et al., 2011) showed that the tripartite motif-containing protein STRIM1 regulates the expression of fgfa in the ectoderm, but not in PMCs, highlighting the separate control of fgfa expression in these two tissues. STRIM1 also regulates otp in the ectoderm whereas VEGF3 and FGFA do not, suggesting that multiple pathways regulate gene expression in the ectoderm overlying the VLCs.

Our studies and those of Duloquin et al. (Duloquin et al., 2007) demonstrate a conserved role for VEGF3 in regulating PMC migration and differentiation in three species of sea urchin. Recent work suggests that evolutionary shifts in VEGF3 expression have been associated with changing patterns of skeletogenesis within the phylum (Morino et al., 2012). In contrast to the highly conserved function of VEGF3, the role of FGFA may vary among species, as this molecule has been implicated in PMC migration and skeletogenesis in P. lividus (Röttinger et al., 2008). In L. variegatus, gastrulation, we do not know whether a VEGF3 concentration gradient directs this phase of PMC migration or whether VEGF3 signaling at the VLCs renders the cells competent to respond to other directional cues.
we observed skeletal phenotypes similar to those reported by Röttinger and co-workers when we injected high concentrations of MO that also appeared to produce general toxic effects. At lower doses, all detectable FGF mRNA was mis-spliced (Fig. 2), but the overall morphology of the embryos was similar to that of controls, and we observed no effects on PMC migration and only subtle effects on skeletal growth. Thus, there might be technical issues that account for the reported differences in the phenotypes of FGF morphants in these two species, and the functions of both VEGF3 and FGFA might be conserved among echinoids.

The downstream components of the VEGF signaling pathway in PMCs are unknown. In other cell types, VEGF receptors signal via the MAPK and PI3 kinase pathways (Schlessinger, 2000), both of which are essential for skeletogenesis in the sea urchin embryo. Bradham et al. (Bradham et al., 2004) showed that inhibiting PI3K blocks the elongation of skeletal rods, but not PMC specification, cell migration, the extension of filopodia, cell fusion or skeletal initiation. The MAPK pathway, by contrast, regulates early PMC specification, thereby affecting all aspects of PMC morphogenesis (Fernandez-Serra et al., 2004; Röttinger et al., 2004). It has been suggested that MAPK signaling may regulate skeletogenesis by two distinct pathways: a Ras-independent pathway necessary for PMC ingression and a Ras-dependent pathway required for PMC migration and skeletogenesis (Fernandez-Serra et al., 2004; Röttinger et al., 2004). As the VEGF pathway does not regulate PMC ingression, it might function by a Ras/MAPK-dependent mechanism. Further research will be necessary to determine the molecular effectors of VEGF signaling in PMCs and the linkages between this pathway and the skeletogenic GRN.


